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ORIGINAL PAPER

# Cytoplasmic membrane fluidity and fatty acid composition of *Acidithiobacillus ferrooxidans* in response to pH stress

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**Abstract** Strain variation in the acidophile *Acidithiobacillus ferrooxidans* was examined as a product of membrane adaptation in response to pH stress. We tested the effects of sub and supra-optimal pH in two type strains and four strains isolated from acid mine drainage water around Sudbury, Ontario, Canada. Growth rate, membrane fluidity and phase, determined from the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene, and fatty acid profiles were compared. The effect of pH 1.5 was the most pronounced compared to the other pH values of 1.8, 3.1, and 3.5. Three different types of response to lower pH were observed, the first of which appeared to maintain cellular homeostasis more effectively. This adaptive mode included a decrease in membrane fluidity and concomitant depression of the phase transition in two distinct membrane lipid components. This was explained through the increase in saturated fatty acids (predominantly 16:0 and cyclopropane 19:0 w8c) with a concomitant decrease in 18:1 w7c fatty acid. The other strains also showed common adaptive mechanisms of specific fatty acid remodeling increasing the abundance of short-chain fatty acids. However, we suspect membrane permeability was compromised due to potential phase separation, which may interfere with energy transduction and viability at pH 1.5. We demonstrate that membrane physiology permits differentiating pH tolerance in strains of this extreme acidophile.

**Keywords** Acidophile · Physiology · Cytoplasmic membrane · Fatty acids · *Acidithiobacillus ferrooxidans* · Membrane fluidity

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## Introduction

*Acidithiobacillus ferrooxidans* is an obligate acidophile commonly found in acid mine drainage (AMD) environments and is also used as a bioleaching agent in the recovery of metals (Leduc and Ferroni 1994; Rawlings 2002). Probably the most widely studied obligate acidophile, *A. ferrooxidans* has been described as tolerating conditions as low as pH 1.0 and as high as pH 6.0, although it is generally agreed that the optimal pH for growth of *A. ferrooxidans* is about 2.0 (Leduc and Ferroni 1994). The variation in range is due to strain heterogeneity and specific growth conditions (Kondrat'eva and Karavaiko 1997) and strains are also known to adapt rapidly to sub-optimal conditions. In both uncontrolled/natural (tailing, mines and mine waste) and bioleaching systems, the pH can affect the overall bacterial activity and resulting leaching rates. Improved knowledge of *A. ferrooxidans* strain adaptation is an important aspect in understanding the intrinsic chemiosmotic kinetics of bacterially mediated bioleaching.

In acidophiles, bioenergetics involves the creation of a proton motive force driven primarily by the large pH

gradient (ΔpH) and to a lesser extent the membrane potential ($\Delta\psi$) which leads to the generation of ATP by the influx of protons across the membrane (Matin 1990; Baker-Austin and Dopson 2007). The cytoplasmic membrane is a crucial permeability barrier that prevents the uncontrolled influx of protons into the cell without which energy transduction could not occur. As numerous factors can compromise the integrity, permeability, and fluidity of the cytoplasmic membrane, mechanisms for maintaining the proton motive force must also be adapted to respond to membrane perturbations during pH stress. In acidophiles, mechanisms for pH homeostasis have structural and functional aspects including the reversed $\Delta\psi$ (negative outside vs. positive inside), highly impermeable cell membranes, and an abundance of secondary transporters (reviewed in Baker-Austin and Dopson 2007). Several studies have reported on the importance of membrane stability and structure for maintaining bioenergetics and survival in acidic environments (Driessen et al. 1996; van de Vossenberg et al. 2000; Konings et al. 2002). A model theory for the homeostasis of proton permeability has been termed the "homeo-proton permeability adaptation" and is maintained constant by controlling membrane lipid composition (Konings et al. 2002). It has been shown that membrane properties might change not simply to maintain optimal membrane fluidity, what is known as homeoviscous adaptation, but rather to maintain the proton permeability of the cytoplasmic membrane at a nearly constant level (van de Vossenberg et al. 2000; Konings et al. 2002).

The understanding of bioenergetics in acidophiles like *A. ferrooxidans* has found some confusion and contradiction in the literature, and continues to challenge classic bioenergetic theory (Ferguson and Ingledew 2008). $H^+$ homeostasis is maintained with a steep proton gradient of about 4.5 across the cytoplasmic membrane while maintaining a near-neutral internal pH ($\sim$6.5) (Cox et al. 1979). The mechanisms for pH homeostasis in *A. ferrooxidans* rely in part on an active maintenance of the proton motive force, although the pH gradient may or may not be energy-dependent as inactive cells appear to maintain the Donnan potential; the electrical potential formed across a membrane without the transmission of current (Cox et al. 1979; Zychlinsky and Matin 1983; Baker-Austin and Dopson 2007). Active maintenance of the ΔpH in *A. ferrooxidans* involves proton extrusion across the cytoplasmic membrane through the action of cytochrome *c* oxidase, however, the pumping activity may involve a different stoichiometry of charge separation than in classical proton pumping models (Ferguson and Ingledew 2008). Any disruption of the $H^+$ pumping capacity or collapse in the membrane potential can significantly increase the influx of protons into the cells (Matin 1990). In Bacteria, the buffering capacity of the cytoplasm may provide some defense against such an influx but this may provide only transient protection, especially in acidophiles (Booth 1985; Matin 1990). Rather, it is the maintenance of an inverted membrane potential (positive inside) by a Donnan potential, typically created through the uptake of potassium that more effectively inhibits the influx of protons by creating a chemiosmotic barrier (Matin 1990; Suzuki et al. 1999; Baker-Austin and Dopson 2007). In *A. ferrooxidans* a few other cellular mechanisms may play a role in maintaining homeostasis in response to pH change. In similar studies, Jerez et al. (1988) reported that a shift to more acidic pH induced the synthesis of heat-shock proteins while Amaro et al. (1991) showed the same pH conditions induced synthesis of a porin-like protein in the outer membrane. The authors raised the question whether these observed responses to pH stress were a direct result of the pH changes or whether they occurred as a result of membrane changes, such as polarization, triggered by external pH changes (Amaro et al. 1991). In a recent study of *A. ferrooxidans* type strain 23270, Chao et al. (2008) reported the differential expression of genes involved in cell membrane structure, transcriptional regulation, and signal transduction in response to pH stress for (pH 1.3) versus optimal values (pH 2.3). The authors suggested that the structure and composition of the cell envelope was likely being modified to decrease proton permeability and mediate pH-induced damage (Chao et al. 2008). The cell envelope of *A. ferrooxidans* and other acidophilic chemolithotrophs are thought to be similar to neutrophilic Gram negative bacteria, however, some studies have show that uncommon membrane properties, such as an overall positive charge may be inherently linked with acidophilic growth (Kar and Dasgupta 1996). Despite the extensive research on *A. ferrooxidans*, little attention has been given to the specific adaptation of the *A. ferrooxidans* cytoplasmic membrane in response to pH.

As the best characterized iron-oxidizing acidophile, *A. ferrooxidans* is a good model for this type of research and the results would help elucidate the mode of adaptation in extremophilic bacteria to stresses such as low pH. In this study growth rate, cytoplasmic membrane polarization, as a measure of cytoplasmic membrane fluidity, the transition temperature midpoints ($T_m$), and fatty acid composition were investigated in six strains of *A. ferrooxidans* in response to sub- (1.5 and 1.8) and supra- (3.1 and 3.5) optimal pH. The overall purpose of the research was to determine whether changes in the membrane characteristics contributed to the ability of *A. ferrooxidans* to tolerate and maintain active metabolism under pH stress. The aim of our research was twofold. The first goal was to determine whether there were differences in the adaptation mechanisms of *A. ferrooxidans* strains that differ phenotypically. The second goal was to determine whether there

was a common adaptation mechanism in *A. ferrooxidans* related to cytoplasmic membrane properties when exposed to variable pH. This study represents the first investigation of an otherwise well-characterized acidophile, providing data on cytoplasmic membrane adaptation in different strains of *A. ferrooxidans* under variable pH conditions. This data will provide additional insight into strain variation within this species, helping to identify reasons for the range of pH over which *A. ferrooxidans* can carry out biooxidation.

## Methods

### Strain selection and characteristics

Experimental analyses were performed using six strains of *A. ferrooxidans*. Four previously isolated strains include D6 and D7 isolated from a uranium mine at the Denison Mines Ltd. and R1 from Rio Algom (Elliot Lake, Ontario, Canada), as well as F1 isolated from tailings at the Falconbridge Nickel Mines Ltd. (now Xstrata Nickel, Falconbridge, Ontario, Canada). These strains were used in our research of various stressors and were chosen on the basis of notably different resistance to certain metals and growth temperatures. Since no previous study of pH-mediated strain variation has been reported, we are comparing the same set of strains for study. *A. ferrooxidans* type strains ATCC 19859 and 23270 were also studied for the effect of pH and used to compare the traits of the lab-adapted type strains to the environmental isolates.

### Experimental conditions and growth rate

All six strains of *A. ferrooxidans* were grown in the liquid medium of Tuovinen and Kelly (1973), TK medium (composition in g/L): $FeSO_4{\cdot}7H_2O$ (33.4). $(NH_4)_2SO_4$ (0.4) $MgSO_4{\cdot}7H_2O$ (0.4), $K_2HPO_4$ (0.4), and adjusted for pH with concentrated $H_2SO_4$. The control for all experiments consisted of a TK culture grown at pH 2.1 and incubated at room temperature (25.0 ± 0.5°C). Shaking speeds varied such that 1 L cultures that were used in fatty acid analyses were incubated on a gyratory shaker at 130 rpm while 40 mL cultures used in fluorescence polarization analyses were incubated at 180 rpm. The experimental values used to test pH stress were below or above the optimal value of pH 2.1, at pH 1.5 and 1.8, or pH 3.1 and 3.5, respectively. The pH stress culture media were adjusted to the desired pH using concentrated $H_2SO_4$ and/or NaOH (20% w/v).

The generation times for each strain were determined under control and experimental conditions to determine the exponential phase of growth prior to further analyses. Due to the production of ferric iron precipitates that prevent common optical density based growth assays, an indirect measure of *A. ferrooxidans* growth rate was determined as follows. Assuming that during the exponential growth phase of *A. ferrooxidans* there is a concomitant exponential transformation of ferrous to ferric iron, the concentration of ferric iron was used as a measure for growth and was determined using the spectrophotometric method described by Karamanev et al. (2002). This method was initially validated by comparing the ferric iron oxidation rate to changes in viable cell numbers using a standard 3-tube most probable number (MPN) quantification. The iron oxidation rate for each strain was determine from triplicate flasks containing 40 mL liquid TK that were inoculated with 1.0 mL exponentially growing cells [average cell count (MPN): $1.2 \times 10^7$] on a gyratory shaker at 180 rpm. The rate of ferrous iron oxidation was determined at regular time intervals by combining a 1.0 mL aliquot of culture with 3 mL of 10% sulfosalicylic acid and brought to 200 mL with distilled water. Absorbance was measured at 500 nm with a Beckman DU-65 Spectrophotometer (Beckman Coulter, California, USA) until no further changes in absorbance were observed. Growth curves were generated by calculating the natural logarithm of the ferric iron concentration from the equation: $A = 0.04 \times C_{Fe^{3+}}$ where $A$ is the absorbance and $C$ the concentration of ferric iron (in mg/L), and plotting these values over time (h) (Karamanev et al. 2002). Generation times were determined by plotting a linear regression through the points exhibiting linearity and the slope ($m$) was then used to calculate generation time ($t_g$), ($t_g = 0.693/m$) (Leduc et al. 1993).

### Fluorescence polarization analysis

The analysis was performed as described by Vincent et al. (2004) with a few modifications. *A. ferrooxidans* cultures were vortexed vigorously and were then allowed to stand for 10 min to settle bulk iron precipitates. The cultures were then decanted and were filtered through Whatman No. 1 filters to remove the finer precipitates. Cells were harvested from the filtered liquid cultures by centrifugation at 10,000×$g$ for 10 min at 5°C, washed three times in 0.01 N $H_2SO_4$. Washed cells were re-suspended in wash solution to an OD 600 nm of 0.2. All further analyses were performed as previously described and outlined here for clarity. A 3.0 mL aliquot of the re-suspended cultures were transferred to a quartz cuvette to which 1.0 µL of 12 mM stock solution DPH in tetrahydrofuran (Molecular Probes, Eugene, Oregon, USA) was added and incubated in the dark for 10 min at room temperature (25°C) to allow the probe to incorporate into the cytoplasmic membrane (Trevors 2003). Measurement of the fluorescence polarization was performed using a

polarization spectrofluorometer equipped with a thermostated cell-holder and magnetic stirrer (Photon Technology International Inc., London, Ontario, Canada). Excitation and emission wavelengths were set to 358 and 428 nm, and the slits widths set to 10 and 12 nm, respectively. Polarizers were set to either the horizontal or vertical positions and amplifiers for the polarized light were set to 1005 and 1040 V, respectively. Data were recorded using FELIX software (Version 1.4 and 3.2 Photon Technology International Inc.).

Single polarization measurements were determined from a 15 s scan of the sample held at the same temperature used during culturing. The degree of polarization was determined from the polarization ratio ($P$, unitless) as described by Shinitzky and Barenholz (1978):

$$P = (I_{VV} - I_{VH}G)/I_{VV} + I_{VH}G$$

where $I_{VV}$ and $I_{VH}$ are the light intensities emitted in the vertical and horizontal directions relative to the beam of excitation (Trevors, 2003). The grating factor ($G$), $[G = (I_{HV}/I_{HH})]$ is instrument dependent and accounts for the different sensitivity of photomultipliers to polarized light measured as the intensities of emitted beams in the vertical ($I_{HV}$) and horizontal ($I_{HH}$) directions when the excitation beam is oriented vertically (Borenstain and Barenholz, 1993). The relationship between fluorescence polarization and membrane fluidity is an inverse one, where increasing polarization ratio values correspond to a more rigid membrane environment and vice versa. The temperature transition midpoints ($T_m$) were determined from a continuous measurement of a sample subjected to a temperature ramp (0–65°C) until the membrane was melted. Temperature transition points are defined as changes in slope of the transition temperature curve. All values are reported as means of triplicate analyses for each experimental variable. Samples of *Escherichia coli* grown in 1/10 strength Nutrient Broth (NB) (Difco, St. Louis, USA) for 24 h at 37°C were used as a positive control for polarization analyses typically having $P$ values of about 0.30 (Vincent et al. 2004).

### Fatty acid composition

Fatty acid composition was determined for cultures grown at control and pH 1.5 only. Each sample consisted of 40–50 mg wet cell weight isolated from TK cultures. The analysis was performed at the Laboratory Services Division at the University of Guelph (Guelph, Ontario, Canada). Their method includes total fatty acids extraction, saponification, methyl esterification, and analysis of the fatty acid methyl esters (FAMEs) by GC-FID using the Microbial ID Inc. (MIDI, Newark, Delaware, USA) microbial identification system as described in Kim et al. (2002).

Fatty acid profiles were organized in simplified groups by chain length and degree of saturation as well as diversity according to the type of branching, cyclization or hydroxylation present in the fatty acids. Relative proportions of fatty acids were determined for each sample as a ratio of saturated to unsaturated fatty acid at each growth condition.

### Statistical analyses

Statistical analyses for fluorescence polarization measurements were determined for comparisons in the responses within each strain to different stresses, and between strains for the same stress using an analysis of variance (ANOVA) followed by a Student–Newman–Kuells (SNK) multiple range test ($\alpha = 95\%$) using Statistica 6.0 (StatSoft Inc. Tulsa, OK, USA) and KaleidaGraph 4.0 (Synergy Software, Pennsylvania, USA). Experiments were performed in triplicate unless otherwise stated.

Percent fatty acid composition of cultures grown at pH 1.5 were compared to the control values (pH 2.1) using an ANOVA followed by an SNK multiple range test ($\alpha = 95\%$) for each strain. All fatty acid profiles were determined in triplicate from three biological repeats with the exception of the type strains for which only two samples were analyzed per growth condition and a single sample for strain 19859 grown at 25°C.

Discriminant function analysis (DFA) was used to determine which fatty acids distinguish between optimal and sub-optimal pH exposed profiles. Log-transformed percent fatty acid data $\log_{10}(x + 1)$ was used in a DFA (Statistica 6.0, StatSoft Inc. Tulsa OK, USA) using the optimal values and pH 1.5 profiles as groups. Two-thirds of the data was chosen for the initial DFA with the remaining fatty acid profiles used for cross-validation of the resulting functions. For pH 1.5 exposure, correlation-based models of fluorescence polarization data using fatty acid composition ($\log_{10}$ transformed) as predictor variables were determined through factor analysis followed by multiple regression (Statistica 6.0, StatSoft Inc. Tulsa, OK, USA).

## Results

### Effect of pH on growth rate

Generation time was used as a measure of total cellular activity in response to pH stress. Growth curves, based on the rate of iron oxidation ($Fe^{2+} \rightarrow Fe^{3+}$), were plotted for each strain and the slope of the exponential phase of growth was used to determine generation time as shown for strain R1 in Fig. 1. This method accurately reflected viable cell counts (MPN) that were initially determined to validate

the method (Fig. 1a). Generation times for cultures grown at 25 ± 0.5°C, pH 2.1 (optimal/control conditions) were similar for the environmental strains (D6, D7, F1, R1) but were significantly different ($p < 0.05$) from the type strains as determined in a related study (Table 1) (Mykytczuk et al. 2007, 2010a, b). Conversely, under pH stress the generation times increased for both sub- and supra-optimal pH with more acidic pH values causing significantly slower growth for all strains ($p < 0.05$) (Table 1). Strain D6 and F1 also showed significantly longer generation times at pH levels of 3.1 and 3.5. In comparing the strains, differences were observed in the generation times under all pH stress conditions. For pH levels just below optimal (pH 1.8) and higher (pH 3.1 and 3.5) the generation times ranged from 9 to 14 h per population doubling. Strain variation was most apparent at pH 1.5 with responses ranging from 14 h for strain R1 to nearly 40 h per generation in strain D7 ($p < 0.05$).

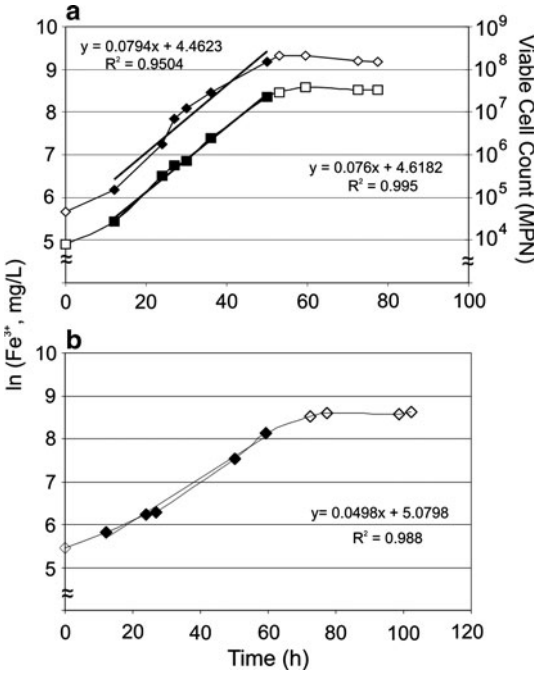


**Fig. 1** Example of growth curves for strain R1 under (**a**) control (pH 2.1), and (**b**) pH stress (pH 1.5) conditions. *Filled squares* represent the exponential phase of growth. Viable cell counts (MPN) agree with the iron oxidation determination as shown in **a** denoted by the (*filled diamonds*) curve

## Fluorescence polarization: cytoplasmic membrane fluidity and phase transition

Membrane fluidity and phase characteristics for pH stress were measured using viable DPH-labelled cells and compared to control values. We have reported that under optimal growth conditions the fluorescence polarization ratios ($P$) for the six environmental strains were inherently different with values ranging from 0.364 ± 0.007 to 0.424 ± 0.008 (Table 2) (Mykytczuk et al. 2010b). With pH stress, the general trend in $P$ values indicated that most of the strains either maintained near-optimal membrane fluidity values or experienced a significant increases in $P$ values, corresponding with a decrease in membrane fluidity ($p < 0.05$) (Table 2). In general, for all of the strains the $P$ values diverged from optimal values to a greater extent as the pH deviated further away from the optimal value of 2.1, and this trend was more pronounced as pH values decreased to 1.5. Specifically, for pH exposures of 1.8, 3.1 and 3.5, the majority of the strains showed moderate changes in $P$ values ranging from around 0.37–0.40 ($p < 0.05$). Strains D6 and D7, however, showed no statistical difference in $P$ values across all pH values although the change in $P$ values was greatest at pH 1.5. In the remaining strains, we noted that the $P$ values at pH 1.5 significantly increased compared to optimal levels but were similar among all strains ranging from 0.410 ± 0.019 for strain D6 to 0.466 ± 0.030 in strain 23270 ($p > 0.05$). Type strain 23270 was the only strain to show significantly higher $P$ values for all sub- and supra-optimal pH values

**Table 1** Mean generation times ($n = 3$ ± SD) of *A. ferrooxidans* strains grown at variable pH

| Strain | Control (25°C, pH 2.1) | pH | | | |
|---|---|---|---|---|---|
| | | 1.5 | 1.8 | 3.1 | 3.5 |
| D6 | $8.4 \pm 0.6^{1,a}$ | $28 \pm 1.4^{2,a}$ | $9.5 \pm 0.7^{1,a}$ | $9.8 \pm 0.2^{1,a}$ | $13 \pm 3.0^{3,a}$ |
| D7 | $9.5 \pm 1.8^{1,a}$ | $38 \pm 5.1^{2,b}$ | $9.5 \pm 0.3^{1,a}$ | $8.6 \pm 1.3^{1,b}$ | $9.6 \pm 0.1^{1,b}$ |
| F1 | $8.4 \pm 0.2^{1,a}$ | $18 \pm 1.2^{2,c}$ | $9.7 \pm 0.1^{1,a}$ | $11 \pm 0.1^{3,a}$ | $14 \pm 1.8^{4,a}$ |
| R1 | $9.1 \pm 0.3^{1,a}$ | $14 \pm 1.8^{2,c}$ | $11 \pm 1.4^{1,b}$ | $10 \pm 0.2^{1,a}$ | $10 \pm 0.8^{1,b}$ |
| 19859 | $11 \pm 0.3^{1,b}$ | $17 \pm 0.9^{2,c}$ | $11 \pm 0.5^{1,b}$ | $11 \pm 0.5^{1,a}$ | $10 \pm 0.8^{1,b}$ |
| 23270 | $14 \pm 0.4^{1,c}$ | $29 \pm 0.7^{2,a}$ | $14 \pm 0.5^{1,c}$ | $14 \pm 0.3^{1,c}$ | $14 \pm 0.2^{1,a}$ |

Mean generation time (h ± SD)

Generation times that are significantly different at the (95% level) by way of ANOVA and an SNK multiple range test are followed by a different number superscript (same strain comparison) or letter superscripts (inter-strain comparison)

**Table 2** Mean fluorescence polarization ratios ($P \pm$ SD, $n = 3$) for six *A. ferrooxidans* strains grown at sub and supra-optimal pH

| Strain | Control (25°C, pH 2.1) | pH stress | | | |
|---|---|---|---|---|---|
| | | 1.5 | 1.8 | 3.1 | 3.5 |
| D6 | $0.380 \pm 0.006^{1,a}$ | $0.410 \pm 0.019^{1,a}$ | $0.385 \pm 0.011^{1,a}$ | $0.400 \pm 0.036^{1,ab}$ | $0.399 \pm 0.019^{1,a}$ |
| D7 | $0.397 \pm 0.003^{1,b}$ | $0.418 \pm 0.014^{1,a}$ | $0.395 \pm 0.002^{1,a}$ | $0.400 \pm 0.002^{1,ab}$ | $0.387 \pm 0.020^{1,a}$ |
| F1 | $0.364 \pm 0.007^{1,c}$ | $0.428 \pm 0.033^{2,a}$ | $0.399 \pm 0.015^{2,a}$ | $0.374 \pm 0.023^{1,a}$ | $0.379 \pm 0.004^{1,a}$ |
| R1 | $0.378 \pm 0.008^{1,a}$ | $0.419 \pm 0.009^{2,a}$ | $0.376 \pm 0.003^{1,a}$ | $0.371 \pm 0.027^{1,a}$ | $0.401 \pm 0.006^{2,a}$ |
| 19859 | $0.424 \pm 0.008^{1,d}$ | $0.442 \pm 0.008^{2,a}$ | $0.441 \pm 0.026^{1,b}$ | $0.414 \pm 0.006^{1,ab}$ | $0.416 \pm 0.032^{1,a}$ |
| 23270 | $0.404 \pm 0.010^{1,b}$ | $0.466 \pm 0.030^{2,a}$ | $0.442 \pm 0.014^{2,b}$ | $0.437 \pm 0.008^{2,b}$ | $0.453 \pm 0.017^{2,b}$ |
| Percent change[A] | | | | | |
| D6 | – | 8.0 | 1.3 | 5.2 | 5.0 |
| D7 | – | 5.2 | 1.0 | 1.0 | 2.5 |
| F1 | – | 18 | 7.1 | 2.7 | 4.1 |
| R1 | – | 11 | 1.0 | 1.9 | 6.1 |
| 19859 | – | 4.2 | 4.0 | 2.4 | 1.9 |
| 23270 | – | 15 | 9.4 | 8.2 | 12 |

Polarization values that are significantly different at the (95% level) by way of ANOVA and an SNK multiple range test are followed by a different number superscripts (same strain comparison) or letter superscripts (inter-strain comparison for same pH level). Multiple letter superscripts indicate value is not significantly different from values corresponding to either letter

[A] Calculated as the absolute value of the expression $(P_{1.5} - P_{2.1}/P_{2.1}) \times 100\%$

and these values were also the highest values recorded among all of the strains. Looking at the percent change we observed that the large change in the $P$ values at pH 1.5 ranged from 4.2% in strain 19859 to 18% in strain F1. A percent change of $\sim$10% in the fluorescence anisotropy ($r = 2P/3 - P$) reflects a greater change in the apparent microviscosity of the membrane by about $\sim$25% (Shinitzky and Barenholz 1978).

The gel-to-liquid-crystalline transition temperature midpoint ($T_m$) analyses indicated how the membrane phase was either being affected directly by the change in extracellular pH, or through active remodeling of the membrane structure by the cell. The phase transition curves were determined over a temperature ramp of 0–55°C for each strain and the changes in slope, or $T_m$, were determined as illustrated for strain R1 in Fig. 2. As with the fluorescence polarization data, the $T_m$ were maintained near-optimal values or decreased in response to pH stress (Table 3). With the exception of two environmental strains, D6 and D7, all other strains showed multiple transition points at pH 1.5 that would indicate active remodeling of the membrane but also the potential for phase separation. In these strains, the significantly lower $T_m$ point occurred about 20°C below optimal values ranging between 2.0 and 6.4°C while the second $T_m$ remained near-optimal values. The single depressed $T_m$ value in strains D6 and D7, occurred at 6.7 and 9.7°C, respectively, also suggesting active membrane remodeling but does not indicate phase separation occurred in response to pH 1.5. As with the fluorescence polarization data, type strain 23270 was the only strain to deviate from

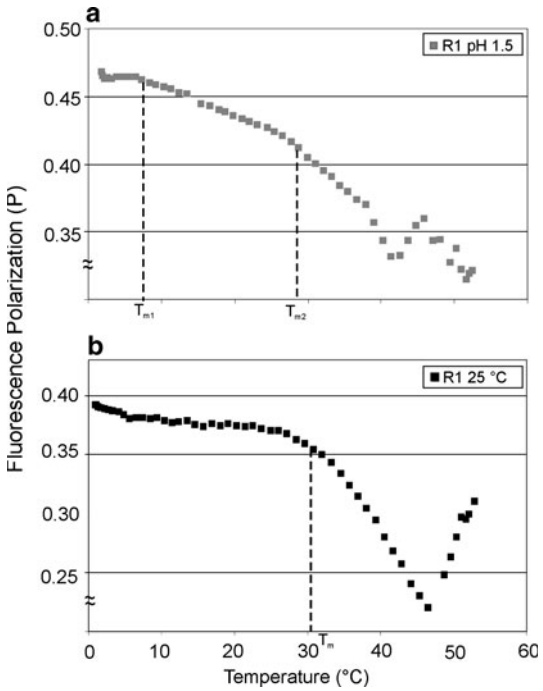


**Fig. 2** Transition temperature ($T_m$) curves for strain R1 grown at (**a**) pH 1.5 and (**b**) pH 2.1. The *dashed lines* represent the transition temperature midpoints

the general trends, showing significant increases in $T_m$ values several degrees higher than the 25°C growth temperature, around 34–39°C in response to all pH levels, which indicates a significant proportion of gel-phase lipids in the membrane. These higher $T_m$ values also correspond

**Table 3** Transition temperature midpoints ($T_m$, °C) for *A. ferrooxidans* strains at different pH levels

| Strain | Control (25°C, pH 2.1) | pH | | | |
|---|---|---|---|---|---|
| | | 1.5 | 1.8 | 3.1 | 3.5 |
| D6 | 28 | 6.7 | 22 | 20 | 19 |
| D7 | 33 | 9.7 | 28 | 29 | 24 |
| F1 | 25 | 1.6, 23 | 20 | 26 | 27 |
| R1 | 31 | 1.2, 26 | 26 | 30 | 29 |
| 19859 | 29 | 2.0, 28 | 27 | 30 | 31 |
| 23270 | 25 | 5.2, 32 | 39 | 37 | 34 |

with the significant increases in membrane *P* values observed in this strain over the range of culture pH.

## Fatty acid composition

Cytoplasmic membrane fluidity and phase characteristics are largely controlled by the fatty acid/lipid composition. We measured total cellular fatty acids in order to determine if *A. ferrooxidans* strains synthesized or modified particular fatty acids in order to compensate for the potential membrane disruption induced by sub-optimal pH 1.5. The determination of total cellular fatty acids included about two dozen unique fatty acid species. These were grouped according to chain length and saturation to show the percent change in abundance under pH 1.5 versus optimal pH conditions for each strain (Fig. 3). Comparisons of total saturated, unsaturated, and cyclic fatty acids as well as the overall percent change in the fatty acid profiles are summarized in Table 4.

Growth at pH 1.5 induced changes in the relative abundance of certain fatty acids in all of the strains (Fig. 3). Among the environmental strains (D6, D7, F1 and R1), an increase in several saturated fatty acids was observed including 12:0 (dodecanoic acid), 14:0 (grouped as tetradecanoic acid, and 14:0 3OH), 16:0 (grouped as hexadecanoic acid, 16:0 2OH and 16:0 3OH) and cyclopropane 19:0 w8c ($p < 0.05$). A similar trend was observed in the aforementioned fatty acids in type strains 19859 and 23270 ($p > 0.05$), however, only increases in the 17:0 saturated fatty acid were significant ($p < 0.05$). In all of the strains, a response to pH 1.5 also brought about decreases in the most abundant fatty acid, 18:1 w7c (*cis*-Δ11-octadecenoic acid) ($p < 0.05$). In all of the strains, a shift towards a greater abundance of short-chain saturated fatty acids would support the observation that decreases in membrane fluidity were the result of fatty acid modulation in response to pH 1.5.

Since individual fatty acid abundance only in part indicates the degree of membrane remodeling, the sum of total fatty acids was used to determine overall changes in membrane structure in response to pH stress (Table 4). Compared to optimal pH of 2.1, a significant shift in the overall proportion of saturated and unsaturated fatty acids occurred in all of the strains ($p < 0.05$). In the environmental strains, 6–14% increases in the saturated fatty acid component were observed, which consequently increased the saturated to unsaturated fatty acid ratios from about 0.4 to 0.6. Nonetheless, the unsaturated fatty pool remained quite high in these strains, ranging from 59 to 64%. Conversely, in the type strains, the relative fatty acid distribution was markedly different under optimal pH conditions compared to the environmental strains and was also quite different following the shift to pH 1.5 despite the common trend towards a higher abundance of saturated fatty acids. In these strains the saturated fatty acid component increased by 10–18% resulting in a membrane with a greater proportion of saturated over unsaturated fatty acids, at a ratio of about 1.4 ($p < 0.05$). The mean unsaturated fatty acid component was 41% for both type strains during pH stress, which was about 20% lower than was found in the environmental strains. The relative abundance of cyclopropane fatty acids also increased in response to pH 1.5, showing significantly higher amounts in strains D6, R1 and 23270 ($p < 0.05$). In the *A. ferrooxidans* strains, only two cyclopropane fatty acids were present with chain lengths 17:0 and 19:0, although the latter accounted for 70–90% of the abundance.

An additional measure of the overall structure of the membranes was determined through the total percent change ($\Delta$), calculated as ($\Delta = \mathrm{SUM}|(\mathrm{FA}_{\mathrm{pH1.5}} - \mathrm{FA}_{\mathrm{control}})|$) where FA represents each fatty acid species measured (Table 4). The results indicated that in addition to having significant shifts in the overall fatty acid distribution in response to the stress of pH 1.5, all of the strains showed large turnover in fatty acid composition (Table 4). The total percent change was greatest in the type strains between 38 and 42% while D6 had the highest turnover among the environmental strains at 37% and the remaining strains had less than 25% turnover. The largest contributors to the percent shift were the most abundant fatty acid species, namely 18:1, and 16:1 w7c (*cis*-Δ9-hexadecenoic acid), along with the fatty acids that showed significant changes in abundance described above; 12:0, 14:0, 16:0, and cyclopropane 19:0. These data illustrate that the observed shift in *P* and $T_m$ in response to pH 1.5 coincides with fatty acid modulation. Specifically, the decrease in membrane fluidity is likely linked to the overall increase in saturated fatty acids. The multiple $T_m$, whereby part of the membrane fatty acids maintained near-optimal values, were likely due to the remaining high proportion of unsaturated fatty acids. The observed depression in the second $T_m$, or the singular $T_m$ in strains D6 and D7, may be related to the slight increase in 16:1 fatty acid (although

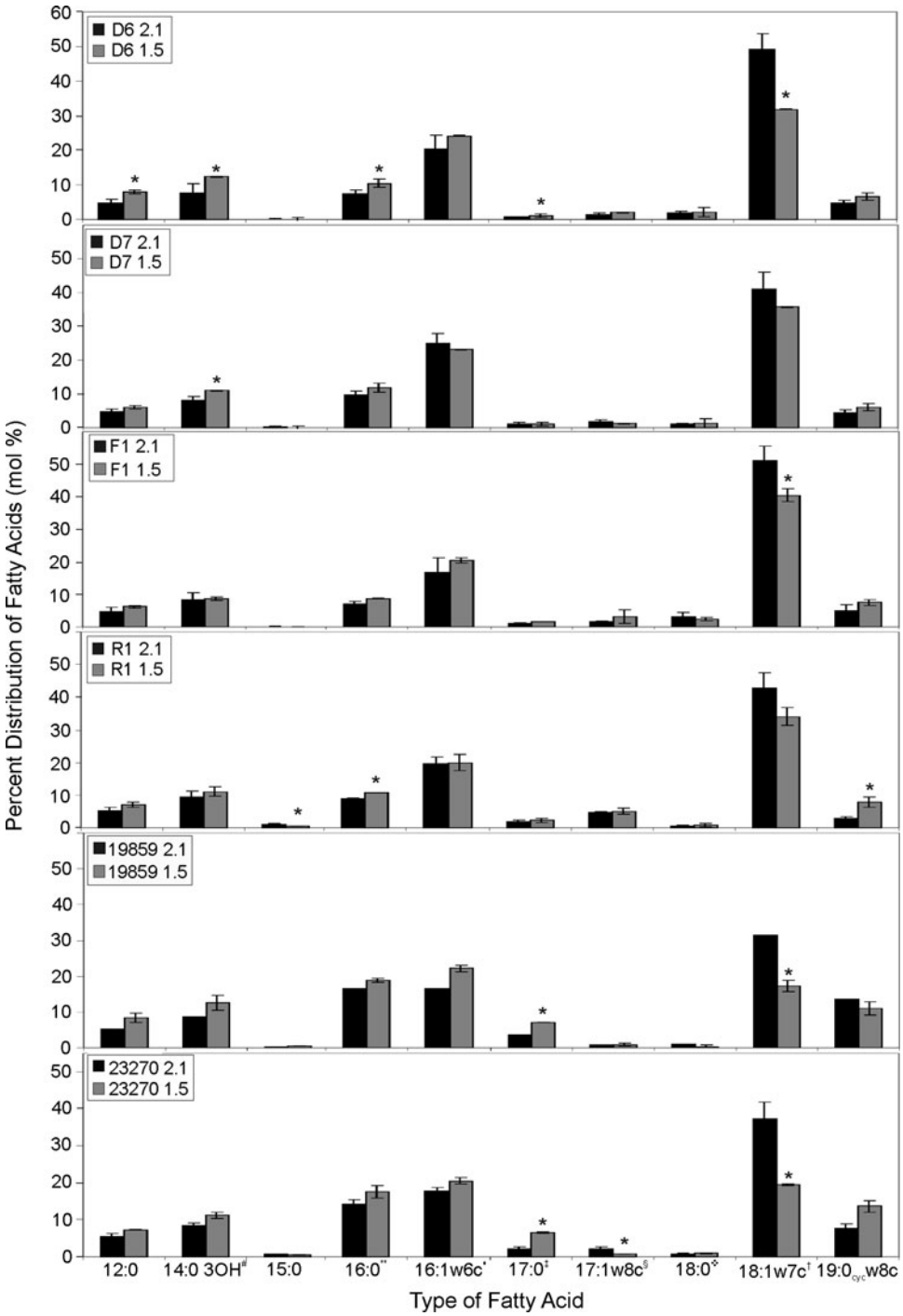


**Fig. 3** Percent distribution of simplified fatty acid groups for six *A. ferrooxidans* strains grown under control conditions (25°C, pH 2.1) or pH 1.5 exposure. Significant differences ($\alpha = 0.05$) in fatty acid abundance are indicated for each strain (*asterisks*). Combined fatty acid groups are shown: (#) 14:0 and 14:0 3OH; (**) 16:0, 16:0 2OH and 16:0 3OH; (◆) 16:1 w6c, 16:1 2OH and 16:1 w5c; (‡) 17:0, 17:$0_{cyc}$, and 17:0 2OH; (§) 17:1 w8c, 17:1 w6c, and 17:1 anteiso; (v) 18:0, 18:0 2OH and 18:0 3OH (†), 18:1 w7c and 18:1 2OH

this shift was not significant) and from the increase in cyclopropane 19:0, that confers a lower melting point than its saturated fatty acid equivalent.

Further differentiation of the fatty acid profiles under pH stress versus optimal profiles was obtained using discriminant function analysis (DFA). The DFA determined which fatty acids (log-transformed) were correlated with the discriminant function and contributed most to separating the pH groups as summarized in Table 5. Since the fatty acid profiles were quite different among the strains, the profiles for the environmental strains were compared using a single DFA while the type strains were compared separately. From the simplified fatty acids groups, only three entered the function comparing the environmental strains at a significant level ($p < 0.05$). The discriminant function was defined as $DF_{(env.strains)} = 38.4 - 26.5(\log_{10}\ 12{:}0) - 23.5(\log_{10}\ 16{:}0) - 12.7(\log_{10}\ 19{:}0)$. Since only two pH groups were being compared the discriminant function

**Table 4** Summary of total percent fatty acids grouped by acyl chain type under control growth conditions (25°C, pH 2.1) or pH stress (pH 1.5)

| Fatty acid group | Strain D6 | | Strain D7 | | Strain F1 | | Strain R1 | | Strain 19859 | | Strain 23270 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C. | pH 1.5 | C. | pH 1.5 | C. | pH 1.5 | C. | pH 1.5 | C[A] | pH 1.5 | C. | pH 1.5 |
| Cyc | 4.8 (0.8)[a] | 7.2 (0.4)[b] | 4.7 (0.8)[a] | 5.5 (0.9)[a] | 5.0 (1.8)[a] | 8.2 (0.7)[a] | 2.7 (0.5)[a] | 7.4 (0.9)[b] | 15[a] | 16 (1.9)[a] | 7.7 (2.1)[a] | 18 (2.4)[b] |
| Total S | 27 (2.7)[a] | 41 (0.6)[b] | 30 (3.5)[a] | 37 (0.7)[a] | 29 (1.7)[a] | 35 (0.2)[b] | 30 (3.4)[a] | 41 (0.7)[b] | 49[a] | 59 (0.8)[b] | 40 (4.2)[a] | 57 (3.8)[b] |
| Total U | 72 (2.1)[a] | 59 (0.6)[b] | 69 (2.8)[a] | 61 (2.6)[a] | 70 (1.5)[a] | 64 (0.2)[b] | 68 (2.7)[a] | 59 (0.7)[b] | 51[a] | 41 (1.2)[b] | 59 (4.2)[a] | 41 (2.1)[b] |
| S/U | 0.3[a] | 0.69[b] | 0.44[a] | 0.61[b] | 0.42[a] | 0.55[b] | 0.45[a] | 0.69[b] | 0.96[a] | 1.44[b] | 0.68[a] | 1.40[b] |
| Total %$\Delta$ | – | 36.5 | – | 18.2 | – | 24.2 | – | 16.8 | – | 37.8 | – | 42.1 |

Values [mean of $n = 3$ (SD)] for each strain compare control (C) versus pH stress (pH 1.5); those that are significantly different at the (95% level) are designated by different letter superscripts (a or b)

Cyc, total cyclic fatty acids; S, total saturated fatty acids; U, total unsaturated fatty acid; S/U, saturated to unsaturated fatty acid ratio with all SD < 0.1; total percent change ($\Delta = \text{SUM}|(FA_{pH1.5} - FA_{control})|$) where FA represents each fatty acid species measured

[A] Profiles determined from a single sample

**Table 5** Summary of discriminant function analysis for fatty acid composition with pH as the grouping variable

**a. Environmental strains**

| | Eigenvalue | % Variance | Wilks' $\Lambda$ | Canonical *R* | *df* | *p* level |
|---|---|---|---|---|---|---|
| Function | 8.94 | 100 | 0.101 | 0.948 | 9 | <0.0006 |
| Variable | Raw coefficient | Standardized coefficient | Loading (correlation) | | | |
| Constant | 38.4 | 0 | – | | | |
| 12:0 | −26.5 | −1.76 | −0.34 | | | |
| 16:0 | −23.5 | −1.37 | −0.28 | | | |
| 19:0 | −12.7 | −1.17 | −0.34 | | | |

**b. Type strains**

| | Eigenvalue | % Variance | Wilks' $\Lambda$ | Canonical *R* | *df* | *p* level |
|---|---|---|---|---|---|---|
| Function | 20.8 | 100 | 0.046 | 0.977 | 5 | <0.0001 |
| Variable | Raw coefficient | Standardized coefficient | Loading (correlation) | | | |
| Constant | −88.8 | 0 | – | | | |
| 16:0 | 21.7 | 2.71 | 0.52 | | | |
| 18:1 | 41.3 | 2.29 | −0.24 | | | |

could not be plotted. A high degree of confidence in the discriminant function was obtained through cross-validation that correctly classified 87.5% of the new cases (Table 6). For the comparison of the type strain profiles, two fatty acids entered the DFA at a significant level ($p < 0.05$) (Table 5). The discriminant function was defined as $DF_{(\text{type strains})} = -88.8 + 21.7(\log_{10}\ 16{:}0) + 41.3(\log_{10}\ 18{:}1)$. Due to the low number of cases, cross-validation of the function could not be completed. In both DFAs, the fatty acids that contributed the most to each function primarily corresponded with those observed to have significant changes in abundance in response to pH or accounted for the largest proportions of the overall percent change in fatty acids described above.

The changes in fatty acid composition were further correlated with the changes in the fluorescence polarization measurements in response to pH stress. Using factor analysis followed by multiple regression, combinations of different fatty acids were determined to be the best predictors of fluorescence polarization at pH 1.5 (Table 7). For the environmental strains the 12:0 14:0, 16:0, and 18:1 w7c fatty acids all loaded significantly onto Factor 1. The resulting regression equation $P_{(\text{env.strains})} = 0.396 + (0.017 \times \text{Factor } 1_{(\text{env.strains})})$ explained 66% of the variance in

**Table 6** Classification matrix for cross-validation of fatty acid profiles not used in initial discriminant function analysis of the environmental strains

| Env. strains | Predicted vs. observed | | |
|---|---|---|---|
| | pH 2.1 | pH 1.5 | % Correct |
| pH 2.1 | 4 | 0 | 100 |
| pH 1.5 | 1 | 3 | 75 |
| Total | 5 | 3 | 87.5 |

**Table 7** Prediction of fluorescence polarization using fatty acids as predictors under pH 1.5 stress

| Factor analysis* | | | | | |
|---|---|---|---|---|---|
| Environmental strains | Factor 1 loadings | | | | |
| 12:0 | 0.858 | | | | |
| 14:0 | 0.851 | | | | |
| 16:0 | 0.828 | | | | |
| 18:1 | −0.976 | | | | |
| Multiple regression | | | | | |
| a. Environmental strains | B | SEB | $\beta$ | $t$ | $p$ |
| Constant | 0.396 | 0.005 | | 80.3 | <0.0001 |
| Factor 1 | 0.017 | 0.006 | 0.617 | 3.32 | 0.004 |
| b. Type strains | | | | | |
| Constant | 1.72 | 0.602 | | 2.87 | 0.045 |
| 18:1 | −0.39 | 0.115 | −1.57 | −3.33 | 0.03 |

* Only significant loadings (Varimax normalized) are shown (>0.700)

fluorescence polarization values $F_{(1,18)} = 11.3$; $p < 0.004$. For the type strains the analysis was completed using only a forward stepwise multiple regression of the fatty acid profiles. Both 16:1 and 18:1 fatty acid were correlated with the $P$ values although only 18:1 was significant. The regression equation $P_{(\text{type strains})} = 1.72 - (0.39 \times \log_{10}18{:}1)$ explained 84.7% of the variance in $P$ values, $F_{(2,4)} = 11.1$; $p < 0.023$. For all strains, the fatty acids that were most strongly correlated with the regression factors and were significant predictors of fluorescence polarization, were predominantly those that showed significant changes in abundance as a product of pH 1.5 growth conditions and/or effectively separated the optimal and pH stress fatty acid profiles, as described.

## Discussion

The purpose of this study was to determine whether changes in the membrane characteristics contributed to the ability of *A. ferrooxidans* to tolerate and maintain active metabolism at sub- and supra-optimal pH. We also investigated whether strain variation, commonly reported among *A. ferrooxidans* strains, was also apparent in response to pH stress and whether membrane characteristics could be used to distinguish between these strains. Acidophiles maintain pH homeostasis by keeping an impermeable cell membrane, a reversed membrane potential through cation transport into the cytoplasm, and by transporting protons back out of the cell once they enter (Baker-Austin and Dopson 2007). Although more attention has been given to the kinetics of the bioenergetic reactions, clear mechanisms that are used to control the flow of protons (membrane channels, proton pumps), or prevent their entry (changes in membrane structure) have not been thoroughly defined across different species of acidophilic bacteria, including *A. ferrooxidans*. Membrane mechanisms have been better defined in acidophilic archaea where the tetraether lipids (as opposed to ester-linked bacterial lipids) form a dense core effectively creating a highly stable and impermeable membrane monolayer (van de Vossenberg et al. 1998). Bacterial lipids cannot form equivalent structures and so must use fatty acid chain branching and saturation as well as altering lipid head groups to adjust the permeability of the cytoplasmic membrane (Driessen et al. 1996). Thus, it was our goal to understand how cytoplasmic membrane characteristics, not previously studied in *A. ferrooxidans*, act as one of the key adaptive mechanisms for pH homeostasis. In using strains that we know have different phenotypic characteristics, such as metal tolerance and psychrotrophic ability (Mykytczuk et al. 2010b) which also correspond to differences in membrane physiology, we expected that if membrane properties were important in pH homeostasis, that they would be apparent in the membrane properties and fatty acid composition of the chosen strains.

### Effects of pH on growth characteristics

Changes in growth characteristics represent an overall measure of physiological activities in bacteria and as such indicate changes in cellular function when rates deviate from optimal values. We plotted growth curves based on iron oxidation rates and found that *A. ferrooxidans* was more inhibited by pH values lower than optimal values, showing significantly longer generation times at pH 1.5. Similar observations have been reported by Chao et al. (2008) where the increase in iron oxidation and cell number were more rapid at pH values of 2.3 and 3.5 compared to those at pH 1.5 and 1.3 while showing no growth at pH 1.0. The authors suggested that the difference in growth rates was pH-dependent but that the viability of the cells was not affected at any of the pH levels tested where growth was observed (Chao et al., 2008). Above optimal pH values, chemiosmotic theory suggests that ferrous iron oxidation rates will decrease (Ingledew 1982) but the cells are still able to grow (Ferguson and Ingledew 2008). The inhibition is believed to be in part linked to the formation of ferric iron precipitates above pH 3.0 which may hinder proton transport processes at the cell surface (Meruane and Vargas 2003). At pH values below optimal, the inter-conversion of the pH gradient and membrane potential, necessary for maintained proton motive force, becomes inoperable in *A. ferrooxidans* (Kar et al. 1996) which would consequently lead to a decrease in the metabolic

activity. From our observations, a shift towards pH 1.5 was more detrimental than values above the pH optimum lending further evidence to support that a more acidic extracellular environment exerts a greater stress on *A. ferrooxidans*' cellular mechanisms than more alkaline conditions. A complementary observation by Amaro et al. (1991) reported that more acidic pH induced a heat-shock-like response in protein expression compared to more basic pH. The degree to which the lowest pH we tested, only 0.6 units lower than optimal values, affected the different strains was apparent in the broad range of generation times observed, unlike the similar patterns that occurred among the strains at higher pH values. The observation that strain R1 only showed an increase of a few hours in its generation time at pH 1.5 as opposed to strain D7 that showed a much higher increase to nearly 40 h per generation clearly illustrated that differences in acid tolerance exist among the studied strains. In studies of metal tolerance in *A. ferrooxidans* strains, the cytochrome *c* oxidase, a component of the iron respiratory supercomplex that transfers electrons to oxygen and consumes intracellular $H^+$ in the process, was found to differ among strains and was believed to contribute to the observed differences in metal resistance (Sugio et al. 2003). As such, it is possible that the differences in generation time observed in the six strains studied here could be related to differences in the efficiency of the bioenergetic machinery, including cytochrome *c* oxidase, under different environmental conditions.

## Membrane physiology changes in response to pH stress

Together, membrane fluidity and phase along with fatty acid data convey a great deal of information on the overall state of the cytoplasmic membrane and were used to determine how *A. ferrooxidans* responds to changes in growth pH. As extracellular $H^+$ increases the higher pH gradient can cause an influx of protons, disrupting the proton motive force, potentially causing acidification of the cytoplasm and interfering with energy transduction (Cobley and Cox 1983). Before the electrochemical balance can be regained through either active efflux or neutralization of the cytoplasmic protons, it is likely that cellular response will include rapid changes to the cytoplasmic membrane to prevent further influx. In bacteria, this can be achieved by incorporating fatty acids with greater chain length that span further across the membrane bilayer and increase membrane order (Hazel and Williams 1990). Additional rigidity can also be imparted by increasing chain saturation and cyclization, or hopanoids (bacterial cholesterol-like molecules) (Driessen et al. 1996). Environmental pH also influences the ionization state of membrane lipids, and at low pH changes in the lipid surface charge, membrane packing, and consequent increase in the gel-to-fluid transition can occur (Hazel and Williams 1990). Cells must react to such membrane lipid perturbations since structural discontinuities caused by phase separation increase the likelihood of membrane integrity and permeability being compromised.

In order to maintain pH homeostasis, we hypothesized that our set of strains, previously shown to have different membrane physiology in response to various environmental conditions, would depict differences in membrane properties when subjected to pH stress. Overall our data illustrated that in *A. ferrooxidans* the response to pH values just below (pH 1.8) and above (pH 3.1 and 3.5) optimal values did not induce as drastic a change in membrane properties as did the most acidic condition of pH 1.5. We chose to direct our analysis on the adaptation to pH 1.5 and did not measure the fatty acid composition from cells grown in supra-optimal pH. We focus the remainder of this discussion on the three different adaptive modes observed in the studied strains in response to pH 1.5.

The first adaptive trend consisted of significant decreases in membrane fluidity along with two distinct phase transition points and a slight decrease in growth rate as observed in the mesophilic environmental strains, F1 and R1 and type strain 19859. As the generation times were only slightly longer than at optimal pH, this adaptive mode appeared to be the most efficient at maintaining cellular homeostasis. From our related studies, we have observed that *A. ferrooxidans* is able to tolerate decreases in membrane fluidity and does not often depict homeoviscous adaptation in response to different stresses (Mykytczuk et al. 2010a, b). In particular, of the strains mentioned above, strain F1 is highly metal tolerant and survives increased metal concentrations while having a more rigid membrane (Mykytczuk et al. 2010a). The decrease in membrane fluidity in response to acidic pH corresponded to a change of 4–18% whereby a change of 18% in *P* values is equivalent to an overall change in the membrane microviscosity of about 30%. This clearly indicated that in these strains, a more acidic pH induced a significantly higher membrane order. Since type strain 19859 had an inherently higher optimal membrane order, the relative change appeared less drastic, but the effective decrease in fluidity was comparable to the other two stains. Similarly, in the lactic acid bacterium *Oenococcus oeni*, acid shock of pH 4.0 and 3.0 induced a rigidification of the membrane, on the order of a 30% increase in the anisotropy at pH 3.0, and the values did not return following a shift back to optimal pH values of 5.5 but no loss in cell viability occurred (Chu-Ky et al. 2005). In response to pH stress, we believe the higher membrane order would increase the effectiveness of the permeability barrier and prevent the influx of $H^+$ into the cell. This appears to be an active modification as the saturated fatty acid component

increased significantly in these strains which would correspond to the higher membrane order observed.

Furthermore, our results of phase transition analyses agreed with the fluidity data in that these three strains experienced dynamic phase changes in the cytoplasmic membrane, and these occurred to a larger extent at pH 1.5 than at higher pH values. During growth at pH 1.5 there was a depression in the gel-to-liquid-crystalline transition temperature by about 20°C which indicated an increase in the portion of the membrane fatty acids were fluid under this growth condition. Although this would seem to contradict the fluidity data suggesting that the membrane is also more fluid under low pH conditions, the fact that the strains showed a significantly more rigid membrane is likely a product of a smaller proportion of membrane lipids undergoing phase changes at the lower transition point. Since only a fraction of lipids might partake in a phase transition, which has been reported to vary from 70 to 90% (McElhaney 1974; Souzu 1986), our results may suggest that the majority of the membrane lipids were involved in the higher $T_m$ and thus the membrane remained quite rigid. The shifts in fatty acid composition that showed an increase in the saturated component, would further support this observation. The slight increase in 16:1 (*cis*-Δ9-hexadecenoic acid) may account for the observed $T_m$ depression due to its melting point of 0.5°C, which is lower than the 15°C melting point of the other abundant unsaturated fatty acid: 18:1 (*cis*-Δ11-octadecenoic acid). With the appearance of multiple transition points, these strains may incur discrete lipid domains and possible phase separation can result. Phase separation potentially leads to a breakdown of the permeability barrier of the cytoplasmic membrane and can decrease its functional efficiency leading to impairment of growth and even cell death (Russell et al. 1995; Beney and Gervais 2001). However, if a smaller portion of fatty acid underwent the second transition in these strains, then this would limit the apparent detrimental effects on cellular homeostasis.

A second type of membrane response was apparent in strains D6 and D7 that only showed slight decreases in membrane fluidity along with a single depressed $T_m$ at pH 1.5. It would appear that this strategy was not as effective at maintaining cellular homeostasis under pH stress, as both of these strains had significant increases in generation times unlike the strains described above. The percent change in fluidity was not as pronounced as in the strains described above and although D6 and D7 also experienced a decrease in fluidity, this represented only a 7 and 14% change in the membrane microviscosity, respectively. The single $T_m$ observed for both strains was not as low as the one described in the first adaptive mode, but would suggest that a larger portion of the membrane fatty acids were remodelled in order to maintain membrane properties closer to optimal values than in the above strains. Although the changes in the fatty acid profiles also showed an increase in the saturated component, as described in the above strains, and account for the increase in membrane order observed, no distinct shift in particular fatty acids was unique to these two strains. It is possible that the same rationale described above also applies to this adaptive mode, such that the depression in $T_m$ is due in part to the increase in the 16:1 fatty acid, but may also be a product of changes in lipid head group. Both strains also had slightly greater depression of the $T_m$ at the other pH values tested compared to the strains described in the first adaptive mode. This indicates that membrane remodeling would also be and important adaptive response at supra-optimal pH in these strains. Both D6 and D7 are psychrotrophic, and we have observed that they have different membrane properties than the other strains we have studied, which are strictly mesophilic (Mykytczuk et al. 2010b). Similarly, the lactic acid bacterium, *Lactobacillus bulgaricus*, was found to have a slight increase in the overall saturated fatty acids and decrease in the cyclic fatty acid in response to lower pH suggesting that the membranes became more rigid in response to more acid conditions (Streit et al. 2008). The authors were unsure of how decreased membrane fluidity allowed for improved cryotolerance but we would suggest that such a change may have been linked to proton permeability, and the strains abilities to tolerate a less fluid membrane, as we have observed in *A. ferrooxidans*.

A third adaptive mode was observed in type strain ATCC 23270 and appeared to be the least effective at maintaining cellular homeostasis in response to pH. Although this strain maintained optimal generation times at all pH levels tested except pH 1.5, the generation time was longer than those observed in the other strains under more acidic conditions. This would suggest that strain 23270 does not carry out iron oxidation as effectively under low pH stress as do the other strains. Strain 23270 also revealed that the significant decreases in membrane fluidity were met with two distinct populations of fatty acids; one that was fluid and the other in gel-phase at pH 1.5 and 25°C. Evidence for gel-phase lipids was also observed at the other pH values tested, unlike the other strains, indicating that this strain can survive with a large component of gel-phase lipids. Although this observation is not common in bacteria, it has been reported that an *E. coli* membrane remained functional with up to 20–55% gel-phase lipids (Morein et al. 1996; Cronan 2003). Also, the fatty acid changes support that an increase in the saturated component up to nearly 60%, accounts for the decreased fluidity and likely accounts for the increases in transition temperatures. This increase in $T_m$ may also be a product of changes in anionic lipid properties that can occur in response to the changes in environmental pH (Hazel and

Williams 1990). In the extremely acidophilic archaea *Picrophilus oshimae* the loss of viability and cell integrity at supra-optimal pH was due to a disruption of the barrier function of the cytoplasmic membrane (van de Vossenberg et al. 1998). Strain 23270 also showed the highest percent turnover in fatty acid composition at pH 1.5 illustrating a more energy intensive adaptive response than in the other strains. The component of fluid-phase lipids that contributed to depressing the $T_m$, again this likely involved the 16:1 fatty acid, are perhaps involved in lipid–protein domains that maintain the functionality of those proteins (Lee 2005) as there is no apparent effect on membrane fluidity. With the two $T_m$ separating the component lipid populations to a greater extent than in the other strains, the possibility of phase separation may be more pronounced in strain 23270. Thus, the likelihood of membrane integrity being compromised causing an increase in the permeability and potential proton influx, is high. This may explain why strain 23270 had significant increases in generation time when exposed to pH 1.5. In studies of bioenergetics in *A. ferrooxidans*, Kar et al. (1996) observed an uncommon positive charge on the membrane surface and they proposed that changes in surface charge following shifts to lower pH-mediated phase separation of the membrane, which could be a possible mechanism responsible for failure in the maintenance of the proton gradient and membrane potential.

## Common response in *A. ferrooxidans* strains under sub-optimal pH

As evidenced by the differences in membrane fluidity and phase data, strain variation in *A. ferrooxidans* is apparent in response to pH stress which is similar to our observations of these strains under metal and temperature stress (Mykytczuk et al. 2010a, b). In addition to investigating strain differences, we also aimed to determine whether there was a general membrane response for all of the *A. ferrooxidans* strains. It is clear that active control of membrane properties in *A. ferrooxidans* was important in response to pH stress. The means by which all of the strains achieved the changes in membrane properties was also of interest and we have illustrated that shifts in specific fatty acids and the relative proportions of fatty acid groups were employed by *A. ferrooxidans* as a common membrane adaptative mechanism.

The fatty acid data indicated that all of the strains employed a diversity of lipids in response to pH stress and the overall fatty acid profiles differed between environmental and type strains. Commonly, the greatest modulation in percent composition came from decreases in 18:1 that would account for the decrease in membrane fluidity observed in all of the strains at pH 1.5. The relative increases in several short-chain saturated fatty acids would also increase membrane order and we suggest that this more rigid membrane is an intrinsic property of the *A. ferrooxidans* cell physiology that permits survival in the extremely acidic AMD environment. Our multivariate analyses made it possible to distinguish between the fatty acid profiles in the environmental and type strains compared to their respective controls. It was necessary to separate the two groups of strains as the type strains had significantly different proportions of saturated and unsaturated fatty acids that skewed the distributions. Despite these differences, for both groups, the 16:0 fatty acid was used in distinguishing of optimal versus pH stress profiles. The ratio of 18:1 and 16:0 appears to be a common adaptive mechanism in bacteria, where shifts towards higher 16:0 fatty acid have been reported for several marine species in response to increased salinity, while the opposite is observed in response to cold temperatures (Hazel and Williams 1990). In the case of *A. ferrooxidans* the shift in abundance towards lower 18:1 and higher 16:0 fatty acids appears to be a general mechanism of membrane modulation in response to acid stress.

The correlation of fatty acid abundance was also successful at accounting for a significant portion of the variance observed in the membrane fluidity data. The fact that the same fatty acids that distinguished between the growth conditions were also those that offered a strong predictive ability of the fluorescence polarization confirm that these fatty acids were the main components in the membrane response to acidic pH stress. Again, it was the shift from a greater abundance of 18:1 fatty acid towards a higher portion of different saturated short-chain fatty acids (12:0, 14:0, and 16:0) that was employed by the *A. ferrooxidans* strains to adapt to pH 1.5.

Another common trait was the relative increase in 19:0 cyclopropane fatty acid that occurred in all of the strains, except 19859 that already had two to three times higher proportions of this fatty acid compared to the environmental strains. We have found in our studies of metal and temperature stress, that changes in the abundance of this cyclic fatty acid are a key component of stress adaptation in *A. ferrooxidans* (Mykytczuk et al. 2010b). Cyclopropane fatty acids, like branched fatty acids in Gram positive bacteria, may confer an intermediate fluidity by forming a less ordered membrane than *trans* unsaturated fatty acids. Cyclization of fatty acids is commonly regarded as a tool to reduce membrane fluidity and decrease permeability to extracellular molecules in bacteria (Brown et al. 1997). It has been recently shown that cyclopropane fatty acids greatly decrease the permeability of *E. coli* membranes and confer increased adaptation to acidic conditions (Shabala and Ross 2008). It is possible that in *A. ferrooxidans* the cyclopropane fatty acids confer an intermediate fluidity

necessary to compensate for the significant increase in membrane order due to an abundance of saturated fatty acids, while maintaining the permeability barrier that might have been compromised by an increased abundance of unsaturated fatty acids.

It is evident in our research that membrane characteristics in *A. ferrooxidans* are a primary factor in stress adaptation. Although *A. ferrooxidans* is the best characterized acidophilic, iron-oxidizing bacterium, there remain several unanswered questions regarding the basic adaptive mechanisms to its native acidic habitat. In the recently published annotated genome of *A. ferrooxidans* ATCC 23270 (Valdés et al. 2008) the genes with functional roles related to the cell envelope were the most abundant, accounting for 7.8% of the genome. This also supports the hypothesis that acidophiles have a more extensive pool of mechanisms to use in extreme pH environments. In the study by Chao et al. (2008), genes for two lipoproteins and two outer membrane proteins were found to increase when challenged by pH 1.3. Similarly, bioinformatic studies of a *Leptospirillum* group II AMD biofilm revealed a large number of genes related to cell membrane biosynthesis which could be indicative of an intrinsic mechanism for acid tolerance that has yet to be defined (Tyson et al. 2004). This increasing body of evidence suggests membrane characteristics are important to the acidophilic existence and likely for adaptability of *A. ferrooxidans* to environmental stress. It would be interesting to compare the bioenergetic parameters to determine whether "homeoproton" adaptation does in fact occur in *A. ferrooxidans* and whether it is correlated with the membrane changes we observed. Another potential target would include cytochrome oxidase differences in the studied strains to determine whether the differences in membrane adaptation we have observed are related to the machinery used to maintain bioenergetics and pH homeostasis in *A. ferrooxidans*.

## Conclusions

It is evident from our results that pH stress in *A. ferrooxidans* induces a cellular response that changes the overall membrane fluidity and phase characteristics through modulations in fatty acid composition. At sub-optimal pH, the increased pH gradient appears to be more challenging to the cell perhaps due to increased $H^+$ leakage across the membrane and despite an active decrease in membrane fluidity aimed at maintaining the permeability barrier. Although pH homeostasis is an essential function in *A. ferrooxidans*, unlike other phenotypes such as metal or temperature tolerance, it appears that variation among strains is also present in response to pH stress. Greater acid tolerance appears in strains that have a more rigid membrane but also modulate membrane fatty acid composition to maintain a functional membrane phase state. The data presented here contribute to the expanding knowledge of *A. ferrooxidans* as a model for understanding the acidophilic mode of growth in bacteria.

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