CHARACTERIZATION OF XANTHOBACTER STRAINS H4-14 AND 25A AND ENZYME PROFILES AFTER GROWTH UNDER AUTOTROPHIC AND HETEROTROPHIC CONDITIONS

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Characterization of *Xanthobacter* strains H4-14 and 25a and enzyme profiles after growth under autotrophic and heterotrophic conditions

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Abstract. All *Xanthobacter* strains studied are versatile autotrophic bacteria, able to grow on methanol and other substrates. Strain 25a, a yellow-pigmented, pleomorphic, Gram-negative bacterium, capable of autotrophic growth on methanol, formate, thiosulfate, and molecular hydrogen, was isolated from an enrichment culture inoculated with soil from a subtropical greenhouse. Subsequent studies showed that the organism also grows on a wide range of multicarbon substrates. Ammonia, nitrate and molecular nitrogen were used as nitrogen sources. The taxonomic relationship of strains H4-14 and 25a with previously described *Xanthobacter* strains was studied by numerical classification. Strain H4-14 was identified as a *X. flavus* strain, but the precise position of strain 25a remained uncertain. It probably belongs to a new species of the genus *Xanthobacter*. The levels of various enzymes involved in autotrophic and heterotrophic metabolism were determined following growth of strains H4-14 and 25a in batch and continuous cultures. The mechanisms involved in controlling ribulose-1,5-bisphosphate carboxylase/oxygenase synthesis in *Xanthobacter* strains appear to be comparable to those observed for other autotrophic bacteria, namely repression by organic compounds and derepression by autotrophic energy sources, such as methanol and hydrogen.

Key words: *Xanthobacter* – Taxonomy – Methylotrophs – Methanol – Calvin cycle – Carbon dioxide fixation – RuBisC/O – Regulation – Continuous culture

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Abbreviations: API, appareils et procédés d'identification; CS, citrate synthase; ED, Entner-Doudoroff pathway; FBP, fructose-1,6-bisphosphate; FDH, formate dehydrogenase; HPS, hexulose-6-phosphate synthase; ICDH, isocitrate dehydrogenase; KDPG, 2-keto-3-deoxy-6-phosphogluconate; MDH, methanol dehydrogenase; PRK, phosphoribulokinase; PQQ, pyrrolo quinoline quinone; RuBisC/O, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuMP, ribulose monophosphate

The genus *Xanthobacter* (Wiegel et al. 1978; Wiegel and Schlegel 1984) comprises aerobic, yellow-pigmented, facultatively autotrophic, hydrogen-oxidizing bacteria, that are able to fix dinitrogen. Three species have been described, namely *Xanthobacter autotrophicus*, *X. flavus* and *X. agilis*, with only the latter species consisting of motile strains (Jenni and Aragno 1987; Jenni et al. 1987).

All *Xanthobacter* strains studied are able to grow on methanol. The enzymology of methanol dissimilation has been studied in most detail in *Xanthobacter* H4-14 (Lidstrom-O'Connor et al. 1983; Weaver and Lidstrom 1985), strain GJ10 (Janssen et al. 1987) and strain 25a (Croes et al. 1986). Methanol oxidation is initiated by a PQQ-dependent methanol dehydrogenase and CO_2 is assimilated via the Calvin cycle. We are interested in the regulation of the synthesis of methanol dehydrogenase and the Calvin cycle enzymes. *Xanthobacter* species are very versatile autotrophic bacteria and appear to be well suited for such studies. Genetic techniques for these organisms have become available in recent years (Wilke 1980; Lehmicke and Lidstrom 1985; Weaver and Lidstrom 1987), allowing a study of regulatory mechanisms at the molecular level.

Although strains H4-14 and 25a strongly resemble *Xanthobacter* species, displaying the characteristic properties mentioned above, their precise taxonomic position is uncertain. As a prelude to further studies on metabolic regulation we have performed standardized phenotypic tests with strains H4-14 and 25a, along with the *Xanthobacter* and *Azotobacter* strains studied by Jenni et al. (1987). The type strain of *Flavobacterium capsulatum* (Leifson 1962) was also included for a comparison. *F. capsulatum* is not able to grow in H_2-lithoautotrophic conditions but it resembles *Xanthobacter* by its pleomorphism, its high G + C contents (contrary to other *Flavobacterium* species) and also by the visible spectrum of its yellow pigment.

In addition, the first results of studies on regulation of synthesis of Calvin cycle enzymes in strains H4-14 and 25a are reported.

Materials and methods

Organisms

Strain 25a was isolated from garden soil from a subtropical greenhouse (University of Groningen) as described by Levering et al. (1981), except that methanol instead of methylamine or trimethylamine was used as sole carbon- and energy source. Strain H4-14 has been described by Lidstrom-O'Connor et al. (1983). *Flavobacterium capsulatum* Leifson 1962, 163^{AL} (DSM 30196, ATCC 14666) was obtained from the DSM. Strains Al-1 (DSM 1721), Al-2 (DSM 1722) and Al-5 (NEU 2150) (which resemble *Azotobacter*; Malik and Schlegel 1980) were included for comparison as representatives of N_2-fixing, H_2-oxidizing bacteria which are not members of the genus *Xanthobacter*. All other strains studied are representatives of *Xanthobacter* species (Fig. 1) and their sources have been described previously (Jenni et al. 1987).

Cultivation conditions for phenotypical tests and data processing

Flavobacterium capsulatum was grown on nutrient agar (Difco). All other strains were grown lithoautotrophically with H_2 and CO_2 as energy and carbon source in mineral medium supplemented with biotin (Jenni et al. 1987, 1988). Cells were resuspended in API-LRA medium (API code 88803) in order to inoculate API galeries 50CH, 50AO and 50AA (API codes 50CH-5030, 50AO-88801, 50AA-88802, API-bioMericux SA, Geneva, Switzerland). Growth was checked after the first and the second week of incubation at 27°C in a water saturated incubator.

The phenotypic data were treated by numerical analysis (Jenni et al. 1987, 1988). The Simple Matching coefficient (S_{SM}; Sokal and Michener 1958), which includes both positive and negative matches, and the Jaccard coefficient (S_j; Jaccard 1908; Sneath 1957a) which does not take negative matches into account, were calculated. Distances between strains were computed as ($1-S_{SM}$) and ($1-S_j$), respectively. Phenograms were plotted from the matrices of distances using the single linkage clustering method (Sneath 1957b).

DNA isolation and G + C mol% determination

These methods were performed as described by Jenni et al. (1987). DNA was purified on urea-hydroxylapatite. The G + C mol% was determined by the Tm method and calculated according to Mandel et al. (1970).

Growth of strains H4-14 and 25a in batch culture

The media and growth conditions used for strain H4-14 have been described previously (Lidstrom-O'Connor et al. 1983; Lehmicke and Lidstrom 1985). Strain 25a was grown in the mineral salts medium (pH 7.0) described by Levering et al. (1981). Nickel was added to a final concentration of 8 µM when molecular hydrogen was used as an energy source. The medium was heat sterilized and after cooling 1 ml/l of the following vitamin solution (filter sterilized) was added (mg/l): thiamin · HCl, 300; biotin, 10. Methanol and gluconate (filter sterilized) and acetate (heat sterilized) were added to final concentrations of 100 mM, 20 mM and 30 mM, respectively. Yeast extract (0.8%, w/v) supplemented with 10 mM succinate was used as rich medium. Solid media contained 1.5% (w/v) agar. For long-term storage glycerol (final concentration 20%, v/v) was added to mid-exponential phase cultures grown in nutrient broth (0.8%, w/v) and stored at −80°C.

Strain 25a was grown in conical flasks filled for 20% of the volume and incubated in a rotary shaker (200 rpm) at 30°C. Cells were harvested at the end of the exponential growth phase. Because of their slimy nature it was necessary to harvest the cells by centrifugation at 17000 × *g* for 30 min at 4°C. The pellet was washed once with 50 mM potassium phosphate buffer pH 7.0 and resuspended in the same buffer to a concentration of 2–3 mg protein/ml and stored at −80°C.

Growth of strain 25a in continuous culture

Strain 25a was grown in carbon-limited continuous cultures in a fermenter (working volume of 1 l) of the type described by Harder et al. (1974). The growth temperature was 30°C and the pH was controlled at 7.0 by automatic adjustment with 1 M NaOH. The medium contained (per l): KH_2PO_4, 1.0 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g; trace element solution (Vishniac and Santer 1957), 0.2 ml. Filter sterilized methanol was used as the carbon- and energy source and added to the medium reservoir at a concentration of 100 mM. For the determination of enzyme activities, cells from these cultures were harvested as described above. The effect of addition of a pulse of acetate (10 mM) directly to the culture vessel was studied after the cultures had reached a steady state, which was assumed to be the case after at least 5 volume displacements.

Preparation of cell-free extracts and enzyme assays

The methods used for preparation of cell-free extracts have been described previously (Dijkhuizen et al. 1978; Lehmicke and Lidstrom 1985). Enzyme measurements were made on a Hitachi model 100-60 spectrophotometer at 30°C. In all assays the observed rate was linear for at least 3 min and was proportional to the amount of extract added. The reaction volumes were 1 ml. Protein was determined by the method of Lowry. Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) was assayed according to van Dijken and Quayle (1977), except that 50 mM Tris-HCl pH 8.5 was used as a buffer. Methanol dehydrogenase (MDH; EC 1.1.9.98) was assayed according to Ghosh and Quayle (1981), except that 125 mM Tris-HCl pH 9.0 and 1.25 mM phenazine methosulphate were used. The combined activities of 6-phosphogluconate dehydratase (EC 4.2.1.12) and KDPG aldolase (EC 4.1.2.14) were assayed according to van Dijken and Quayle (1977), except that 100 mM Tris-HCl pH 7.5 was used as buffer and reduced glutathione and lactate dehydrogenase at concentrations of respectively 3 mM, and 2 units per ml. The following enzymes were assayed according to published methods: Citrate synthase (CS, EC 4.1.3.7), Srere (1969); isocitrate lyase (EC 4.1.3.1), Dixon and Kornberg (1959); isocitrate dehydrogenase (ICDH, EC 1.1.1.42), Levering and Dijkhuizen (1985); fructose-1,6-bisphosphate (FBP) aldolase (EC 4.1.2.13) and hexulose phosphate synthase (HPS), Levering et al. (1981); ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisC/O; EC 4.1.1.39) and NAD-dependent formate dehydrogenase (FDH; EC 1.2.1.2), Dijkhuizen et al. (1978); phosphoribulokinase (PRK; EC 2.7.1.19) and hydrogenase, Lehmicke and Lidstrom (1985).

Results

Isolation of strain 25a

During attempts to isolate facultative RuMP cycle methylotrophs (Levering et al. 1981) using methanol as sole source of carbon and energy, bacteria forming yellow-pigmented colonies were frequently obtained. HPS, the key enzyme of the RuMP cycle, could not be detected in cell-free extracts prepared from these organisms. Instead, RuBisC/O activity, indicative for the operation of the

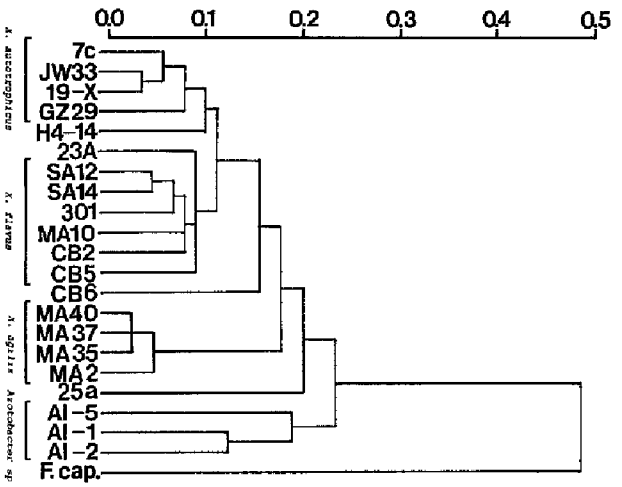

Fig. 1. Phenogram obtained from the API results presented in Table 1. The scale indicates the distance ($1\text{-}S_{SM}$), i.e. 0.0 equals 100% similarity

Calvin cycle, was consistently found to be present in cells grown on methanol. One of these isolates, designated strain 25a, was used for further studies.

Characterization of strains 25a and H4-14

A preliminary characterization of strain H4-14 has been reported by Lidstrom-O'Connor et al. (1983). Strain 25a was capable of autotrophic growth on methanol, formate, hydrogen and thiosulfate (both at 30 and at 37° C). Growth on hydrogen was nickel dependent. In addition, the organism could grow heterotrophically on a number of organic substrates in liquid medium: acetate, lactate, dihydroxyacetone, pyruvate, glycerol, succinate, citrate, gluconate, yeast extract and nutrient broth. Substrates which did not support growth were: erythritol, fructose, glucose, lactose, malonate, oxalate, ribose, sorbose, sorbitol, sucrose, xylitol and xylose. Ammonium chloride, nitrate and molecular nitrogen were used as nitrogen sources. Biotin was required for growth. Strain 25a was Gram-negative, as judged from a positive aminopeptidase reaction, and formed pleomorphic rod-shaped cells. These properties suggest that strain 25a belongs to the genus *Xanthobacter* (Wiegel et al. 1978; Wiegel and Schlegel 1984). Also the G + C contents of DNA of strain H4-14 and strain 25a (67–68 mol%) agree well with those reported for other *Xanthobacter* strains (Jenni et al. 1987). Cells of strain 25a showed the irregular appearance characteristic for other *Xanthobacter* strains, with refractile inclusions at both ends of the cells. Also the visible spectrum of its pigment (not shown) was in accordance with those of other *Xanthobacter* strains. Interestingly, cells of strain 25a were clearly motile after prolonged periods (> 10 days) of growth in liquid media under autotrophic conditions, or with gluconate.

API analysis of strains H4-14 and 25a

An API analysis was used to further establish the taxonomic positions of strains 25a and H4-14 (Table 1). The phenogram shown in Fig. 1 was computed from the data in Table 1 by single linkage clustering with the ($1\text{-}S_{SM}$) distance. This phenogram looks very similar to those obtained by classical phenotypic analysis and DNA :DNA hybridization studies of *Xanthobacter* strains (Jenni et al. 1987). This indicates that the API substrate assimilation tests are applicable for the classification of new strains of the genus *Xanthobacter*. The same method was applied successfully for the taxonomy of *Alcaligenes eutrophus* (Jenni et al. 1988). A limitation of this method (also noticed by Jenni et al. 1988) is that negative growth with a few substrates in API galeries turns out to be positive when tested in aerated liquid cultures. Examples of this with strain 25a are acetate, glycerol, gluconate, which did not support grow in API tests but the organism clearly was able to assimilate these substrates in liquid cultures (see above).

In Fig. 1 strain H4-14 is positioned between *X. autotrophicus* and *X. flavus* with a distance of 0.1 ($1\text{-}S_{SM}$). Using the complete linkage clustering (not shown), strain H4-14 is related preferentially to *X. flavus*. Strain H4-14 shares with the members of the *X. flavus* species the biotin requirement, the ability to grow on aspartate, n-butyrate, 3-hydroxy-butyrate, 2-keto-gluconate, 2-keto-glutarate, glycolate, oxalate, propionate, and inability to use N-caproate and mesotartrate.

Strain 25a is related with each of the described species of *Xanthobacter* (Fig. 1), presenting an overall similarity of 80% [i.e. ($1\text{-}S_{SM}$) = 0.2]. This fairly high relationship could imply that strain 25a is a member of a new *Xanthobacter* species, but the numerous negative responses (Table 1) could also lead to misinterpretation. When using the Jaccard coefficients (S_j), not taking the negative matches into account for the calculation of distances (Jenni et al. 1987), strain 25a is not more related to *Xanthobacter* than to *Flavobacterium capsulatum* (not shown).

Enzyme profiles of strain H4-14

The levels of RuBisC/O, PRK and hydrogenase were determined in cells of strain H4-14 grown on a variety of substrates (Table 2). Cells grown on $H_2/CO_2/O_2$ contained significant amounts of all three enzymes, but no activity was detectable in cells grown with organic compounds. Addition of hydrogen to the gas atmosphere of the latter cultures resulted in hydrogenase levels comparable to those observed in autotrophically-grown cells. Under these conditions the activities of RuBisC/O and PRK varied strongly with the organic substrate used (Table 2). Cells grown with succinate and fructose showed no activities, glycerol-grown cells showed intermediate levels while gluconate-grown cells possessed activities

Table 1. Phenotypic characteristics (API tests) of strains A: 7C; B: JW33; C: 19-X; D: GZ29; E: 23A; F: SA12; G: SA14; H: MA10; I: 301; J: CB2; K: CB5; L: CB6; M: MA40; N: MA37; O: SA35; P: MA2; Q: AL-5; R: AL-1; S: AL-2; T: H4-14; U: 25a; V: F. cap.
−: no growth; +: growth; ~: very weak response; b: black colored

Tests	Strains and results																					
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
API 50 CH																						
Glycerol	+	+	−	+	+	+	+	~	+	~	+	+	−	−	−	−	+	+	+	+	−	+
Erythritol	−	−	−	−	−	~	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Ribose	~	−	~	−	−	−	−	−	−	−	−	−	−	−	−	−	−	~	+	−	−	−
D-Glucose	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+	+	−	−	+
D-Fructose	+	+	+	+	+	+	+	+	−	−	−	+	−	−	−	−	+	+	+	+	−	+
D-Mannose	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
α-Methyl-D-glucoside	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	~	+	−	−	−
Arbutine	b	b	b	b	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Salicine	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	+
Cellobiose	−	−	−	+	−	−	−	~	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Maltose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+
Sucrose	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+
Trehalose	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Melezitose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+
Starch	−	−	−	−	−	~	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+
Glycogen	+	+	−	+	−	~	−	−	−	−	−	−	~	−	~	−	~	+	+	−	−	−
D-Turanose	−	−	−	−	−	~	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
D-Lyxose	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Arabitol	−	−	−	−	−	~	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Gluconate	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	~	~	~	+	−	+
2-Keto-gluconate	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	~	+	−	+	−	−
5-Keto-gluconate	+	+	−	+	+	+	+	+	+	+	~	+	~	~	~	~	+	−	−	−	−	−
API 50 AO																						
Acetate	~	~	+	+	~	+	~	+	~	−	−	+	+	+	+	+	~	~	−	−	−	−
Propionate	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	−	−	~	+	−	+
Butyrate	+	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+	−	+	−	+	−	−
Isobutyrate	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	−	−	−	+	−	−
n-Valerate	+	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+	+	−	−	+	−	−
Isovalerate	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	−	−	−	+	−	−
n-Caproate	+	+	+	+	~	−	−	−	−	~	−	~	−	−	−	+	−	~	−	−	−	−
Oxalate	+	+	+	+	+	+	+	+	+	−	−	−	−	+	~	~	−	−	−	+	−	−
Succinate	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	−	−	−	+	+	+
Maleate	~	+	~	~	+	~	~	~	~	~	+	~	−	−	−	−	−	−	−	−	−	−
Fumarate	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	−	+	~	+	~	+
Glutarate	+	+	+	+	~	+	+	+	+	+	+	−	+	+	+	+	−	−	−	+	~	−
Adipate	~	−	~	+	~	+	+	+	+	+	+	−	+	+	+	−	−	−	−	−	~	−
Pimelate	−	−	−	+	−	−	−	−	−	−	−	−	+	+	+	+	~	+	~	−	−	−
Suberate	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−	~
Azelate	−	−	−	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	~
Sebacate	−	−	−	+	−	−	−	−	−	−	−	+	+	+	+	+	~	−	−	−	−	+
Glycolate	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	−	+	+	−	−
DL-Lactate	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	~	~	+	+	+
DL-Glycerate	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	+	~	+
DL-3-Hydroxybutyrate	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
D-Malate	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	~	−
L-Malate	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	~	+	+	+
Mesotartrate	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	~
Levulinate	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	+
2-Keto-glutarate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	+	−
Citraconate	~	−	~	−	+	−	~	+	+	~	−	−	−	−	−	−	−	−	−	−	−	−
Itaconate	−	−	~	−	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−
Mesaconate	−	−	−	−	−	+	+	−	−	−	−	+	+	+	−	+	−	−	−	−	+	−
Aconitate	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
Citrate	+	+	+	+	+	−	−	−	+	+	+	+	−	−	−	−	−	−	−	+	~	−
Phenylacetate	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−
Benzoate	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
o-Hydroxy-benzoate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
m-Hydroxy-benzoate	+	−	−	−	−	−	−	−	−	+	−	−	+	+	+	−	+	+	+	+	−	−
p-Hydroxy-benzoate	+	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	+	~	~	+	−	+
D-Mandelate	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−
L-Mandelate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
iso-Phthalate	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Table 1. Continue

Tests	Strains and results																				
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
API 50 AA																						
L-Alanine	~	−	~	−	−	−	−	~	~	−	−	−	−	−	−	−	−	+	−	−	+
L-Cysteine	~	~	~	~	~	~	~	~	~	+	~	~	+	~	~	~	−	−	−	−	−
L-Phenylalanine	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−
L-Tyrosine	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	+
L-Aspartate	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	+	+	+	+	−	+
L-Glutamate	+	+	+	+	+	+	+	+	+	+	+	~	−	−	−	−	+	+	+	+	−	+
Betaine	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	~	+	−	−	+
Acetamide	−	−	−	−	−	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
Ethylamine	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Benzylamine	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−

Substrates utilized by none of the strains:

API 50 CH: D-arabinose, L-xylose, adonitol, methyl-xyloside, L-sorbose, dulcitol, inositol, mannitol, sorbitol, methyl-D-mannoside, amygdaline, esculine, melibiose, inuline, xylitol, D-tagatose, D-fucose, L-arabitol

API 50 AO: heptanoate, caprylate, pelargonate, caprate, malonate, D-tartrate, L-tartrate, phthalate, tere-phthalate

API 50 AA: glycine, D-α-alanine, L-norleucine, DL-norvaline, DL-2-aminobutyrate, L-methionine, D-tryptophan, L-tryptophan, trigonelline, L-lysine, L-citrulline, DL-kynurenine, creatine, β-alanine, DL-3-aminobutyrate, DL-4-aminobutyrate, DL-5-aminovalerate, 2-aminobenzoate, 3-aminobenzoate, 4-aminobenzoate, urea, butylamine, amylamine, ethanolamine, diaminobutane, spermine, histamine, tryptamine

Substrates utilized by Flavobacterium capsulatum (DSM 30196) only:

API 50 CH: L-arabinose, D-xylose, galactose, rhamnose, N-acetyl-glucosamine, lactose, D-raffinose, β-gentiobiose, L-fucose

API 50 AA: L-leucine, L-isoleucine, L-valine, L-serine, L-threonine, L-histidine, L-ornithine, L-arginine, L-proline, sarcosine, glucosamine

Table 2. Regulation of RuBisC/O, PRK and hydrogenase in *Xanthobacter* strain H4-14

Growth conditions	Enzyme activities[a]		
	RuBisC/O	PRK	Hydrogenase
H_2/CO_2, 25% air[b]	59	45	270
Succinate, fructose, glycerol, gluconate (20% air)	<1	<1	<1
Succinate, 80% H_2, 20% air	<1	<1	290
Fructose, 80% H_2, 20% air	<1	<1	330
Glycerol, 80% H_2, 20% air	20	15	335
Gluconate, 80% H_2, 20% air	55	40	300
Gluconate, formate, 20% air	55	50	nd

[a] nmol · min^{-1} · mg^{-1} of protein

[b] Mixture (v/v) of 75% H_2/CO_2 (H_2:CO_2 = 6:1) and 25% air

nd, not determined

Organic substrates, 0.2% (w/v)

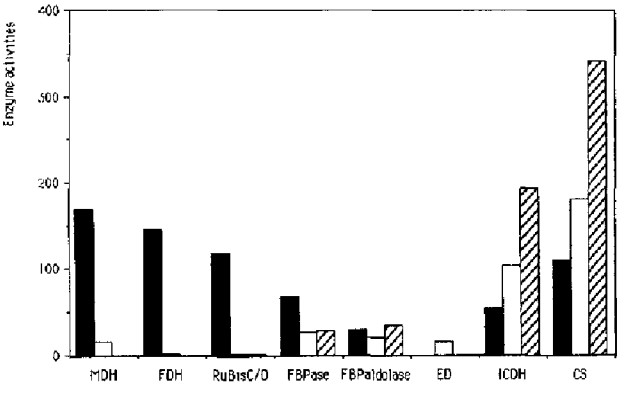

Fig. 2. Enzyme profiles of *Xanthobacter* strain 25a after growth on methanol (100 mM; ■), gluconate (15 mM; □) and acetate (30 mM; ▨) in batch cultures. CS, citrate synthase; ED, Entner-Doudoroff pathway enzymes; FBP aldolase, fructose-1,6-bisphosphate aldolase; FBPase, fructose-1,6-bisphosphatase; FDH, formate dehydrogenase; ICDH, isocitrate dehydrogenase; MDH, methanol dehydrogenase; RuBisC/O, ribulose-1,5-bisphosphate carboxylase/oxygenase. Enzyme activities: nmol · min^{-1} · mg^{-1} of protein. Specific activities of ICDH: ×0.1

equal to those in cells grown on H_2 and CO_2. These enzymes activities were also present in gluconate-grown cells when an alternate energy source, formate, was substituted for H_2. The levels of methanol dehydrogenase (MDH) and formate dehydrogenase (FDH) in *Xanthobacter* H4-14 have been reported previously (Weaver and Lidstrom 1985).

Enzyme profiles of strain 25a grown in batch cultures

Following growth of strain 25a in batch cultures on gluconate, acetate or methanol (with doubling times of 3.3, 2.4 and 4.5 h, respectively), the activities of various enzymes were determined in extracts prepared from late exponential growth phase cells (Fig. 2). MDH, FDH and RuBisC/O were only present in high activities after

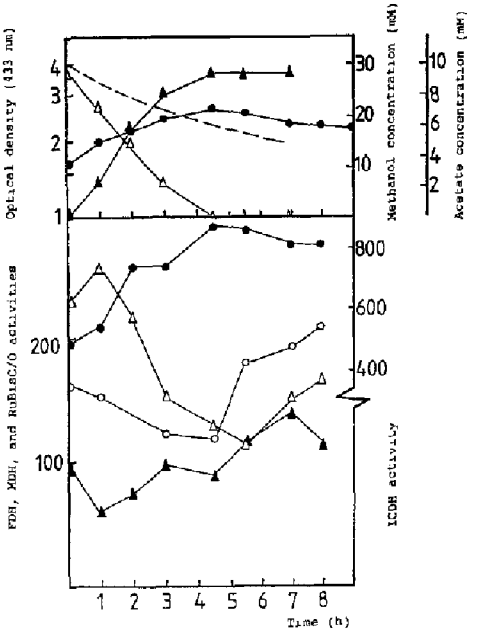

Fig. 3. Addition of acetate (10 mM) to a methanol-limited (S_R = 100 mM) continuous culture of *Xanthobacter* strain 25a growing at D = 0.10 h^{-1}. *A* ●, optical density; △, acetate concentration; ---, wash-out kinetics for acetate; ▲, methanol concentration. *B* △, methanol dehydrogenase; ▲, formate dehydrogenase; ○, RuBisC/O; ●, isocitrate dehydrogenase. Enzyme activities: nmol · min^{-1} mg^{-1} of protein

growth on methanol. Minor activities of MDH were detected in gluconate-grown cells. Although no formaldehyde dehydrogenase activity could be detected in extracts of methanol-grown cells, whole cells were able to oxidize formaldehyde. This activity was absent in acetate- or gluconate-grown cells. FBPase and FBP aldolase activities were present after growth on each of these substrates. In methanol-grown cells the activity of FBPase was twice as high as in extracts of acetate- or gluconate-grown cells. This may be a reflection of the involvement of this enzyme in the regeneration of RuBP, the acceptor molecule for CO_2 in the Calvin cycle, during growth on methanol. The combined activities of 6-phosphogluconate dehydratase and KDPG aldolase, indicative for the operation of the Entner-Doudoroff pathway, were only present in extracts of gluconate-grown cells.

The levels of the TCA cycle enzymes citrate synthase (CS) and isocitrate dehydrogenase (ICDH) varied strongly. Lowest activities were observed after growth on methanol. Almost four times higher activities were detected in acetate-grown cells, reflecting a shift in the role of the TCA cycle from merely biosynthetic to dissimilatory and biosynthetic. Interestingly, isocitrate lyase activity could not be detected in extracts of acetate-grown cells of strain 25a.

Effect of the addition of acetate to a methanol-limited continuous culture of strain 25a

To study the regulation of the methanol metabolism in more detail, strain 25a was grown in a continuous culture (D = 0.10 h^{-1}) with methanol (S_R = 100 mM) as the limiting substrate. After reaching steady state conditions, acetate was added directly to the culture (at zero time) to a final concentration of 10 mM (Fig. 3). Acetate utilization started immediately, which resulted in an increase in optical density and accumulation of residual methanol. After the disappearance of acetate from the culture vessel (4.5 h after its addition), the optical density slowly decreased to reach the original steady state value after 20 h. Over the same period methanol was consumed to completion (not shown).

The acetate pulse had a dramatic effect on the enzyme levels in the culture. MDH activity decreased at a rate comparable to the wash-out rate, suggesting a complete cessation of enzyme synthesis. RuBisC/O activity also decreased but not at the wash-out rate, while the activity of FDH remained fairly constant. Acetate utilization was reflected by an initially strong increase in ICDH levels. All activities returned to their steady state values after the complete utilization of acetate in the culture vessel (t = 20 h; not shown).

Discussion

Strain 25a displays the characteristic properties of members of the genus *Xanthobacter* (autotrophic growth on hydrogen, methanol and formate; nitrogen fixation; yellow cellular pigmentation; pleomorphic appearance; G + C mol% of 67–68; Wiegel et al. 1978; Wiegel and Schlegel 1984; Jenni et al. 1987). Also the API analysis data indicated a fairly high relationship between this isolate and the three *Xanthobacter* species that have been described, but its precise taxonomic position remained unclear. Strain 25a has certain features in common with *X. autotrophicus* and *X. flavus* (growth on nutrient broth and gluconate, autotrophic growth at 37°C, pleomorphism and slime production; Jenni et al. 1987). A most striking difference, however, is its motility. The only motile *Xanthobacter* species described sofar is *X. agilis*, but strain 25a differs from this species in the above mentioned characteristic properties (Jenni and Aragno 1987). In conclusion, strain 25a is clearly not a member of the species *X. autotrophicus*, *X. flavus*, or *X. agilis*. Although it is possible than strain 25a is a member of a new *Xanthobacter* species, its precise relationship with the genus *Xanthobacter* needs further investigation. We suggest that strain 25a remains a *Xanthobacter* sp. until further data are available.

On the basis of a preliminary characterization, Lidstrom-O'Connor et al. (1983) suggested that strain H4-14 is most similar to *X. flavus*. The data obtained in the present study further support this conclusion.

A study of enzyme profiles in *Xanthobacter* strains H4-14 and 25a showed that the synthesis of RuBisC/O (and PRK) in both strains is under strict metabolic control, and regulated in a similar fashion as observed in other autotrophic bacteria (Dijkhuizen and Harder 1984; Tabita 1988). Synthesis of these Calvin cycle enzymes is

completely repressed under heterotrophic growth conditions. Derepression occurs during growth on methanol or $H_2/CO_2/O_2$ and following addition of an "autotrophic" energy source to cultures growing with organic substrates that cause a lesser degree of repression (i.e. gluconate). Interestingly, addition of acetate to the culture vessel of a methanol-limited chemostat of *Xanthobacter* strain 25a only caused a partial repression of RuBisC/O synthesis (Fig. 3). In contrast, addition of acetate to thiosulfate- and formate-limited cultures of *Thiobacillus versutus* (Gottschal and Kuenen 1980) and *Pseudomonas oxalaticus* (Meijer and Dijkhuizen 1988), respectively, resulted in complete repression.

The results further indicate that synthesis of MDH, FDH, and RuBisC/O in *Xanthobacter* sp. 25a is not coordinately regulated. MDH and FDH are both present at high levels during growth in a methanol-limited chemostat, but only synthesis of MDH became completely repressed following addition of acetate (Fig. 3). The repression of MDH by gluconate is less severe (Fig. 2). The data therefore suggest that MDH is induced by methanol, or products derived, and repressed to various degrees by "heterotrophic" substrates. A similar regulatory pattern for this enzyme was observed in *Xanthobacter* strain H4-14 (Weaver and Lidstrom 1985) and *Paracoccus denitrificans* (de Vries et al. 1988).

The Entner-Doudoroff system is inducible by gluconate in *Xanthobacter* strain 25a. This was also observed in *Nocardia opaca* (Probst and Schlegel 1973) and *P. oxalaticus* (Gordon and McFadden 1980). No activity of the glyoxylate cycle enzyme isocitrate lyase could be detected in *Xanthobacter* strains following growth on acetate. Thus, as is the case in a number of other bacteria (Claassen et al. 1986), the pathway(s) used in these organisms for assimilation of acetate still remains to be established.

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