

The influence of cultivation methods on *Shewanella oneidensis* physiology and proteome expression

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Abstract High-throughput analyses that are central to microbial systems biology and ecophysiology research benefit from highly homogeneous and physiologically well-defined cell cultures. While attention has focused on the technical variation associated with high-throughput technologies, biological variation introduced as a function of cell cultivation methods has been largely overlooked. This study evaluated the impact of cultivation methods, controlled batch or continuous culture in bioreactors versus shake flasks, on the reproducibility of global proteome measurements in *Shewanella oneidensis* MR-1. Variability in dissolved oxygen concentration and consumption rate, metabolite profiles, and proteome was greater in shake flask than controlled batch or chemostat cultures. Proteins indicative of suboxic and anaerobic growth (e.g., fumarate reductase and decaheme *c*-type cytochromes) were more abundant in cells from shake flasks compared to bioreactor

cultures, a finding consistent with data demonstrating that “aerobic” flask cultures were O₂ deficient due to poor mass transfer kinetics. The work described herein establishes the necessity of controlled cultivation for ensuring highly reproducible and homogenous microbial cultures. By decreasing cell to cell variability, higher quality samples will allow for the interpretive accuracy necessary for drawing conclusions relevant to microbial systems biology research.

Keywords Controlled cultivation · Systems biology · Proteomics · *Shewanella*

Introduction

The cultivation of microorganisms has been performed for more than a century beginning with Louis Pasteur (1879), Robert Koch (1881) and R. J. Petri (1882) (Sedgwick 1916; Gest 1987), with ever increasingly sophisticated methods becoming available over time. These methods range from using undefined medium (e.g., boiled meat extracts) in shake flask cultures where growth rate and substrate utilization (Narang et al. 1997) as well as proteomic profiles (Valentine et al. 2005; Wunschel et al. 2005) can vary widely, to defined culturing media combined with state-of-the-science bioreactors (Nethe-Jaenchen and Thauer 1984; Vasconcelos et al. 1994; Elias et al. 2005).

A considerable amount of physiological (Keltjens et al. 1990; Dolla et al. 2000; Elias et al. 2004) and high-throughput functional genomic data (Beliaev et al. 2002; Thompson et al. 2002; Wan et al. 2004) has been generated from microbial samples produced using shake flask batch cultures. Although results may be statistically defensible, the inherent short-comings of this cultivation technique are that essential parameters directly impacting organism physiology

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are poorly described and difficult to control. These parameters include nutrient availability, metabolite production, specific growth rate, and poor mixing leading to cultural/environmental heterogeneity. Poor mass transfer kinetics can lead to unwanted limitations in dissolved gasses for use by the organism (such as oxygen or hydrogen) and the buildup of gaseous metabolic byproducts, such as CH₄ and CO₂ that can have secondary effects on cellular metabolism. For example, the accumulation of dissolved CO₂ and the production of dissolved organic acids can significantly alter pH in poorly buffered systems (Ji et al. 1995; Narang et al. 1997; Mayville et al. 1999; Miller and Bassler 2001). Fluctuations of pH in bacterial cultures induce physiological responses in various organisms (Snoep et al. 1990; Blankenhorn et al. 1999; Stancik et al. 2002; Yohannes et al. 2004). Hence, the conditions of cultivation at the beginning of growth can be far different than those when the cells are harvested for analysis. Furthermore, the time of cell harvest during different “phases of growth” will also impact biochemical reaction rates (Wright and Holland 2003) and the complement of cellular proteins (Lipton et al. 2002; Wunschel et al. 2005). In actuality, the functional characteristics and relative abundance of proteins measured are derived from a heterogeneous cell population including cells in various stages of growth that are potentially expressing a range of metabolic states within relatively short time periods. We argue that the term “aerobic shake flask,” therefore, is more an operational description than an accurate description of the physiological growth conditions.

In bioreactors, all relevant physiological parameters, including pH, dissolved oxygen (DO₂), temperature, agitation, and the incoming gas flow rate and composition can be monitored and controlled. Because the nutrient medium can be of variable composition and added to the bioreactor at discreet rates, cells can be cultured with varying degrees of electron donor/carbon source or electron acceptor limitation and over a range of growth rates. The former has important ramifications given the effects of these limitations on cell physiology, while being able to address the issue of different growth rates enables studies that more closely mimic the variable and potentially long generation times typical of many habitats including sedimentary and aquatic environments. A complete mass (fermentation) balance can also be obtained by measuring biogenic gases produced during the cultivation by using in-line mass spectrometry (MS) in addition to the byproducts of carbon and energy source metabolism and the amount of produced biomass. The utilization of such advanced technologies are allowing for unprecedented insights into the metabolic complexities of microbiological systems.

Controlled cultivation technologies, such as chemostats and turbidostats, minimize culture heterogeneity through continual and thorough agitation and by monitoring and

controlling all culture parameters. This mixing substantially increases the mass transfer rates of both the influent gas stream and those gases resulting from microbial metabolism, thus creating a more homogenous gas profile throughout the culture (Pin et al. 2002) and reducing the gas effect on pH. Any change in pH that occurs during cell growth due to non-gaseous metabolic by-products is compensated for by monitoring probes and the addition of acid or base (typically HCl or NaOH) via computer controlled pumps. While there is a greater upfront capital investment for bioreactors, the investment is minor relative to the expense associated with high-throughput functional genomics techniques.

Systems biology seeks to integrate high-throughput and comprehensive analytical techniques such as DNA and RNA microarrays, proteomics and protein interaction analyses, and metabolite measurements with computational biology (i.e., modeling) to describe the structure of the system and responses to individual perturbations. Such knowledge can be used, for example, to predict systems level responses to environmental changes/perturbations. In our opinion, a key element of systems microbiology research is the use of controlled cultivation techniques to generate samples under well-defined conditions where variations in intra- as well as inter-culture variability, are minimized.

The work described herein was designed to quantitatively assess the relative suitability and limitations of various cultivation approaches for systems biology research. Cells cultivated in shake flasks, controlled batch bioreactors and chemostats were subjected to targeted as well as comprehensive proteomic analysis via two-dimensional gel electrophoresis (Beliaev et al. 2002; Abboud et al. 2005) and the MS based accurate mass and time (AMT) tag approach (Conrads et al. 2002; Smith et al. 2002), to determine the extent of biological variation. Recent reports have used controlled cultivation to determine the “metabolic flux” with different carbon, electron donor or nitrogen sources (Daran-Lapujade et al. 2004; Kolkman et al. 2005, 2006; Adams et al. 2006). However, this is the first report documenting the metabolic differences with varied electron-acceptor between cultures. Finally, while others have utilized steady state cultures and cite advantages similar to those mentioned above, this is, to our knowledge, the first report that assesses and quantifies the effect of cultivation methods on microbial physiology and cell proteome.

Materials and methods

Materials

Acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Urea, dithiothreitol (DTT),

and CaCl_2 were obtained from Sigma-Aldrich (St Louis, MO, USA) while thiourea, trifluoroacetic acid, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate and ammonium bicarbonate were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Sequencing grade, modified trypsin was purchased from Promega (Madison, WI, USA) while ammonium formate was obtained from Fluka (St Louis, MO, USA). Water was purified using a Barnstead Nanopure Infinity water purification system (Dubuque, IA, USA).

Cell cultivation and harvesting

Shake flask cultures

Shewanella oneidensis MR-1 (ATCC 700550) were cultivated in a defined medium as described elsewhere (Elias et al. 2005) except that 18 mM lactate was used instead of 6 mM and the PIPES concentration was increased to 30 mM since there was no pH control. Shake flask cultures (0.15 l) were grown aerobically at 30°C with constant shaking at 150 rpm. Duplicate cultures were inoculated at 2-h intervals over 20 h. After an additional 4 h of incubation the optical density (OD; A_{600}), pH and DO_2 were recorded and culture samples for organic acid analysis were taken via syringe, filtered (0.22 μm) and stored at -80°C until analyzed. The cultures that had grown for 24 h were in the late log phase of growth and were then harvested as previously described (Elias et al. 2005). First, 100 ml of culture was centrifuged and the resulting pellet dehydrated for dry weight biomass measurement (MA100 moisture analyzer Sartorius, Inc.; <http://www.sartorius.com/>). Then, the remaining cells were pelleted by centrifugation (27,000 $\times g$; 8 min; 4°C). The resulting pellets and approximately 2–3 ml of the supernatant were transferred to cryovials (1.0 ml) and centrifuged (9,000 $\times g$; 4 min; 4°C). After removal of the supernatant, the cryovials containing packed cell pellets were immediately stored at -80°C for later analysis by 2DE and AMT MS.

Controlled batch cultures

Cells were cultured in controlled batches using Bioflow model 110 reactors (New Brunswick Scientific, Edison, NJ, USA). For the direct proteome comparison, lactate was used at 18 mM as in the shake flasks but the PIPES concentration was lowered to 3 mM since pH control was present. Cells were grown with oxygen at 20% of dissolved saturation (DO_2) using a combination of air and N_2 gas while pH was constantly maintained at 7.00 ± 0.03 by the addition of 2 N HCl or 2 N NaOH. The temperature was a constant 30°C and the reactors were stirred at 400 rpm to minimize mass transfer limitations of O_2 and CO_2 . Samples were

taken for organic acid analysis as described above until the late log phase of growth at $A_{600} \sim 0.6$. At this point cells were harvested for the analyses as described above for the shake flasks.

Chemostat cultures

Steady state cultures were attained using Bioflow model 3000 reactors (New Brunswick Scientific, Edison, NJ, USA) with the same medium and identical parameters and controls as described for the controlled batch cultures. Once the optical density (OD; A_{600}) was approximately 0.6, fresh medium was pumped into the reactor at a dilution rate of 0.1/h and a constant 3 l volume maintained. Culturing continued until a constant OD and acid addition rate (i.e., steady state) was attained. Samples for organic acids were collected periodically and the cells harvested as above.

Organic acid quantitation

The organic acids lactate, acetate, pyruvate, formate and fumarate were measured using isocratic flow (0.5 ml/min) with both high performance liquid chromatography (HPLC) and ion chromatography (IC). The HPLC (Agilent 1100 series HPLC with a 300×78 mm Rezex organic acid column (Phenomenex, Torrance, CA, USA); 60°C; 35 min run time) used a dilute acid mobile phase of 0.005 N H_2SO_4 . Quantification was accomplished by injecting the sample (25 μl) and using either a diode array detector or a fixed wavelength detector at 210 nm (4 nm bandwidth). Samples were prepared by filtering 1 ml (0.2 μm) and acidifying with 10 μl of 2.5 N H_2SO_4 . Lactate had a retention time of 18.8 min and detection limit of ~ 0.1 mM while pyruvate, acetate, and formate retention times were 13.4, 22.1, and 20.2 min, respectively with a detection limit each of ~ 0.2 mM. For IC (Dionex DX-500) analysis was performed for the above organic acids as previously described (Senko et al. 2002; Elias et al. 2004).

2D-gel proteomic analysis

Frozen cell pellets were mixed with 2 vols. of a solution containing 9 M urea, 2% 2-mercaptoethanol, 2% ampholytes (pH 8–10, BioRad), and 2% Nonidet P40 (a nonionic detergent). The cell lysate was then ultracentrifuged (435,000 $\times g$; 10 min) using a Beckman TL100 tabletop ultracentrifuge, and the soluble denatured proteins were recovered in supernatants from centrifugation. Protein concentrations were determined using a modification of the Bradford protein assay (Ramagli and Rodriguez 1985). Aliquots of sample containing 40 μg of protein were separated in the first dimension by isoelectric focusing using polyacrylamide gels containing 50% pH 5–7 with 50% pH 3–10

carrier ampholytes (Anderson and Anderson 1978a). After 14,000 V-h, the first-dimension gels were equilibrated with sodium dodecyl sulfate (SDS) and the proteins were separated by SDS-polyacrylamide gel electrophoresis as described (O'Farrell 1975) using a linear gradient of 10–17% acrylamide (Anderson and Anderson 1978b). Proteins were then detected by staining with silver nitrate (Giometti et al. 1991). The 2DE images were digitized using an Eikonix1412 scanner interfaced with a VAX 4000-90 workstation. The images were then transferred to a PC, converted to TIFF format, and then processed for spot detection and pattern matching using the Progenesis software (Nonlinear USA). One 2DE image was used as a reference pattern for the experiment. All patterns in the experiment were matched to the reference pattern so that the protein spots were given identification numbers. Statistical analysis of the relative abundance of each matched protein spot across the data set was done using a two-tailed Student's *t*-test as previously described (Giometti and Taylor 1991). Proteins to be identified were cut from two to three replicate gels stained with Coomassie Blue R250 (approximately 200 µg of protein was loaded on each gel), and the proteins were digested in-gel with trypsin (Promega sequence-grade trypsin, 12.5 ng/µg). The resulting peptides were eluted from the gel pieces by extracting three times, first with equal parts of 25 mM ammonium bicarbonate and acetonitrile, then twice with equal parts of 5% (v/v) formic acid and acetonitrile. The eluted tryptic peptides were desalted and concentrated with a commercial ZipTip C₁₈ pipette tip (Millipore). Peptide samples were then loaded onto a 365 × 100 µm fused silica capillary (FSC) column packed with 10 µm POROS 10 R2 packing material (PE Biosystem) at a length of 10–15 cm. Peptides were separated with a 30-min linear gradient of 0–60% solvent containing 80% acetonitrile and 0.5% acetic acid, and then entered into an LCQ ion trap mass spectrometer (Finnigan MAT). Tandem mass spectra were automatically collected under computer control during the 30-min LC-MS runs. MS/MS spectra were then directly subjected to SEQUEST database searches (Eng et al. 1994; Sadygov et al. 2002) by correlating experimental MS/MS spectra to predicted protein sequences in the *S. oneidensis* MR-1 open reading frame database.

Mass spectrometry proteomic analysis

Cell lysis and tryptic digestion

Whole cell lysis was achieved by bead beating while tryptic digestion was achieved with sequencing grade-modified trypsin as previously described (Elias et al. 2005) and digested for 5 h at 37°C using a 1:50 (w/w) trypsin-to-protein ratio. Samples were quick frozen in liquid N₂ and

stored at –80°C until analyzed. Protein concentration was determined by the BCA assay kit (Pierce, Rockford, IL, USA).

Mass spectrometric analysis

The capillary LC system used was described previously (Elias et al. 2005) using 5,000 psi reversed-phase packed capillaries at ~1.8 µl/min (150 µm i.d. × 360 µm o.d.; Polymicro Technologies) (Shen et al. 2001, 2002) with two mobile phase solvents consisting of 0.2% acetic acid and 0.05% TFA in water (A) and 0.1% TFA in 90% acetonitrile/10% water (B). The LTQ-FT data was processed using the PRISM data analysis system as described previously for LC-FTICR-MS data (Elias et al. 2005). Since the separation systems for both the LTQ-FT and the LCQ analyses were identical, peptide confirmation was based on both the calculated (from the mass tag database) and measured mass (from the FTICR analysis) of the peptide matching to within 6 ppm and the elution times matching to within 5%.

All samples were analyzed as previously described (Lipton et al. 2002; Elias et al. 2005) using an LTQ-FT (Thermo-Finnigan, MI, USA). The mass spectrometer measurements were analyzed with SEQUEST (Eng et al. 1994) using our current database as previously described (Elias et al. 2005). Mass spectra were acquired with approximately 10⁵ resolution.

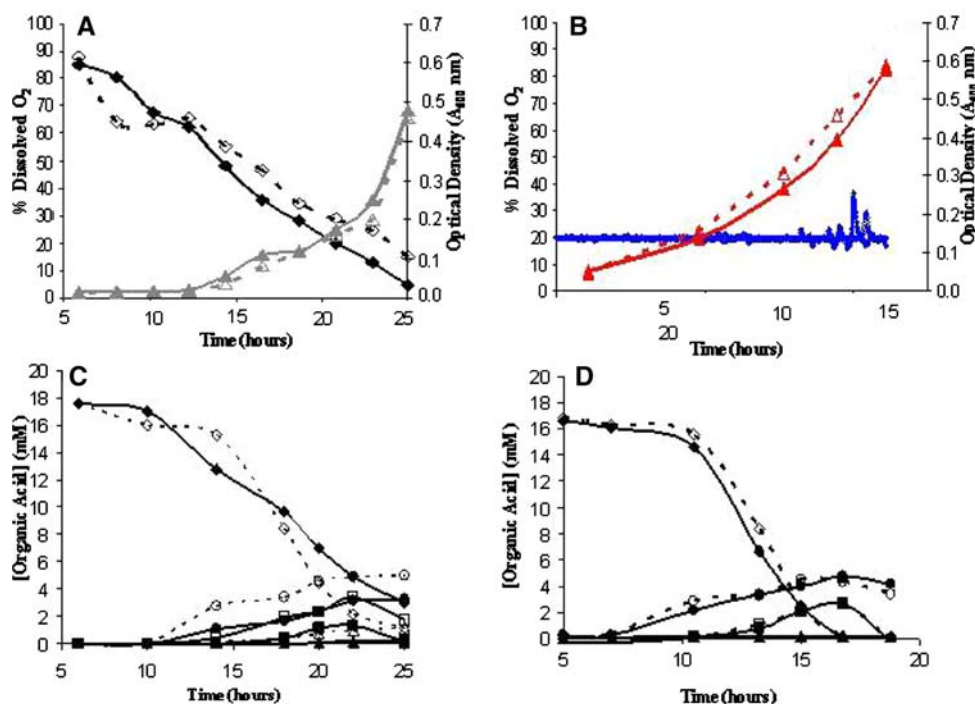
The peptide identifications were determined using the spatially localized confidence scoring (SLiC) algorithm (Norbeck et al. 2005) that incorporates a number of constraints and estimates the confidence of each peptide identification by yielding a score of 0–1. Recent work in our laboratory shows that a SLiC score of at least 0.7 is sufficiently rigorous (unpublished) and is used herein. Further, at least one high-confidence “unique” peptide (i.e., mapping to only one possible parent protein) and a total of two peptides was required for protein identification in each analysis.

Results

Effect of cultivation method on growth and metabolism

Duplicate cultures were grown in; (1) shake flasks (uncontrolled batch) (2) controlled batch and (3) continuous flow reactors operating as steady state chemostats all with O₂ as the sole terminal electron acceptor. Shake flask cultures exhibited an extended lag phase (approximately 13 h), which compared with the shorter lag phase ((5 h) in controlled batch cultures (Fig. 1a, b). The extended lag and slow growth rates ((0.15/h) in shake flasks correlated with elevated DO₂ concentration that gradually decreased from

Fig. 1 A comparison of duplicate uncontrolled versus duplicate controlled (*right*) batch culturing of *S. oneidensis* MR-1. **a** The inconsistency in decreasing DO₂ (*dark filled diamond, open diamond*) and growth (*dark filled triangle, open triangle*) in the uncontrolled cultures is apparent when compared to those for controlled batch cultures (**b**) using the same symbols. This variation is reflected in the (**c, d**) organic acid profiles. The lactate (*dark filled diamond, open diamond*), pyruvate (*dark filled square, open square*) and acetate (*dark filled triangle, open triangle*) all exhibited more similar profiles between culture replicates in the controlled batches



nearly 100% of air saturation to approximately 20% of air saturation (Fig. 1a). Below 20% DO₂ growth rates increased to approximately 0.275/h, which is close to the growth rate observed for controlled batch culture operated at DOT 20% of air saturation (Fig. 1a, b). By definition, chemostat cultures operating at steady state were maintained at a constant growth rate (0.1/h) and 20% DO₂ throughout the course of the experiment (data not shown).

Despite the similarity in growth rates between the duplicate flask cultures (Fig. 1a), the decrease in DO₂, i.e., the O₂ consumption rates, were dissimilar. These differences correlated with variations in the organic acid concentration profiles in shake flask cultures (Fig. 1c). Although the order of appearance and disappearance of the organic acids was the same between the flask culture replicates, the rate of lactate consumption varied significantly as did the transient concentrations of pyruvate and acetate. The differences in these profiles presumably reflect the metabolic variability between cultures at any given point along the growth curve, and may result from increased variability in the mass transfer kinetics in poorly mixed cultures compared to controlled batch.

In comparison, maintaining a constant DO₂ concentration in the controlled batch cultures resulted in a shorter lag phase with nearly identical growth rates (Fig. 1b). Cells in well-mixed controlled batch cultures were continuously exposed to a constant 20% DO₂ concentration, as opposed to variable DO₂ concentrations typical in “aerobic” flask cultures that in fact become O₂-limited as cell densities increase due to poor mass transfer rates. This resulted in

much greater consistency in organic acid concentration profiles between the replicates (Fig. 1d).

Relating cultivation data to metabolic activity

Not only does controlled cultivation result in more consistent metabolic activities within and between cultures as shown above, but the very nature of highly instrumented and controlled cultivation provides important characterization data that help to relate cellular metabolism with the environmental conditions experienced by the culture. In a controlled batch experiment designed to illustrate these relationships, cell growth (Fig. 2a) was correlated with the DO₂ consumption and the amount of acid needed to maintain a constant pH (Fig. 2b), as well as the organic acid profiles (Fig. 2c). Figure 2a illustrates the increase in optical density measured during the course of the experiment. The solid circles indicate optical density of samples taken directly from the culture, while solid squares represent optical density measurements on the same samples after the addition of 10 μM Na-EDTA. This treatment destabilized cell aggregates (flocs) by chelating calcium, which can cross-link polysaccharides in extracellular polymeric substances. The ratio of flocculated:deflocculated cultures is termed the flocculation index (FCI) and is represented by the open triangles. Hence, flocculation indices above 1 indicate the presence of cell aggregates while values at or slightly below 1 indicate the absence of flocculated cells. The cultures lacked flocculated cells during the first 15 h of growth (Fig. 1a). Acid addition (Fig. 2b, red line) during

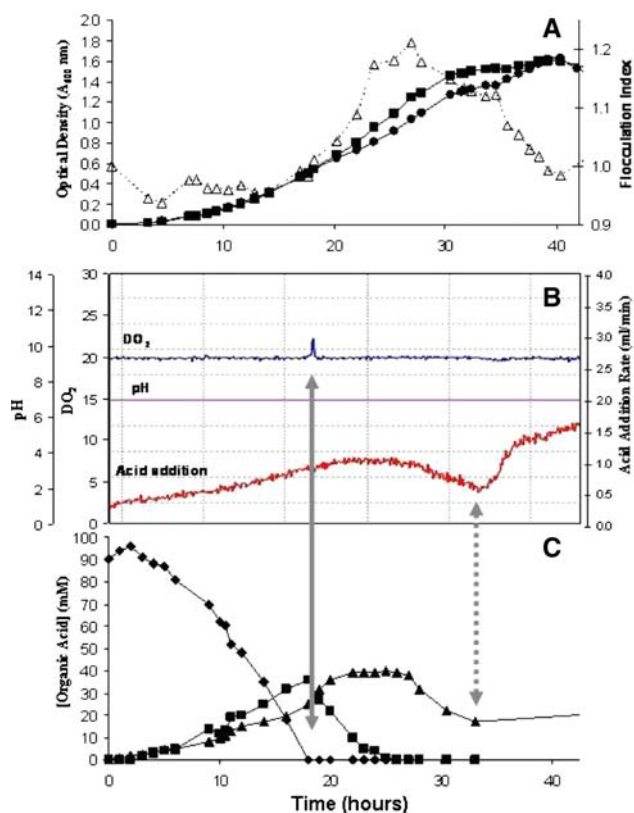


Fig. 2 Alignment of the (a) growth, (b) controlled parameters and (c) organic acid profile for *S. oneidensis* MR-1 cells grown in controlled batch to indicate consequent shifts in metabolism. **a** The growth of flocculated (dark filled square; after EDTA addition), and non-flocculated cells (dark filled circle) were initially compared to profile the degree of flocculation (dark filled triangle) during growth. When coordinated with changes in (b) pH, DO₂ and acid addition and (c) the appearance and exhaustion of the organic acids lactate (dark filled diamond), pyruvate (dark filled square), acetate (dark filled triangle), changes in metabolism can be identified. These include the DO₂ spike (solid arrow) signaling lactate exhaustion with the concomitant increase in cell flocculation as well as the decreased acid addition rate (broken arrow) showing major carbon source exhaustion and the concomitant disappearance of flocculation

this period directly tracked increases in optical density. Lactate served as the primary electron donor and carbon source, and decreased while pyruvate and acetate accumulated in the medium (Fig. 2c).

The point at which lactate was completely consumed (18 h, solid gray arrow) corresponded with a transient spike in DO₂ (Fig. 2b, blue line). This peak resulted from a rapid decrease in O₂ consumption rate and a delayed response of the control loop used by the reactor to maintain constant DO₂ values. The coordinated decrease in respiration rate and the complete consumption of lactate as the primary electron donor also correlated with the onset of flocculation, as indicated by an increased FCI. The organic acid analyses clearly indicated that pyruvate assumed the role of primary electron donor with no apparent change in acetate

concentration. Cell flocculation peaked at ~25 h, just as the last of detectable pyruvate was consumed. The subsequent decrease in the FCI was bimodal. The initial decrease in the FCI corresponded to a slow decline in alkalinity production as measured by decreasing acid addition to maintain pH. When the concentration of acetate fell to ~15 mM and the FCI entered its second phase and decreased sharply, acid addition increased dramatically. This was likely a result of both the decrease in microbial metabolism and the aeration of the bioreactor. During active growth, lactate will be mineralized to both CO₂ and bicarbonate with the CO₂ being stripped from the culture via aeration and bicarbonate modestly increasing the alkalinity of the culture. As the metabolic rate of the culture decreases due to nutrient limitation, CO₂ and bicarbonate generation will also decrease, but the CO₂ will continue to be removed from the system while increasing amounts of bicarbonate will form carbonic acid and CO₂. This will result in a net loss of protons from the system, thus increasing the pH and cause the increased addition of acid to the culture.

Correlating protein expression with culture conditions

Significant differences were observed between the proteomes of cells from uncontrolled “aerobic” flasks and controlled aerobic batch reactors when analyzed by 2DE and MS. In general, proteins predicted to be expressed in cells growing under low oxygen tensions or anaerobically [e.g., fumarate reductase (Myers and Myers 1992; Maier et al. 2003), OmpW, glutamine synthase, decaheme cytochrome *c* MtrA, MtrC, and OmcA (Beliaev et al. 2002, 2005; Fang et al. 2006) were exclusively expressed or detected with increased abundance in samples from shake flasks when compared to those from controlled batch and chemostat cultures. Fumarate reductase (spot 432, SO0970), an enzyme required for anaerobic growth with fumarate (Maier et al. 2003), was detected in high abundance in 2DE patterns of lysate proteins from cells grown in the flask cultures, but was not detected in samples from aerobic controlled batch reactors (Fig. 3). Similarly, the outer membrane protein OmpW (spot 1945, SO1673), and a hypothetical protein (spot 1275, SO3549) were present in 2DE patterns of proteins from flask culture lysates, but not detected in aerobic controlled batch reactors. A significant increase in the number of peptides corresponding to OmpW and fumarate reductase (8.1 × and 6.3 × higher, respectively, in the flask samples compared to controlled batch samples) was observed in the MS-based AMT tag analyses of these lysates (Table 1), confirming the 2DE results. It is presently unclear why OmpW and the hypothetical protein abundances increased in low DO₂, but our laboratory has observed this trend in the former protein several times (unpublished). In addition, peptides representing the multiheme cytochromes

Fig. 3 Two-dimensional gel electrophoresis patterns of whole cell lysate proteins from *S. oneidensis* MR-1 grown in shake flasks or continuous batch cultures. The proteins indicated by *spot number* correspond to those identified as varying with statistical significance between the two different culture methods (see Table 1). The gel images are oriented with the basic proteins to the *right*, acidic proteins to the *left*, high molecular weight toward the *top* and low molecular weight toward the *bottom*. Flask: shake flask cultures (two replicate experiments); CB, controlled batch cultures (two replicate experiments)

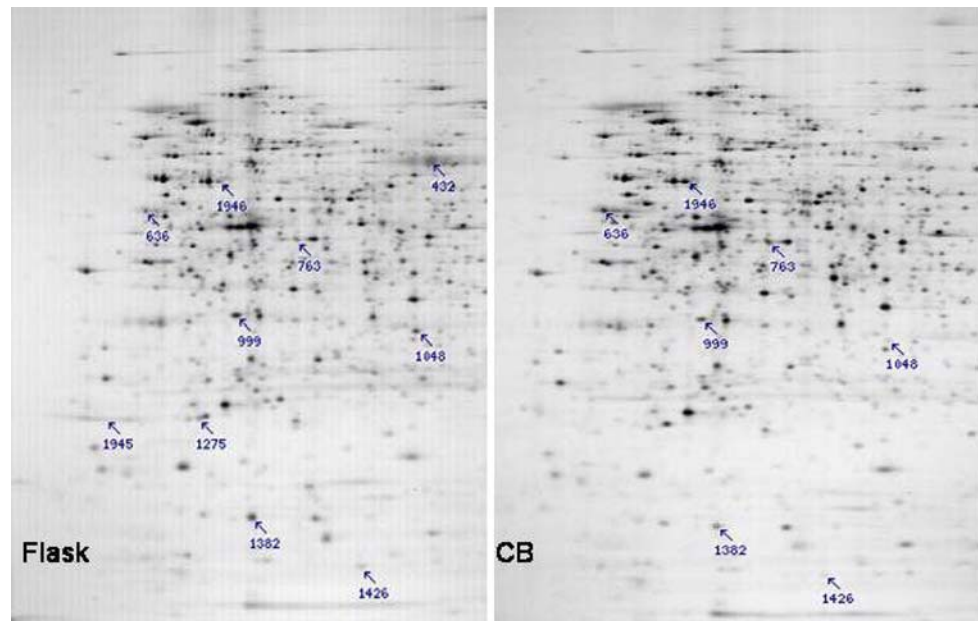


Table 1 Differential protein detection between flask and controlled batch cultures as determined by 2DE and MS

ORF	Protein name	Spot number	2DE				MS		
			Avg flask	Avg CB	<i>P</i> -value	Flask/CB ratio	Avg flask	Avg CB	Flask/CB ratio
SO0970	Fumarate reductase	432	4900	ND	–	–	106 ± 34	17 ± 2 ^b	6.3
SO4349	Ketol-acid reductoisomerase	636	1148	3150	0.003	0.4	22 ± 6	139 ± 26	0.2
SO1931	2-Oxoglutarate dehydrogenase	763	495 ^a	951	0.024 ^a	0.5	36 ± 1	46 ± 4	0.8
SO3237	Flagellin	999	2711	1223	9.9E-05	2.2	108 ± 36	73 ± 14	1.5
–	ND	1048	1196	196	8.8E-04	6.1	–	–	–
SO3549	Hypothetical protein	1275	648	ND	–	–	–	–	–
SO3681	Universal stress protein family	1382	2600	701	3.5E-06	3.7	51 ± 12	13 ± 2	3.9
–	ND	1426	313	ND	–	–	–	–	–
SO1673	OmpW	1945	2927	ND	–	–	62 ± 24	8 ± 1	8.1
SO4410	Glutamine synthetase, type I	1946	1001	1747	1.8E-04	0.6	58 ± 12	63 ± 3	0.9
SO1776	MtrB						7 ± 1	4 ± 1	2.1
SO1777	Decaheme cytochrome <i>c</i> MtrA						8 ± 1	2 ± 0	5.5
SO1778	Decaheme cytochrome <i>c</i> MtrC						36 ± 6	18 ± 1	2.0
SO1779	Decaheme cytochrome <i>c</i> OmcA						21 ± 3	11 ± 1	1.9

ORF open reading frame (www.ncbi.nih.gov/), *Protein name* annotation for open reading frame (www.ncbi.nih.gov/), *Spot number* spot number assigned by Progenesis software using images shown in Figs. 3 and 5, *Avg flask* average integrated spot density in 2DE images (3–4 images per sample) from shake flask samples, *Avg CB* average integrated spot density in 2DE images (3–4 images per sample) from continuous batch culture samples, *P-value* *P*-value from two-tailed Student's *t*-test analysis of average integrated density values, *Flask/CB ratio* ratio of average integrated density values for flask and continuous batch culture samples

^a Note that the difference in spot 763 is a function of the variability in the protein between the two flask cultures. In Flask 1, the average integrated density was 1020 with a coefficient of variation (CV) of 12.5% whereas in Flask 2, the average integrated density was 316 with a CV of 31.6%. The 495 represents the averaging of integrated densities from all of the patterns from these two flasks together with a resulting CV of 60%

^b The average MS abundance data ± the standard deviation resulting from triplicate analysis of each biological sample

MtrA, MtrC, and OmcA and outer membrane β -barrel protein MtrB, were observed in two- to fivefold higher abundance in the AMT tag analysis of samples from shake flasks versus those from controlled batch cultures. These observa-

tions strongly suggest the use of shake flask cultures to assess changes in protein expression patterns related to aerobic versus anaerobic metabolism could yield inaccurate results.

Chemostats provide enhanced experimental consistency

Chemostat cultures provide the highest level of control of any known cultivation technology (Monod 1950; Novick and Szilard 1950; Wimpenny 1985). To evaluate the reproducibility of chemostat cultures, duplicate bioreactors were operated in steady state with *S. oneidensis* MR-1 under 50% DO₂ as above and the growth and carbon metabolism parameters were measured. Each of the aerobic cultures attained an initial maximum optical density of ~0.70 by 20 h and eventually achieved a steady state cell density at 0.53 (A_{600 nm}) (Fig. 4). The two reactors were highly similar in terms of growth and acid addition rates, as well as organic acid concentrations at the time of sampling of 100 h. Lactate, acetate, fumarate and succinate were all below detectable limits suggesting that the cultures were in fact carbon-limited during steady state growth. Pyruvate was present in both chemostatic cultures at 0.14 ± 0.02 mM. Dry weight biomass measurements confirmed the highly similar cell densities of 338.0 ± 4.3 mg/l with only a 1.3% variance between the reactors. In comparison, the duplicate controlled batch and shake flask cultures used in this study yielded similar biomass amounts but varied by 2.3 and 17.6%, respectively. These results support our contention of greater biological variability among replicate flask cultures relative to bioreactor cultures.

Culture and proteome variability

When the intercultural variability of the proteomes of cells from duplicate shake flasks, controlled batch bioreactors, and chemostat cultures was assessed using 2DE and MS,

notable differences were identified. For 2DE analysis, digital images of the gels were processed for spot detection and pattern matching with one 2DE image as a reference pattern and all others matched to it to give the protein spots identification numbers. Statistical analysis of the relative abundance of each matched protein spot across the datasets employed a two-tailed Student's *t*-test as previously described (Giometti and Taylor 1991). Differences in 2DE spot patterns were observed between the replicate flask cultures (Fig. 5a, d), including one unidentified protein (spot 771; *P* < 0.004). In contrast, there was relatively little variability (*P* < 0.05) in 2DE proteome profiles from the replicate gels from either the controlled batch or chemostat cultures.

Data from MS-proteome analysis was consistent with the 2DE results. Triplicate samples from duplicate cultures from shake flask, controlled batch, and chemostats were analyzed and Venn diagrams (Fig. 6) were constructed to illustrate the similarities and differences between biological and analytical replicates based on the cultivation method. A comparison of protein detection consistency between the biological duplicates showed that the flask grown cells (Fig. 5a) exhibit a higher degree of variability. Flask A averaged 972 proteins detected from triplicate analyses while flask B averaged 1,029 proteins with only 770 proteins (77%) in common. The consistency of detected proteins between biological replicates was higher in the controlled batch (84%, Fig. 5c) and chemostat cultures (88%, Fig. 5e). This was not due to a lack of consistency in the analytical replicates since protein detection consistency was between 88 and 89% for all three cultivation methods (Fig. 5d–f). Secondly, there was an overall decrease in the

Fig. 4 *Shewanella oneidensis* MR-1 cultured in duplicate bioreactors at 20% DO₂. The same inoculum culture was used for both bioreactors. Cells were harvested from each reactor when the optical density and organic acid concentrations were similar. The average dry weight biomass was 338 ± 4.3 mg/l

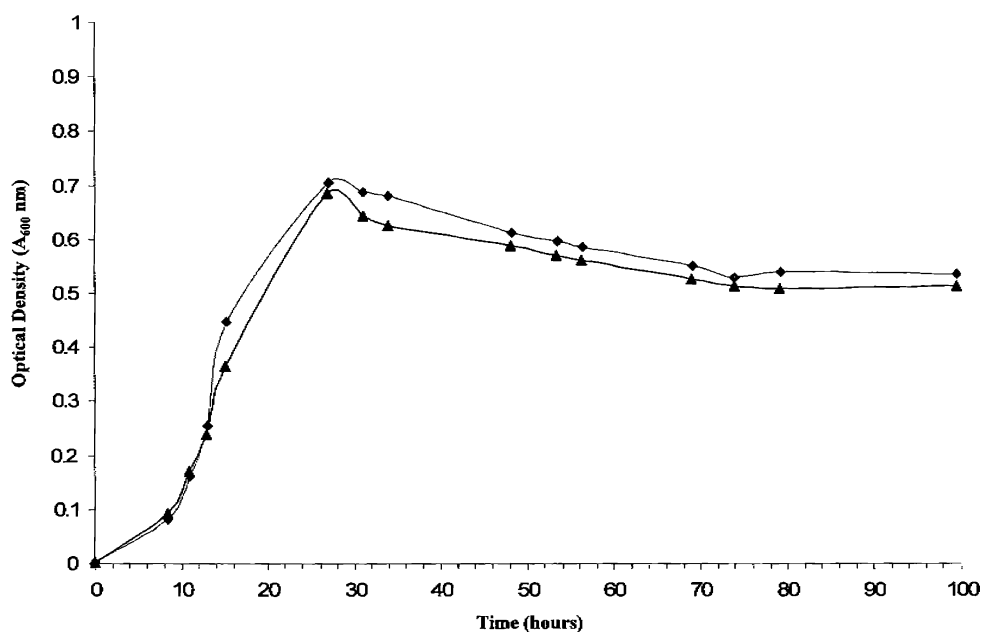
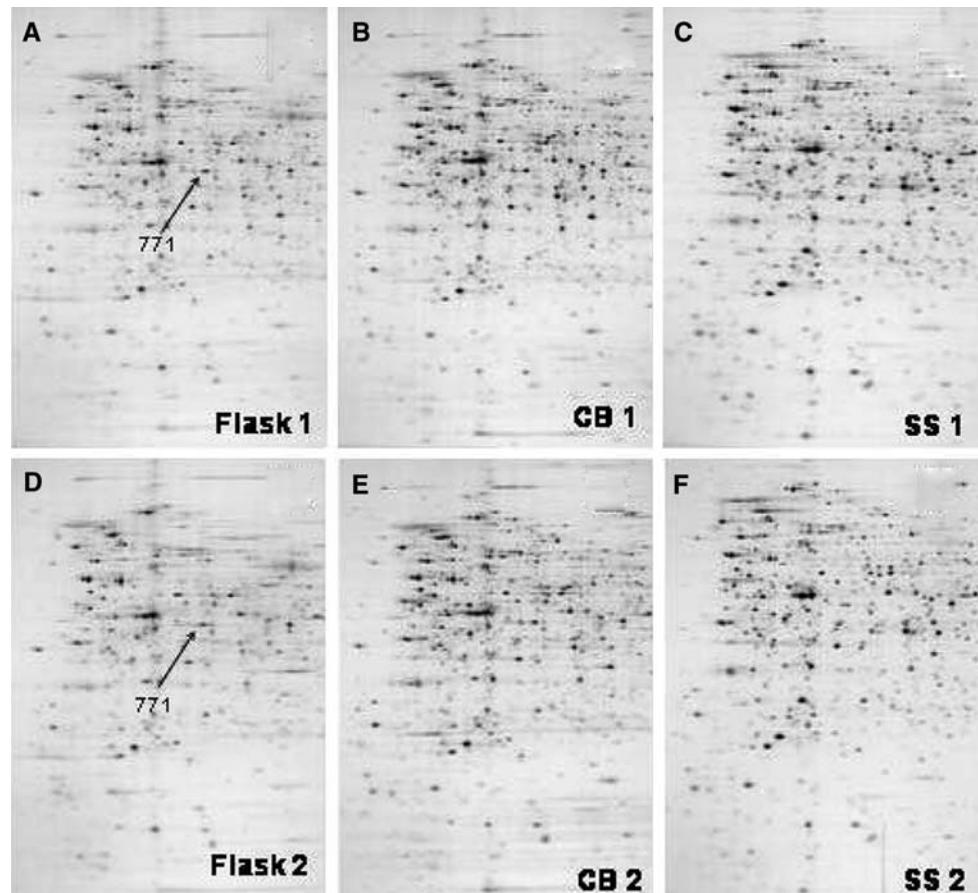


Fig. 5 2DE protein patterns from biological replicates of the three growth methods. The within method replicates show few significant differences. The chemostat and controlled batch replicates showed no quantitative differences with a statistical significance of $P < 0.05$ whereas the flask culture replicates showed one quantitative difference (Spot 771) with a difference of $P < 0.004$. *SS* steady state culture, *CB* controlled batch culture. The gel images are oriented with the basic proteins to the *right*, acidic proteins to the *left*, high molecular weight toward the *top* and low molecular weight toward the *bottom*



number of proteins detected from the flask grown (770) to the controlled batch (713) and to the chemostat (571) cultures. This can be explained by the presumed homogeneity of the culture increasing as the degree of control over pH and DO_2 increased thus allowing the entire culture to experience similar environmental conditions.

Discussion

The advantages of controlled cultivation have been demonstrated for many different research applications in microbiology, including the determination of carbon, nitrogen or electron flow (Vasconcelos et al. 1994; Daran-Lapujade et al. 2004; Kolkman et al. 2005, 2006; Adams et al. 2006), kinetic constants for specific reactions (Nethe-Jaenchen and Thauer 1984), alternate roles for traditionally well-defined enzymes (Snoep et al. 1990), and whole cell protein fingerprinting (Wunschel et al. 2005). While there have been statements touting the advantages of controlled cultivation, an in depth investigation of the differences in metabolism between cultivation methods has not been performed. The closest exception to this reported that microarray results between three chemostatic cultures from three

different laboratories were significantly more reproducible compared to a previous study that performed a similar comparison using shake flask cultures (Piper et al. 2002). However, the authors reported that there were differences in harvesting and other procedures which may well have skewed the findings. To this end, the experiments reported here were specifically directed toward determining whether or not controlled cultivation results in a more homogenous culture with regard to both targeted and comprehensive proteome profiles from three cultivation methods in our laboratory. Our results demonstrate that variations in DO_2 concentration and consumption rates between replicate flask cultures in the late log phase of growth can impact metabolism. This was evidenced by the increased lag time in the uncontrolled flask cultures relative to the controlled cultivations. The data suggests that this may be an effect of oxygen toxicity when DO_2 levels are above 20%. This has been recently tested in *S. oneidensis* MR-1 using sealed culturing vessels and levels above 21% headspace O_2 were found to be inhibitory (McLean et al. 2007). Two possible explanations exist for this observation. It is possible that the extended lag and initial slow growth rate was the result of oxygen toxicity at DO_2 values above 20%. However, the uncontrolled batch cultures contained 30 mM PIPES buffer

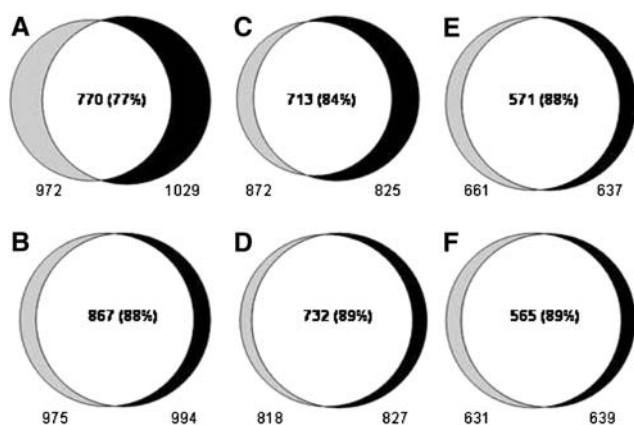


Fig. 6 MS proteomic analysis of the same duplicate (*left*) shake flask, (*middle*) controlled batch and (*right*) chemostat grown cells that were analyzed by 2DE. The consistency in the proteins detected between biological duplicates from shake flask (**a**), controlled batch (**c**) and chemostat (**e**) cultures shows the increased similarity in proteomes with increased control over culturing parameters. This was not an effect of the instrumentation since analytical replicates from each of the shake flask (**b**), controlled batch (**d**) and chemostat (**f**) cultures consistently had (88% of the detected proteins in common. The values below each Venn diagram circle are the number of detected proteins for each biological (**a, c, e**) or analytical (**b, d, f**) replicate. The value in the overlap region is the number of proteins in common between the replicates with the corresponding percentage for ease of comparison. The percentage shown is the number of proteins in common divided by the average of the two replicates presented

to aid in the maintenance of pH during growth while the controlled batch and chemostatic cultures contained only 3 mM PIPES. Hence, there would have been a large difference in the ionic strength of the two media and so while DO_2 toxicity may be the reason for the extended lag phase, other reasons cannot be ruled out. Separately, differences in acetate concentrations of the replicate shake flask cultures compared to controlled batch and chemostat cultures indicated that such differences may well have promoted additional biological variability.

The difference in acetate concentrations between replicate shake flask cultures compared to the consistent acetate concentrations in controlled batch and chemostat cultures has particular implications for *S. oneidensis* strain MR-1. MR-1 can use lactate and pyruvate as an electron donor during anaerobic growth, but not acetate (Lovley et al. 1989; Neelson and Myers 1992). Thus, variations in the concentration of acetate and the variable DO_2 uptake rates in shake flask cultures will cause a temporal shift in carbon source usage and electron flux. Such shifts can be expected to result in variable metabolic, transcriptomic and proteomic profiles between independent shake flask cultures if only a small number (1–3) of samples are taken, since one culture may have exhausted a carbon source and consequently shifted metabolism and electron flow while the “biological replicate” has not.

Given the general metabolic flexibility of bacteria including *S. oneidensis* MR-1 and their ability to respond rapidly to changes in environmental conditions, changes in the expressed proteins (proteome) would be expected as a result of exposing cultures to different environmental conditions. Through the use of comprehensive proteome measurements, the results presented here demonstrate that chemostat cultures provide the most consistent source of cellular material for biochemical measurements involving microbial cells with a minimum contribution to those measurements from biological variation. Two independent methods of proteome measurement, 2DE and AMT MS, both showed that flask cultures yielded protein samples with more culture-to-culture variability than observed in either controlled batch cultures or continuous chemostat cultures, primarily among proteins thought to be associated with anaerobic or microaerobic metabolism. A similar effect was found with respect to differential regulation of genes via microarrays when the results of two different investigations were compared from yeast cells, showing less discrepancy between chemostatic cultures (Daran-Lapujade et al. 2004).

Many of the proteins present in higher abundance in the aerobic shake flask cultures relative to the controlled batch or chemostat cultures in this study have previously been reported to be up-regulated in anaerobic compared to aerobic cultures (Beliaev et al. 2002, 2005; Fang et al. 2006). MtrA,B,C and OmcA are involved in metal-reduction in anaerobic cultures supplied with metals as terminal electron acceptors (Beliaev and Saffarini 1998; Beliaev et al. 2001; Myers and Myers 2002; Pitts et al. 2003) while the outer membrane protein OmpW and fumarate reductase have both been observed to be significantly more abundant in anaerobic cultures supplied with iron or fumarate as electron acceptor than in aerobic cultures (Fang et al. 2006). Thus, the shake flask cultures in the experiments described here must have included cells experiencing a lack of oxygen due to the nutrient depletion that is an inherent complication of such cultures, resulting in a greater range of expressed proteins.

Achieving a systems-level understanding of microorganisms, including the underlying metabolic and regulatory networks that control cell physiology and the ability to predict responses to perturbations, is indeed an ambitious goal. Systems biology takes full advantage of the state-of-the-science technology and genomic information, with the scientific as well as the practical benefits expected to be plentiful. Our results emphasize that functional genomics can greatly benefit from well-defined and homogeneous cell cultures provided by controlled cultivation techniques. The use of controlled cultivation techniques ensures that the large and comprehensive datasets used in systems biology research are derived from well-characterized and homogeneous

biological samples, thus reducing the inherent variability that often complicates drawing biological conclusions and will allow for more accurate and robust identifications of metabolic and regulatory networks.

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