Variability in *Halothiobacillus neapolitanus* type strain cultures

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Abstract Numerous microbial species are reported to utilize oxidation and/or reduction of sulfur containing compounds in the energy producing portions of their metabolism. *Halothiobacillus neapolitanus* cultures obtained from different commercial sources appear to display considerable variability in terms of growth rate, carbonate consumption and activity of individual enzymes.

Keywords *Halothiobacillus neapolitanus* · Sulfur oxidation · Freeze-drying · Viability

Introduction

Halothiobacillus neapolitanus, formerly known as *Thiobacillus neapolitanus*, is a moderately neutrophilic obligate chemolithoautotroph reported to utilize a wide variety of reduced and partially reduced inorganic sulfur compounds as its sole source of metabolic energy [1–3]. Similar capabilities have been identified in a number of genera [4–6]. Until recently, the general consensus was that there was a fundamentally common sulfur oxidation pathway operating in all of the thiobacilli and metabolically similar chemolithotrophs. However, recent successes in the preparation and partial fractionation of cell-free extracts capable of sulfur compound oxidation have indicated that different organisms may exhibit considerable diversity in the detailed molecular mechanisms for sulfur oxidation [6–9]. A thiosulfate oxidizing multienzyme system (TOMES) has recently been well characterized in *Paracoccus pantotrophus* GB-17 that appears to oxidize thiosulfate directly to 2 moles of sulfate with no detectable free intermediates [10–15]. This has led to the ‘S2’ or ‘paracoccus-like’ designations for microbes which oxidize thiosulfate directly to sulfate. Oxidation of thiosulfate by other species of microbes appears to involve the formation and/or disproportionation of polythionate sulfur species such as tetrathionate, giving rise to the ‘S4’ or ‘polythionate-forming’ designation [16–17].

The *Paracoccus* *sp.* system seems to represent a well characterized example of the multienzyme complex or ‘S2 pathway’ method of thiosulfate oxidation. We propose to use the individual enzymes of the sulfur oxidizing bacterium *H. neapolitanus* as a model system for the investigation of the mechanism of action of biological sulfur transformations in those microorganisms which display discrete intermediates during the oxidation of thiosulfate (i.e., the

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'S4' pathway). We chose this species specifically because the individual steps within the overall oxidation scheme are reported to be independent of one another and allow individual enzyme activities to be isolated, purified and studied at the molecular level [16–17]. In order to complete these investigations, it is necessary to have a culture source that displays consistent properties/behaviors. We report here variability in growth patterns, carbonate consumption and properties of individual purified proteins of *H. neapolitanus* cultures purchased from different sources.

Materials and Methods

Culture growth *Halothiobacillus neapolitanus* type strain cultures were purchased as either freeze-dried culture from the American Type Culture Collection (ATCC #23641) in Arlington, VA, USA or as a live culture from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ #15147) in Braunschweig, Germany. Liquid growth media was prepared as previously described by Jones [18]. Solid media was prepared in a like fashion with the addition of 15g/L of agar to Solution 1 prior to autoclaving. Protein assay stock solution was obtained from BioRad Life Sciences, Hercules, CA, USA. All other reagents were of reagent grade or better.

Freeze dried cultures from ATCC were rehydrated using ATCC medium 290 according to the provided instructions and incubated in a rotary shaker at 30°C and 180rpm. Once established, as determined by turbidity of culture, the ATCC culture was subcultured to liquid medium and incubated under the previous conditions [18]. Live culture obtained from DSMZ was immediately transferred to 50ml of sterile media and incubated as per the ATCC culture. Upon reaching $OD_{600} \sim 0.10$, both cultures were colony purified by streaking on plates and incubating at 30°C with 5% CO_2 . Single, well isolated colonies were selected and transferred to 2000ml flasks containing 600 ml of liquid media and shaken at 30°C for 24hrs. When cultures reached an OD_{600} of ~ 0.10 they were used to inoculate 8L of media A in a New Brunswick BioFlo 110 fermentor configured as a chemostat. Cultures were grown as previously reported utilizing thiosulfate as the sole energy source [18–19].

Enzyme assay Thiosulfate dehydrogenase enzyme activity was measured spectrophotometrically by following ferricyanide reduction at 420nm at 30°C, using an extinction coefficient of $1.04 \text{ mM}^{-1}\text{cm}^{-1}$ [18–19]. The reaction mixture contained 50 mM PIPES pH 6.8, 2 mM potassium ferricyanide, 2 mM sodium thiosulfate, and enzyme solution (0.05 ml) in a total volume of 2 ml. The reaction was initiated by the addition of enzyme. Enzyme activity was expressed as

the amount of enzyme that reduced 1 μmol of ferricyanide per minute.

Protein assay Protein concentration in cell free extracts was estimated by the method of Bradford [20] using bovine serum albumin as reference standard.

Protein purification Cytochrome c-554 was purified from cells grown as previously described [21].

Protein sequencing and mass spectrometry N-terminal amino acid sequencing was performed by automated Edman degradation at the Texas Tech University Biotechnology Core Facility in Lubbock, Texas USA. Protein mass was determined by MALDI-TOF mass spectrometry at the Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas USA.

16S RNA analysis The genomic DNA for both ATCC and DSMZ cultures was prepared using the Qiagen DNeasy Kit (Qiagen). This genomic DNA, as well as libraries constructed from 3–5 Kb fragments of genomic DNA, was used as template for PCR amplification. The PCR amplification was performed with a common set of primers designed using published sequences for *H. neapolitanus* 16S RNA (see Fig. 1) [22–25]. The amplified products were detected by agarose gel electrophoresis with ethidium bromide staining.

Results and Discussion

Differences in growth patterns between cultures from the two different sources become apparent almost immediately. This is evident starting with the colony purification procedure. All cultures were initially grown to equivalent optical densities in liquid media and then a single 10 microliter loopful was used to streak plates for colony isolation. An approximately 2-fold difference in the time necessary for visible colonies to appear was evident, with the DSMZ and earlier ATCC cultures being faster (see Table 1). Maximum optical densities of cultures grown in liquid media followed a similar trend with DSMZ and earlier ATCC cultures

Accession	position #	
	325	350
AB308268	GCAACCCTGATCCAGCAATGCCGCGT	
AY686547	GCAACCCTGATCCAGCAATGCCGCGT	
AF173169	GCAACCCTGATCCAGCAATGCCGCGT	
M79418	GCAACCCTGATCCAGCAATGCCGCGT	
M79419	-----	
M79420	-----	
Primer	-----ACCCTGATCCAGCAATGCCG-----	

Fig. 1 Alignment of published 16S rRNA sequences from position 325 to 350 (M79419 and M79420 are fragments of M79418) and the PCR primer used for analysis

showing 2.5 to 4 times higher optical densities. The most obvious difference is evident in the carbonate consumption with an almost 8 to 1 difference in the amount of carbonate required to maintain pH during chemostat operation.

In order to minimize the possibility that the observations from the ‘abnormal’ ATCC cultures were due to obtaining a mislabeled or otherwise incorrectly identified culture, multiple samples were ordered from each vendor over a period

of several years. In addition, PCR was used for analysis of 16S rRNA from representative samples of cultures displaying the abnormal growth characteristics (see Fig. 2). An approximately 500 bp band was produced using all cultures. In a similar attempt to substantiate culture identity, cytochrome c-554 was purified from both the ATCC cultures in question and the DSMZ culture and found to be identical to that previously reported by Trudinger, et al [26] in terms of mass ($m/z = 9839.58$), spectra of the reduced form (data not shown), and N-terminal sequence (AGDAAAGKTLTY).

Since the PCR data seems to support the identity of the culture as indeed being *Halothiobacillus neapolitanus* and the purified protein seems to be identical to that previously reported, it seems reasonable to conclude that the ATCC cultures bearing lot numbers 3362376 and 3967479 may represent propagation of spontaneous mutants or may be the result in some change in preparation or processing of the cultures.

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Fig. 2 Agarose gel analysis of 16S rRNA-PCR products. lane 1, 2-log DNA ladder; lane 2, PCR product from ATCC 23641 lot# 3362376 genomic DNA template; lane 3, PCR product from ATCC 23641 lot# 3967479 genomic DNA template; lane 4, PCR product from DSMZ 15147 genomic DNA template; lane 5, PCR product from DSMZ 15147 DNA library; lane 6, ATCC 23641 genomic DNA lot# (R)96-12 positive control; lane 7, negative control -PCR product from *E. coli* HB101 genomic DNA; lane 8, 2-log DNA ladder

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Table 1 Growth characteristics of *H. neapolitanus* cultures from different sources

Source	Lot Number	Time to visible colonies (hrs)	Wet cell mass (mg/L)	Maximum optical density (OD600)	Thiosulfate dehydrogenase specific activity (u/mg)	mmoles carbonate consumed per minute	
ATCC	(R)96-12	24±2	527±60	0.28±0.03	0.39±0.10	2.42±0.092	n=3
	3362376	46±8	55±38	0.11±0.02	0.37±0.17	0.7±0.008	n=11
	3967479	44±4	118±64	0.09±0.04	0.33±0.12	0.61±0.019	n=3
DSMZ	n/a	26±1.5	728±88	0.33±0.04	0.44±0.16	2.57±0.111	n=5

n/a – not available

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