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Complete assimilation of cysteine by a newly isolated non-sulfur purple bacterium resembling *Rhodovulum sulfidophilum* (*Rhodobacter sulfidophilus*)

Abstract A rod-shaped, motile, phototrophic bacterium, strain SiCys, was enriched and isolated from a marine microbial mat, with cysteine as sole substrate. During phototrophic anaerobic growth with cysteine, sulfide was produced as an intermediate, which was subsequently oxidized to sulfate. The molar growth yield with cysteine was 103 g mol^{-1}, in accordance with complete assimilation of electrons from the carbon and the sulfur moiety into cell material. Growth yields with alanine and serine were proportionally lower. Thiosulfate, sulfide, hydrogen, and several organic compounds were used as electron donors in the light, whereas cystine, sulfite, or elemental sulfur did not support phototrophic anaerobic growth. Aerobic growth in the dark was possible with fructose as substrate. Cultures of strain SiCys were yellowish-brown in color and contained bacteriochlorophyll *a*, spheroidene, spheroidenone, and OH-spheroidene as major photosynthetic pigments. Taking the morphology, photosynthetic pigments, aerobic growth in the dark, and utilization of sulfide for phototrophic growth into account, strain SiCys was assigned to the genus *Rhodovulum* (formerly *Rhodobacter*) and tentatively classified as a strain of *R. sulfidophilum*. In cell-free extracts in the presence of pyridoxal phosphate, cysteine was converted to pyruvate and sulfide, which is characteristic for cysteine desulfhydrase activity (L-cystathionine γ-lyase, EC 4.4.1.1).

Key words Cysteine · Phototrophic bacteria · Anaerobic degradation · Non-sulfur purple bacteria · *Rhodovulum (Rhodobacter) sulfidophilum* · Microbial mats

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Introduction

Since Andreae (1985) discovered a significant impact of methylated sulfur compounds from marine environments on the global sulfur cycle, interest in the cycling of organosulfur compounds has increased. Salt marshes exhibit high rates of dimethylsulfide, dimethyldisulfide, and methanethiol emission (Cooper et al. 1987; Steudler and Peterson 1984). Sources of these methylated organosulfur compounds are dimethylsulfoniopropionate, an osmolyte of marine algae, and sulfur-containing amino acids (Taylor 1993). Some phototrophic bacteria growing with organosulfur compounds have been isolated from microbial mats. Dimethylsulfide is oxidized to dimethylsulfoxide by purple bacteria (Zeyer et al. 1987; Visscher and Van Gemerden 1991, Hanlon et al. 1994). Different phototrophic bacteria have been shown to degrade mercaptomalate and mercaptoacetate (Visscher and Taylor 1993). The purple sulfur bacterium *Thiocapsa roseopersicina* oxidizes only the sulfhydryl group of either mercaptomalate or mercaptoacetate, yielding fumarate or acrylate as end product. In contrast, the non-sulfur purple bacterium *Rhodopseudomonas* sp. utilizes both the carbon skeleton and the sulfhydryl group of these organic thiols (Visscher and Taylor 1993).

Little is known about the fate of amino acids as substrates in microbial mats. When anoxic marine sediments are amended with methionine or homocysteine, methanethiol and 3-mercaptopropionate are found as products (Kiene et al. 1990). When cysteine is added to slurries of marine sediments, 3-mercaptopyruvate, mercaptoacetate, and methanethiol are produced (Kiene et al. 1990). Several anaerobic bacteria are known to degrade cysteine, e.g., clostridia (Woods and Clifton 1937; Cardon and Barker 1947), other fermenting bacteria (Desnuelle et al. 1940), and sulfate-reducing bacteria (Forsberg 1980). Since these studies were performed with complex growth media, it was not proven whether these bacteria used cysteine as sole source of carbon and energy. Cysteine degradation was studied also with several aerobic heterotrophic bacteria of the genera *Es-*

cherichia, *Enterobacter*, *Serratia*, *Bacillus*, and *Salmonella* (Ohkishi et al. 1981). These bacteria possess cysteine desulfhydrase activity, which converts cysteine to pyruvate, ammonium, and sulfide. The cysteine desulfhydrase (EC 4.4.1.1) of *Salmonella typhimurium* has been purified and characterized as a pyridoxalphosphate-containing enzyme (Collins and Monty 1973; Kredich et al. 1973).

In the present study, we describe the complete assimilation of cysteine in a defined mineral medium by a non-sulfur purple bacterium resembling *Rhodovulum (Rhodobacter) sulfidophilum*.

Materials and methods

Sources of organisms

The enrichment culture was inoculated with a sample from laminated microbial mats at Great Sippewisset Salt Marsh, Cape Cod, Mass., USA. *Rhodobacter sulfidophilus* (strain W4, LMG 5201^T), now *Rhodovulum sulfidophilum* (Hirashi and Ueda 1994), was obtained from the LMG Culture Collection (Lab. voor Microbiologie, Universiteit Gent, Gent, Belgium).

Media and growth conditions

The brackish water mineral medium used for enrichment and cultivation contained per liter of distilled water: KH_2PO_4, 0.5 g; NaCl, 10 g; NH_4Cl, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $MgCl_2 \cdot 6H_2O$, 0.5 g; $CaCl_2 \cdot 4H_2O$, 0.1 g. After sterilization, $NaHCO_3$ (30 mM), trace elements solution SL10 (1 ml l^{-1}; Widdel et al. 1983), and vitamin solution (1 ml l^{-1}; Pfennig 1978) were added, the pH was adjusted to 6.8, and the medium was dispensed into screw-capped bottles or test tubes with rubber seals, as described by Pfennig and Trüper (1991). Substrates were added from sterile stock solutions. The L-cysteine stock solution prepared with boiled, anoxic water was filter-sterilized and maintained under a N_2 atmosphere. Sulfide was added to the cultures from a neutralized sulfide solution (Siefert and Pfennig 1984). Cultures were incubated at 28° C in front of a tungsten lamp at a light intensity of 14 W m^{-2} (Li 200 SB pyranometer sensor, 400–1,100 nm; Li-Cor, Lincoln, Neb., USA). Growth was followed by measuring the optical density at 650 nm, either with a UV 1100 spectrophotometer (Hitachi, Tokyo, Japan) or with a Spectronic 20 photometer (Milton Roy, Rochester, N.Y., USA).

Isolation and characterization

Pure cultures were obtained by repeated application of deep-agar-dilution series (Pfennig and Trüper 1991). Purity of cultures was checked microscopically and by growth tests in complex medium (AC medium, Difco, Ann Arbor, Mich., USA) at one-tenth normal strength. To study the salt requirement, different volumes of a concentrated salt solution (20 g NaCl and 3 g $MgCl_2 \cdot 6H_2O$ per 100 ml) were added to the medium. Absorption spectra of living cells were recorded in 3.5 ml of a dense cell suspension containing 5 g sucrose (Pfennig and Trüper 1991). Spectra were recorded with a UV 300 spectrophotometer (Shimadzu, Kyoto, Japan) equipped with an end-on photomultiplier. Carotenoids were extracted with methanol-acetone (2:7, v/v) and separated on silica gel 60 thin-layer plates (Merck, Darmstadt, Germany) with 10% (v/v) acetone in petroleum ether (Schmidt 1971; Züllig 1985). Carotenoids of *Rhodovulum sulfidophilum* were isolated for comparison.

Enzyme assay

Cells were harvested in the late exponential growth phase by centrifugation for 20 min at 24,000 × *g* in a Sorvall RC-2B centrifuge. Cells were washed with 50 mM Tris-HCl (pH 8.6), and 40 μg ml^{-1} DNase I and 2 mg ml^{-1} $MgCl_2 \cdot 6H_2O$ were added to the cell suspension prior to disruption of the cells by French pressure cell treatment at 138 MPa. Enzyme assays for cysteine desulfhydrase (L-cystathionine γ-lyase, EC 4.4.1.1) activity were carried out discontinuously under anoxic conditions at 30° C. The assay mixture contained: 2.5 mM L-cysteine, 0.4 mM pyridoxal 5′-phosphate and 0.2–8 mg ml^{-1} protein in 50 mM Tris-HCl, pH 8.6 (Collins and Monty 1973). Samples were withdrawn by syringes and analyzed for pyruvate, sulfide, and cysteine as described below. Protein was determined with bicinchoninic acid (Smith et al. 1985) and bovine serum albumin as standard.

Chemical analyses

To increase its stability, cysteine was derivatised with monobrombiman prior to quantification (Fahey and Newton 1987). The resulting thioether was analyzed by HPLC (System Gold, Beckman, Munich, Germany) with a reversed-phase Ultrasphere-ODS column (4.6 × 150 mm) and a mobile phase consisting of varying amounts of 0.25% (w/v) acetic acid adjusted to pH 4.0 with NaOH, and methanol (Fahey and Newton 1987). Peaks were detected by a UV detector (Beckman 167) at 242 nm. Sulfate and thiosulfate were determined by ion chromatography. The system was equipped with an anion-exchange column LCA A03 (Sykam, Gilching, Germany), a suppression column Dowex 50 WX 16 (Serva, Heidelberg, Germany), and a conductivity detector S3110 (Sykam). The anions were eluted with a mobile phase consisting of 10% (v/v) acetonitrile, 5 mM Na_2CO_3, and 50 mg l^{-1} 4-hydroxybenzonitrile. For quantification of pyruvate, an Interaction ORH-801 organic acids column (300 × 6.5 mm) packed with a cation-exchange polymer (Interaction Chemicals, Mountain View, Calif., USA) was used, eluting isocratically with 5 mM H_2SO_4. Peaks were detected by a refraction index detector ERC-7512 (Sykam). Sulfide was determined colorimetrically as described by Cline (1969).

Chemicals

Chemicals were obtained from Aldrich (Steinheim, Germany), Boehringer (Mannheim, Germany), Fluka (Neu Ulm, Germany), Merck (Darmstadt, Germany), and Sigma (Deisenhofen, Germany). All chemicals were of p.a. quality. Gases were obtained from Messer Griesheim (Ludwigshafen, Germany).

Results

Enrichment and isolation

Enrichment cultures in brackish water mineral medium containing 2 mM cysteine as sole substrate were inoculated with about 5 g wet mass of a sample from microbial mats at Great Sippewisset Salt Marsh. After 2 weeks of incubation, microbial growth was indicated by the development of a yellow-brown cell suspension. After several transfers, short motile rods dominated. These bacteria were isolated by agar dilution series with 2 mM cysteine. In these tubes, colonies appeared after 9 days, which were red-colored close to the gas phase, but yellow-brown deep in the agar. When red or yellow-brown colonies were picked and diluted separately in agar series, red and yellow-brown colonies developed again in both cases in the upper and lower parts, respectively, indicating that the differently colored colonies were formed by the same bacteria. From one of these colonies, strain SiCys was isolated.

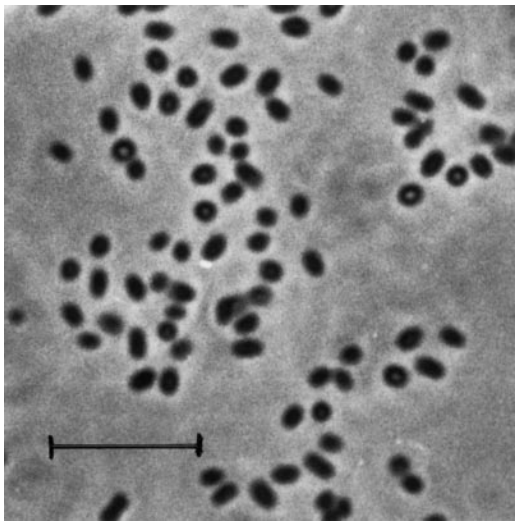

Fig. 1 Phase-contrast photomicrograph of strain SiCys. *Bar* 5 μm

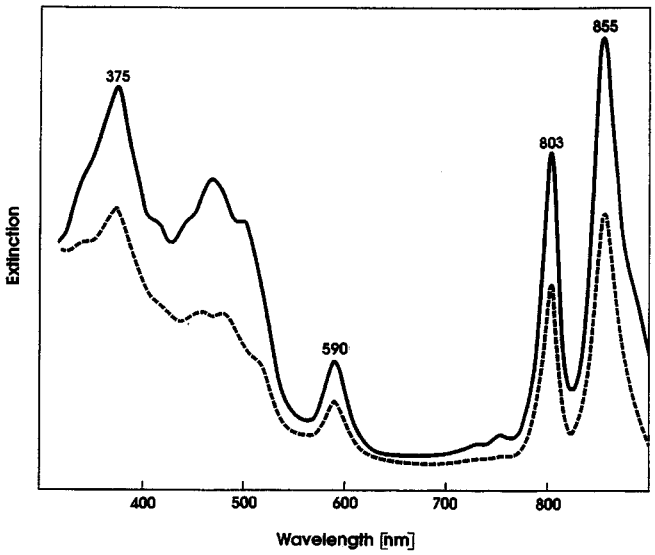

Fig. 2 Absorption spectra of living cells of strain SiCys (—), and *Rhodovulum sulfidophilum* (---). Each strain was grown with 10 mM acetate plus 4 mM cysteine. The protein content was 5.6 μg ml^{-1}

Morphology and photosynthetic pigments

Cells of strain SiCys were short motile rods, 0.7 × 1 μm in size, occasionally in short chains of 4–5 cells (Fig. 1). The color of dense cultures grown anaerobically in the light was yellow-brown and turned red after contact with air. The same phenomenon was observed with the type strain of *Rhodovulum sulfidophilum*. In vivo absorption spectra of strain SiCys and *Rdv. sulfidophilum* were similar and exhibited both the typical absorption spectra of bacteriochlorophyll-*a*-containing phototrophs (Fig. 2; maxima at 375, 590, 803, and 855 nm). The maxima between 450 and 550 nm indicated the presence of carotenoids of the spheroidene series. After methanol-acetone extraction, thin-layer chromatography, and comparison of absorption spectra, spheroidene, spheroidenone, and OH-spheroidene were identified as the most abundant carotenoids of both strain SiCys and *Rdv. sulfidophilum*.

Table 1 Comparison of the utilization of substrates by strain SiCys and by *Rhodovulum sulfidophilum*. Data for *Rhodovulum sulfidophilum (Rhodobacter sulfidophilus)* strain W4 are from Hansen and Veldkamp (1973) or from this study (*n.d.* not determined)

Substrates tested for support of phototrophic growth	Strain SiCys	*Rhodovulum sulfidophilum*
H2/CO2, formate, acetate, pyruvate, succinate, L-alanine, cysteine, HS^-, $S_2O_3^{2-}$	+	+
L-Aspartate, $S_2O_3^{2-}$	–	–
Fructose	+	–
S^0	–	+
Serine	+	n.d.
Cystine, L-threonine, L-methionine	–	n.d.

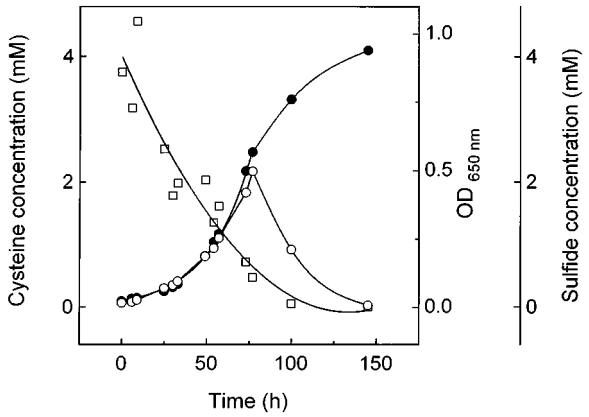

Fig. 3 Growth of strain SiCys with 4 mM cysteine. Cysteine, (□), $OD_{650\ nm}$ (●), Sulfide (○)

Physiological characterization

Strain SiCys grew with cysteine at 30° C and 14 W m^{-2} at a doubling time of 21 h ($\mu = 0.79\ d^{-1}$); growth with sulfide was considerably slower (doubling time about 30 h). Growth occurred at temperatures between 25 and 39° C and within a pH range of 5.5–7.5, with an optimum at 37° C, pH 6.5, and salt concentrations of 10 g l^{-1}. Salt concentrations between 1.4 and 23 g l^{-1} allowed growth.

Anaerobically in the light, strain SiCys and the type strain of *Rdv. sulfidophilum* utilized several organic acids and inorganic sulfur compounds (Table 1). Both strains grew with sulfide, thiosulfate, or cysteine. On these substrates, no sulfur globules were formed. No growth occurred with cystine, the poorly soluble product of cysteine oxidation. Strain SiCys grew aerobically in the dark with fructose as substrate.

Figure 3 shows a typical growth curve of strain SiCys with cysteine as sole substrate. During cysteine degrada-

Table 2 Molar growth yield and stoichiometry of cysteine degradation by strain SiCys

[a] Calculations are based on correlation of OD with dry cell material. The conversion factor used was 0.1 $OD_{650\ nm}$ = 47.2 mg dry cell matter l^{-1}

Cysteine added to the cultures (mM)	Sulfate produced (mM)	$OD_{650\ nm}$	Cell material formed[a] (mg l^{-1})	Molar growth yield (Y_s) (g mol^{-1})	Electron recovery
1	0.6	0.220	103.9	103.9	96%
2	1.9	0.434	205.0	102.5	95%
3	2.9	0.655	309.4	103.1	95%

tion, 2 mM sulfide accumulated, and was consumed subsequently. Besides sulfide, small amounts of thiosulfate (0.2 mM) were found as an additional intermediate. The yield depended linearly on the cysteine concentration between 1 and 15 mM, and decreased at higher cysteine concentrations. Strain SiCys assimilated carbon and electrons of cysteine completely, with sulfate as oxidized product (Table 2), according to the following equation:

$$17C_3H_7O_2NS + 28H_2O + 21CO_2 + NH_4^+ \rightarrow 18 <C_4H_8O_2N> + 17SO_4^{2-} + 35H^+$$

The molar growth yield (Y_S) with cysteine was 103 g mol^{-1}. Growth yields obtained with alanine and serine were lower, 67 g mol^{-1} and 61 g mol^{-1}, respectively, in accordance with the following equations:

$$17C_3H_7O_2N + 5H^+ \rightarrow 12 <C_4H_8O_2N> + 3CO_2 + 5NH_4^+ + 4H_2O$$

$$17C_3H_7O_3N + 7H^+ \rightarrow 10 <C_4H_8O_2N> + 11CO_2 + 7NH_4^+ + 9H_2O$$

Cysteine desulfhydrase activity

Cell-free extracts of strain SiCys converted cysteine to pyruvate and sulfide. Pyruvate was formed in stoichiometric amounts, whereas sulfide was produced substoichiometrically, compared to cysteine consumption. The sulfide production rate depended linearly on the protein concentration between 0.3 and 1.3 mg protein ml^{-1}. In controls without pyridoxal 5′-phosphate or without protein, no sulfide was produced. The rates of sulfide production were 21–33 nmol min^{-1} (mg protein)$^{-1}$, which is in good agreement with the in vivo turnover of cysteine in growing cultures [11 nmol min^{-1} (mg protein)$^{-1}$].

Discussion

Strain SiCys is to our knowledge the first anoxygenic phototrophic bacterium known to utilize cysteine. During phototrophic growth in the light, cysteine was completely assimilated into cell material, with sulfate as oxidized byproduct. Other amino acids, such as alanine and serine, were also completely assimilated. Alanine is more reduced than serine; therefore, alanine oxidation yields more reducing equivalents than serine oxidation. When strain SiCys grew with cysteine, sulfide was found as an intermediate, which was subsequently oxidized to sulfate. Visscher and Taylor (1993) obtained similar results for the oxidation of an organosulfur compound by another non-sulfur purple bacterium. *Rhodopseudomonas* sp. grew with mercaptomalate and oxidized both the carbon and the sulfhydryl moiety. In cell suspensions, sulfide was observed as an intermediate during mercaptomalate degradation. In contrast to the non-sulfur purple bacteria, the sulfur purple bacterium *Thiocapsa roseopersicina* used only the sulfhydryl group of mercaptomalate or mercaptopropionate for growth, and the organic residues were not further degraded (Visscher and Taylor 1993). The same holds true for dimethylsulfide oxidation to dimethylsulfoxide by the purple bacteria *Thiocystis* sp., *Thiocapsa roseopersicina*, and *Rdv. sulfidophilum* (Zeyer et al. 1987; Visscher and Van Gemerden 1991; Hanlon et al. 1994).

The first step in cysteine degradation by strain SiCys appears to be a desulfhydrase reaction (L-cystathionine γ-lyase, EC 4.4.1.1) converting cysteine to sulfide, pyruvate, and ammonia. In crude cell extracts, we found pyruvate and sulfide as products of cysteine degradation. The reaction was pyridoxal-5′-phosphate-dependent. Sulfide was not detected in stoichiometric amounts compared to cysteine consumption, perhaps because of background activities of transaminases, which might lead to an increased cysteine consumption without concomitant sulfide production. While Collins and Monty (1973) found a 1:1 stoichiometry with a fivefold-enriched cysteine desulfhydrase fraction of *Salmonella typhimurium*, other authors did not observe equimolar formation of pyruvate and sulfide by enriched enzyme fractions or crude extracts (Guarneros and Ortega 1970; Kredich et al. 1973; Morra and Dick 1991).

Strain SiCys is a short motile rod and cells divide by binary fission. The major photosynthetic pigments are bacteriochlorophyll *a*, spheroidene, spheroidenone, and OH-spheroidene. This allows affiliation of strain SiCys with the genus *Rhodovulum* (formerly *Rhodobacter;* Imhoff and Trüper 1991). Most physiological properties of strain SiCys resemble those of *Rhodovulum sulfidophilum* (Hansen and Veldkamp 1973). Both strains grow at elevated salt concentrations and use organic compounds and also sulfur compounds for phototrophic growth. Sulfide is oxidized to sulfate without formation of sulfur droplets. Thiosulfate and cysteine are used by both strains. While *Rdv. sulfidophilum* grows slowly with sulfur (Hansen and Veldkamp 1973), strain SiCys did not use sulfur at all as substrate for phototrophic growth. Both strains are able to grow aerobically in the dark. Other species of the genera *Rhodobacter* and *Rhodovulum* that form sulfate during sulfide oxidation differ from strain SiCys in other respects (Holt et al. 1994): *Rba. veldkampii*

and *Rdv. adriaticum* are non-motile and unable to use hydrogen for phototrophic growth. With *Rba. euryhalinus*, no aerobic growth has been observed.

For strain SiCys, the tolerance to high cysteine concentrations (15 mM) is remarkable since the non-sulfur purple bacteria described so far (Visscher and Taylor 1993) tolerate only smaller amounts (5 mM) of thiols. This might be an effect of sulfide toxicity since sulfide is produced as an intermediate during the degradation of the thiol compound. The tolerance towards high sulfide concentrations is an important feature of members of the genus *Rhodovulum*, while strains of the genera *Rhodopseudomonas* and *Rhodobacter* usually are more sensitive to sulfide (Hansen and Veldkamp 1973; Hirashi and Ueda 1994). Cysteine degradation by strain SiCys is a further example of an important metabolic role that sulfur compounds may play as substrates for phototrophic bacteria in microbial mats.

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