Lithotrophy to Organotrophy Conversion in *Thiobacillus A2*

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*Thiobacillus A2* grown on thiosulphate lacks the capacity to transport succinate into the cells but is otherwise fully capable of oxidatively metabolizing succinate at a rapid rate. On the other hand, succinate-grown cells lack elements of the thiosulphate oxidation system; specifically, a special c-type cytochrome. Adaptation of thiosulphate-grown cells to succinate is characterized by a 30–40 min pause in growth during which time an efficient succinate transport system is produced and net cytochrome synthesis ceases. Resumed synthesis of cytochromes *a* and *b* parallels resumption of growth in cells newly adapted to succinate. By contrast, net synthesis of the characteristic c-type cytochrome of lithotrophic cells does not resume and the overall capacity to respire thiosulphate declines.

**INTRODUCTION**

The presence of specialized electron transport machinery in lithotrophic bacteria has been suggested more than once (Kelly, 1967; Rittenberg, 1969; Carr, 1973; Smith & Hoare, 1977). Consistent with this notion is the exceptionally high content of cytochromes found in obligate lithotrophs (Milhaud *et al.*, 1958; Trudinger, 1961; Tikhonova *et al.*, 1967), and in facultative lithotrophs after growth on their characteristic inorganic substrate (Charles, 1971; Kula *et al.*, 1975). Accordingly, a versatile chemolithotroph such as *Thiobacillus A2* (Taylor & Hoare, 1969) might be expected to elaborate two separate and distinct respiratory chains — one for reduced sulphur and the other for reduced carbon substrates. Alternatively, electron flow from inorganic sulphur donors may utilize the same basic set of intermediate carriers as in the case of organic substrates, plus some novel ones as well. Evidence for the second of these two possibilities is presented in this communication.

**METHODS**

*Bacteria.* The nalidixate-resistant (*nax*) mutant of *Thiobacillus A2* used throughout these studies was obtained by plating a large number (>10⁹) of cells on nutrient agar containing nalidixic acid (50 µg ml⁻¹) and further purifying one of the several colonies which spontaneously arose after several days at 30 °C. This mutant, henceforth referred to as strain A2N, grew normally in either the presence or absence of nalidixic acid. It was routinely maintained on thiosulphate agar (Taylor & Hoare, 1969) in the absence of the drug. Strain A2NS-1, a mutant with 90% deficiency in succinate dehydrogenase function, was obtained from a chemically mutagenized A2N population by penicillin selection in the presence of succinic acid, and screening for colonies with identical appearance on thiosulphate and thiosulphate-succinate agar. One such colony, which represented a cell line capable of growth on malate, but unable to form colonies on succinate agar in 36 h, was further purified. Similar procedures were used to isolate strain A2NP. This latter derivative of A2N is a double mutant, auxotrophic for proline and also unable to utilize it as a general source of energy and carbon. Strain A2NP was always cultured in the presence of L-proline (50 µg ml⁻¹).

*Culture conditions.* The basal medium of Taylor & Hoare (1969), adjusted to pH 8.4 and devoid of phenol red, was used for culture on either thiosulphate or succinate. Additional changes in the composition of this medium during culture on sodium thiosulphate were the elevation of the phosphate input from 30 to 100 mM and supplementation with sodium bicarbonate to a final concentration of 12 mM. The freshly prepared and
filter-sterilized NaHCO₃ was added just prior to inoculation of the medium to an initial density of 10⁴ cells ml⁻¹. The corresponding inoculum size for culture on succinate was 10⁴ cells ml⁻¹. Inocula were always prepared from cultures harvested while still in the exponential phase of growth on the relevant substrate.

Inoculated flasks containing one-fifth of their capacity in culture volume were shaken at 250 rev. min⁻¹ and 30 °C in a New Brunswick G7C gyrotory incubator bath. Growth was monitored by iodometric titration of the thiosulphate consumption (Roy & Trudinger, 1970), or by following the increase in turbidity at 540 nm during culture on succinate. All cultures were routinely measured for viable cell numbers on nutrient agar with and without nalidixic acid. Comparative plating, as above, served as a check against contamination by heterotrophs when organic energy-carbon sources were employed. Large-scale (i.e. 10–15 litre) thiosulphate cultures were aerated by sparging from a compressed air source.

Exponential phase organisms (2–5 × 10⁴ ml⁻¹) on 20 mM-sodium thiosulphate in the complete absence of organic matter are referred to as 'lithotrophic cells'. The term 'organotrophic cells' designates an analogous population pregrown on 20 mM-sodium succinate.

**Lithotrophic to organotrophic transition.** Lithotrophic cells were washed several times with phosphate buffer (50 mM, pH 8.4) and then resuspended at 2–4 × 10⁶ ml⁻¹ (100 μg cell protein ml⁻¹) in standard mineral salts medium without substrate. Such suspensions were already at cell densities as high or higher than the maximum obtainable on excess thiosulphate. After equilibration to 30 °C with shaking for 10–15 min, sodium succinate (20 mM, pH 8.4) was added to the suspension which was then aerated for an additional 3 h. Samples were withdrawn at intervals during this period and the cells were analysed for their protein and cytochrome contents, for their ability to respire succinate and for thiosulphate-promoted oxygen consumption.

**Preparation of cell-free extracts.** Washed cells were suspended at various protein concentrations in 0.1 M-potassium phosphate buffer (pH 7-0) containing 1 mM-MgCl₂, and 8 ml samples were disrupted by sonication using a Braun Labsonic disintegrator at 75 W. Pauses (40 s) were allowed for heat dispersion between each of five 20 s pulses. DNAase I and RNAase I ( Worthington, each 1 μg ml⁻¹) were added to the disrupted organisms which were centrifuged at 4 °C for 10 min at 2000 g, and then for 30 min at 20000 g. The resulting pellet was discarded. The 20000 g supernatant, referred to as the 'crude extract', was further fractionated by centrifuging at 4 °C for 2 h at 144000 g. The 144000 g supernatant, called the 'soluble fraction', was either frozen at −40 °C or used immediately, as was the 144000 g pellet or 'membrane fraction' after resuspension in 1 vol. phosphate/MgCl₂ buffer. Storage at −40 °C had no noticeable effect on any tested parameter over a period of several months.

**Uptake of [¹⁴C]succinate by intact cells.** Exponential phase cells (see above) were washed, resuspended in substrate-free medium at densities equivalent to 0-15–0-3 mg cell protein ml⁻¹, equilibrated at 30 °C for 10 min with strong aeration and then made 0-1 mM in sodium [2,3-¹⁴C]succinate [0-5 mCi mmol⁻¹ (18.5 MBq mmol⁻¹); Amersham]. Samples (0-1 ml) were removed from the mixtures at 15 s intervals over 2 min and immediately diluted into ice-cold 0.5 mM-phosphate buffer (pH 8.4). Diluted cells were collected on membrane filters (Millipore, 0-45 μm pore size), washed twice on the filters with 5 ml portions of cold buffer, dried, immersed in 3 ml toluene containing 1% (w/v) PPO and 0-05% (w/v) POPOP (Wang & Willis, 1965) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

**Spectrophotometry.** Cell suspensions containing 2–3 mg cell protein in 0-5–3-5 ml medium or buffer were scanned from 650 nm at a speed of 200 nm min⁻¹ and an overall scan time of 45 s in a specially constructed single-beam spectrophotometer linked to a Hewlett-Packard 2100A minicomputer. Readings were taken automatically at 0-83 nm intervals during the scan. The spectral band width of the double monochromator (a modified Cary 15 instrument) was 2 nm at 550 nm. The light path, cuvette and detector were arranged to allow measurement of spectra in densely-scattering samples as described by Butler (1972). Absolute concentrations of cellular cytochromes were estimated from computer-generated second-derivative absorption spectra (differentiating intervals of 6-3 nm and 8-0 nm). As shown by Butler & Hopkins (1970), such second-derivative spectra give rise to sharper peaks and enhanced resolution of multicomponent systems. Relative c-type and a-type cytochrome concentrations were directly estimated from a-peak heights at 548 nm (or 550 nm after growth on succinate) and 603 nm, respectively. However, the peak height of the a-band of cytochrome b could not be directly measured in thiosulphate-grown cells because of masking by the much larger amounts of cytochrome c₅₅₈ present. Instead, the relative concentration of the b-type cytochrome was estimated from the negative peak of its long-wavelength second-derivative side-band at 573 nm. Interference by c-type cytochrome was negligible at 573 nm (see Fig. 4).

**Substrate respiration in whole cells.** Oxygen consumption was measured polarographically at 30 °C with an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) in 0-05 M-phosphate buffer (pH 8.4) containing 50–500 μg cell protein in a total volume of 1·6 ml. Mixtures were allowed to equilibrate in the presence of inhibitors of respiration which were added, as indicated, 10 min prior to the addition of succinate or thiosulphate. Respiratory inhibitors were dissolved in water just prior to use except in the cases of 2-nonyl-4-hydroxyquinoline N-oxide (NHQNO) and dicumarol. These were freshly prepared in NaOH solution at pH 10.5 to give stock concentrations of 0-5 mg ml⁻¹ and 0-05 M, respectively. Control trials established that the effects obtained with these agents were not due to addition of the alkali to the test system.
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Table 1. Capacity to respire succinate in lithotrophic and organotrophic cells

Reaction mixtures contained the following amounts of protein. Lithotrophic (thiosulphate-grown) cells: intact cells, 0.30 mg; crude extract, 1.93 mg; membrane fraction, 0.56 mg; soluble fraction, 1.79 mg. Organotrophic (succinate-grown) cells: intact cells 0.10 mg; crude extract, 2.64 mg; membrane fraction, 2.90 mg; soluble fraction, 2.33 mg.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Succinate respiration* [nmol O₂ consumed min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lithotrophic cells</td>
</tr>
<tr>
<td>Intact cell suspension</td>
<td>3</td>
</tr>
<tr>
<td>Crude lysate</td>
<td>25</td>
</tr>
<tr>
<td>Membrane-rich 144 000 g pellet</td>
<td>75</td>
</tr>
<tr>
<td>Soluble 144 000 g supernatant</td>
<td>0</td>
</tr>
</tbody>
</table>

*Succinate respiration was measured in the presence of 20 mM-succinate and has been corrected for endogenous respiration.

Table 2. Effect of inhibitors on oxygen uptake during oxidation by cell-free extracts

Reaction mixtures contained 2–3 mg protein (crude extract). Each value represents the average of at least three independent determinations.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor concn (mM)</th>
<th>Inhibition of succinate respiration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amytal</td>
<td>1</td>
<td>Lithotrophic extracts: 7, 6</td>
</tr>
<tr>
<td>Atabrine</td>
<td>1</td>
<td>Organotrophic extracts: 32, 43</td>
</tr>
<tr>
<td>Dicumarol</td>
<td>1</td>
<td>80, 72</td>
</tr>
<tr>
<td>NHQNO</td>
<td>0.035*</td>
<td>48, 73</td>
</tr>
<tr>
<td>Cyanide</td>
<td>2</td>
<td>100, 100</td>
</tr>
</tbody>
</table>

*Added to about 3-2 μg (mg protein)⁻¹.

RESULTS

Succinate metabolism in thiosulphate- and succinate-grown cells

Intact cells grown on thiosulphate were unable to respire succinate at a high rate immediately. However, extracts prepared from lithotrophic cells had almost the same capacity for membrane-associated succinate respiration as did extracts derived from succinate-grown cells (Table 1). Furthermore, comparative studies with standard respiratory inhibitors in extracts of both cell types established the existence of a similar energy-linked pathway to oxygen in each case. Thus, succinate oxidation is coupled to a flavoprotein carrier (atabrine sensitivity) and not to a pyridine nucleotide (amytal insensitivity) (Table 2). Based on dicumarol sensitivity, a carrier of the quinone type also appears to be involved (Brodie, 1967). Moreover, NHQNO and cyanide sensitivities together indicate involvement of a cytochrome chain with carriers of the b-, c- and a-type. Direct evidence for the presence of a-, b- and c-type cytochromes in Thiobacillus A2N is presented below. Therefore, the inability of whole cells to rapidly metabolize succinate after preliminary culture on thiosulphate was probably due to insufficient capacity to transport succinate.

Changes in succinate metabolism associated with transfer of thiosulphate-grown cells to medium containing only succinate

Suspensions of thiosulphate-grown cells (Fig. 1a) took up [¹⁴C]succinate, but only at one-tenth of the maximal rate exhibited by succinate-grown cells (Fig. 1b). Cyanide inhibited
the uptake of succinate by both cell types, thus implying a requirement for metabolic energy. The apparent rate of succinate uptake by thiosulphate-grown cells was approximately 8 nmol min\(^{-1}\) mg\(^{-1}\) over a period of 2 min, whereas the corresponding value for succinate-grown cells was 100 nmol min\(^{-1}\) mg\(^{-1}\). This difference, together with the results previously discussed, suggests that the rate of succinate permeation across the cell membrane may be limiting. One prerequisite for growth of lithotrophic cells on succinate would therefore be the acquisition of additional uptake capacity.

When *Thiobacillus* A2N cells were transferred to minimal medium containing succinate as sole energy and carbon source after pregrowth on thiosulphate (see Methods), a lag of 30–40 min was always observed before growth resumed at a rate characteristic for succinate (Fig. 2a, c). During this lag period, the succinate uptake capacity increased more than 10-fold (Fig. 2a). Also, both the prior rise in the specific rate of succinate uptake and the subsequent resumption of rapid growth failed to occur when protein synthesis was inhibited by chloramphenicol (Fig. 2a).

The kinetics of enhancement of both the rate of uptake of succinate (Fig. 2a) and the rate of respiration of succinate (Fig. 2c) in wild-type cells were essentially identical to the kinetics of enhancement of the rate of succinate uptake in the succinate dehydrogenase-deficient mutant (Fig. 2b). In this case, exposure to succinate after pregrowth on thiosulphate was not accompanied by resumption of growth with succinate utilization. The lower extent of induction of uptake in the mutant compared with the wild-type (Fig. 2a, b) probably arises from the energetic nature of the lesion in the mutant strain. These results suggest that *de novo* synthesis of an efficient substrate transport system precedes resumption of growth by lithotrophic cells on the organic energy-carbon source.

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**Fig. 1.** Comparison of the capacity for succinate uptake by lithotrophic and organotrophic cells. (a) *Thiobacillus* A2N cells (97 μg ml\(^{-1}\)) pregrown on 20 mM-thiosulphate and (b) strain A2N cells (80 μg ml\(^{-1}\)) pregrown on 20 mM-succinate: uptake of \(^{14}\)C\-succinate (●); uptake after 10 min pre-equilibration in the presence of 2 mM-cyanide (○).
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(a) Alteration of the specific rate of succinate uptake with time after exposure of thiosulphate-grown A2N cells to succinate: uptake of $[^{14}C]$succinate (●); uptake of $[^{14}C]$succinate in the presence of 100 µg chloramphenicol ml$^{-1}$ (○); growth as measured by relative protein increase (▲); protein increase in the presence of 100 µg chloramphenicol ml$^{-1}$ (●).

(b) Alteration of the specific rate of succinate uptake with time after exposure of thiosulphate-grown strain AZNS-1 cells to succinate: uptake of $[^{14}C]$succinate (●); growth as measured by relative protein increase (▲).

(c) Alteration of the specific rate of succinate respiration with time after exposure of thiosulphate-grown strain A2N cells to succinate: succinate-promoted oxygen consumption (●); growth as measured by relative protein increase (▲).

**Loss of capacity to oxidize thiosulphate during adaptation of lithotrophic cells to growth on succinate**

The rapid rise in the capacity to respire succinate observed when lithotrophic cells of strain A2N were exposed to succinate was not paralleled by an equally rapid decline in the capacity to respire thiosulphate. No loss of capacity to oxidize thiosulphate occurred until the cells had already resumed growth on succinate some 30–40 min after their first contact with the organic substrate (Fig. 3). However, once initiated, the overall capacity to respire thiosulphate per unit volume of culture declined more rapidly than could be explained by a process of simple growth dilution (Fig. 3). Exactly the same result was obtained when proline-requiring strain A2NP cells were examined in the presence of 50 µg L-proline ml$^{-1}$. However, in the absence of the required amino acid, cells of strain A2NP neither resumed growth nor lost any of their initial capacity to oxidize thiosulphate over a period of many
hours (Fig. 3). Thus, the process leading to elimination of the capacity to oxidize thiosulphate appears to include both growth dilution and programmed destruction of at least some elements of the lithotrophic energy system.

**Changes in cytochrome distribution during adaptation of lithotrophic cells to growth on succinate**

Absorption spectra of intact lithotrophic and organotrophic cells under aerobic conditions are presented in Fig. 4. Such spectra were characterized by absorption maxima at around 560 nm, with a shoulder around 548–550 nm and a small peak in the vicinity of 600–608 nm. Therefore, in agreement with others (Kula et al., 1975), it was concluded that cytochromes of the α-, β- and c-type were present. Given the inhibitory effect of NHQNO and cyanide on the respiration of succinate by extracts of the two cell types (Table 2), there is reason to suppose that all three cytochrome types are functional. However, as revealed by the dithionite reduction spectra shown in Fig. 5, the cytochrome contents of lithotrophic and organotrophic cells appear to be different. For example, the α-absorption peak of the prominent cytochrome c band in lithotrophic cells is at 548 nm (Fig. 5, trace A/8). Table 3 shows that the cytochrome c content of lithotrophic cells is about 10 times greater than that of organotrophic cells. The extraordinarily high cytochrome c content of lithotrophic cells masked the presence of a substantial peak of reduced cytochrome b at or near 560 nm (Fig. 5, trace A/8) and necessitated indirect measurement of this latter pigment (see Methods). Such measurements showed the cytochrome b content of lithotrophic cells to be identical with that of organotrophic cells (Table 3). At sufficiently high sensitivities (Fig. 5, traces A and B) it was also possible to show that both cell types were equally rich in cytochrome a (Table 3). In fact, the only demonstrable difference, albeit a very striking one, was in cytochrome c548 content.

The above findings predict that specific cytochrome a and b contents in thiosulphate-grown cells should remain unchanged during the entire process of transition to
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Fig. 4. Endogenous negative second-derivative absorption spectra of lithotrophic and organotrophic cells. The measurement scale was arbitrarily chosen to equal 1 unit per mm of pen deflection at maximum sensitivities of the system. The two traces derive from spectra taken under aerobic conditions of strain A2N cells after growth on thiosulphate (A) and succinate (B). In each case 2.3 mg cell protein was present in the test cuvette.

Fig. 5. Reduced cytochrome distributions in lithotrophic and organotrophic cells. The traces are the second-derivative dithionite reduction spectra after growth of strain A2N cells on thiosulphate (A) and succinate (B). The B/2 and A/8 traces are the result of, respectively, 2-fold and 8-fold signal attenuation by the computer in order to bring the curves on to scale. Scale measurement and cell protein content were as in the legend to Fig. 4.

Table 3. **Cytochrome content of lithotrophic and organotrophic cells**

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Specific cytochrome content* [arbitrary units (mg cell protein)^{-1}]</th>
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</thead>
<tbody>
<tr>
<td>Thiosulphate</td>
<td>( a_{503} ) 13, ( b_{560} ) 15, ( c_{548 \text{ or } 550} ) 380</td>
</tr>
<tr>
<td>Succinate</td>
<td>( a_{503} ) 13, ( b_{560} ) 15, ( c_{548 \text{ or } 550} ) 40</td>
</tr>
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</table>

* Calculated from data in Fig. 5.

organotrophy. From Fig. 6, it is evident that the cytochrome \( b \) content remained constant during the lag period immediately after exposure of thiosulphate-grown cells to succinate, and then increased in a manner indistinguishable from that of total cellular protein when growth resumed. A similar situation also prevailed with respect to cytochrome \( a \). In Fig. 6, apparent acceleration in the rate of cytochrome \( a \) increase relative to that of total protein (i.e. growth) is not significant and fell within the expected range of experimental error. In sharp contrast, synthesis of cytochrome \( c_{548} \) did not resume with growth and its concentration per unit
Fig. 6. Quantitative cytochrome changes associated with transition of lithotrophic cells to organotrophy. Samples withdrawn from transition cultures (see Methods) were washed, concentrated to 4–6 mg cell protein ml⁻¹ and divided into two parts. One part was analysed for protein and the second was subjected to second-derivative spectral analysis after chemical reduction with dithionite. Each point is the average of five separate experiments with strain A2N. Protein contents of cultures have been normalized to the zero time value for each experiment, as have the concentrations of any given cytochrome. Absolute cytochrome measurements were made as described in Methods and the legend to Fig. 5. Protein (●); cytochrome b (□); cytochrome a (△); cytochrome c₅₅₈ (▲).

volume of culture remained constant over the course of the experiment. Consequently, it would appear that loss of cytochrome c from thiosulphate-grown cells following exposure to an organic substrate is simply due to dilution of pre-existing molecules as a consequence of growth.

DISCUSSION

The results obtained in this study indicate that carriers mediating electron transfer from organic sources to oxygen in *Thiobacillus A2* are generally similar to those found in other bacteria (Haddock & Jones, 1977). According to our studies on inhibition of respiration, electron flow from succinate to oxygen probably involves passage through flavoprotein, quinone, b-type, c-type and a-type cytochromes. Furthermore, absorption spectra indicate that this respiratory chain is terminated by a set of conventional 'abc' cytochromes. In addition, growth yield data pertaining to batch culture of strain A2N on succinate (Loya, 1979) established that *Thiobacillus A2* oxidatively exploits succinate at least as effectively as all other bacteria (Payne, 1970; Payne & Wiebe, 1978). An efficient linkage of the above electron-transfer system to phosphorylation is thereby suggested. Indeed, addition of components to accommodate entry of electrons into the chain from donors more (or less) reduced than succinate would appear to offer a general explanation of organotrophic energy metabolism in *Thiobacillus A2*.

Most, if not all, of the elements of the organotrophic system discussed above are apparently present at reasonably high levels in thiosulphate-grown cells. Our data (Loya, 1979) and those of others (Taylor & Hoare, 1971; Peeters *et al.*, 1970) show comparable concentrations of succinate dehydrogenase and respiration of succinate in cell-free extracts regardless of whether growth was first carried out in succinate or thiosulphate. Furthermore,
the entire ‘abc’ set of carriers is present in lithotrophic cells. In fact, as shown in the transfer experiments, at least cytochromes a and b are constitutively synthesized without regard to the organic or inorganic nature of the substrate. The inability of lithotrophic cells to oxidize succinate immediately seems, therefore, merely due to insufficient substrate transport capacity. In fact, a potential to respire succinate appeared in lithotrophic cells after exposure to succinate coincident with the increase in capacity to take up succinate. It is nevertheless clear that additional inductive changes must take place before growth of lithotrophic cells on an organic energy source can proceed. For example, activities of phosphoenolpyruvate carboxylase (Smith et al., 1980) and α-ketoglutarate dehydrogenase (Peeters et al., 1970) are known to be low after growth on thiosulphate and high during organotrophic culture. Although the ‘organotrophic’ system may be necessary for lithotrophic energy metabolism, other factors are also required. Cells adapting to thiosulphate must synthesize increased amounts of cytochrome c$_{548}$. This carrier was the only one of those tested whose synthesis was not found to recommence in parallel with growth during the adaptation period, and the cells subsequently developed on succinate without high levels of c$_{548}$. Further, patterns of the overall loss of the capacity to respire thiosulphate during adaptation to succinate, as well as growth dilution of the cytochrome c$_{548}$, made it obvious that at least one of the components involved in lithotrophic energy conservation is unique.

Cells grown on thiosulphate contain all the necessary components for extracting energy from an organic substrate, and, in addition, a special c-type cytochrome specifically involved in thiosulphate respiration. Work relevant to the role of this unique cytochrome in thiosulphate oxidation will be presented elsewhere.

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