

Methanol Metabolism in *Corynebacterium* sp. XG, a Facultatively Methylotrophic Strain

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Corynebacterium sp. XG is a facultative methylotroph. Determination of the activities of several enzymes and use of mutants unable to grow on methanol indicated that in this Gram-positive strain methanol was metabolized through the serine pathway. Synthesis of the enzymes of this pathway was induced when cells were grown in the presence of methanol or its catabolic products.

INTRODUCTION

Bacteria able to grow on C₁ compounds are heterotrophic and facultative or obligate methylotrophs. Two cyclic routes are known for assimilation of C₁ substrates in such organisms: the ribulose monophosphate (RuMP) pathway and the serine pathway. While C₁ compounds enter the RuMP cycle at the level of formaldehyde, the serine pathway utilizes formaldehyde plus carbon dioxide. A third route of assimilation, the Calvin cycle, is found only in autotrophic methylotrophs, which assimilate carbon from the reduced C₁ growth substrate after oxidation to carbon dioxide (see reviews by Colby *et al.*, 1979; Anthony, 1982; Large, 1981).

The increasing interest in micro-organisms utilizing methanol has led to the isolation of a large number of novel methylotrophs. This has created the basis for comparative investigations of the peculiarities of methanol metabolism in methylotrophs differing in taxonomic position. Duménil *et al.* (1983) described a bacterial strain growing on methanol as carbon and energy source, as well as on a range of other compounds, i.e. sugars and organic acids. This facultative methylotroph has been classified into the genus *Corynebacterium* on the basis of its morphological and physiological characteristics (Duménil *et al.*, 1983), including the mol% G + C value and the presence of corynomycolic acids in its cell wall (J. Asselineau, personal communication). It was named *Corynebacterium* sp. XG.

No information on the metabolism of methanol in strain XG was available (Andriantsoa *et al.*, 1984; Laget *et al.*, 1987). As corynebacteria are widely used in industrial fermentations, such a strain growing on C₁ compounds could be of interest from a biotechnological perspective. Thus we decided to investigate the assimilatory pathway for methanol metabolism and its regulation in *Corynebacterium* sp. XG.

METHODS

Media and growth conditions. Strain XG was grown in mineral salts medium FB4, which contained (g l⁻¹): (NH₄)₂SO₄, 3; (NH₄)₂HPO₄, 2; KH₂PO₄, 1.18; Na₂HPO₄.2H₂O, 2.13; CaCl₂.2H₂O, 0.01; FeSO₄.7H₂O, 0.01; MnSO₄.H₂O, 0.025; CoCl₂.6H₂O, 0.02; MgSO₄.7H₂O, 0.9; NaCl, 5; pH 7.0. Sodium succinate was sterilized separately and used at a concentration of 1.6% (w/v). Methanol (99.8%, v/v) was filter-

Abbreviation: RuMP, ribulose monophosphate.

sterilized and used at a concentration of 1% (v/v). Methylamine, serine and sodium formate were used at 0.4% (w/v). All liquid cultures were incubated at 30 °C on a rotatory shaker in Erlenmeyer flasks. For plating, agar medium M1 was used (Duménil *et al.*, 1983), modified by omitting yeast extract, and supplemented with substrates as above.

Isolation of mutants. Mutants unable to grow on methanol were isolated using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; 1 mg ml⁻¹) in 50 mM-Tris/maleate buffer pH 6, resulting in more than 98% death after 2 h treatment. After NTG treatment and growth in FB4-succinate medium, cells were washed and resuspended in FB4-methanol medium containing penicillin G (1000 units ml⁻¹). After three cycles of penicillin treatment, cells were plated on M1 medium plus succinate and mutants were identified by replica plating.

Preparation of cell-free crude extracts. Cells in the exponential phase were centrifuged, washed twice in ice-cold 0.02 M-sodium phosphate buffer pH 7, resuspended in the same buffer (containing 5 mM-MgCl₂) to a final concentration of about 0.4 g wet wt ml⁻¹ and then sonicated at 4 °C for periods of 30 s at 60 W (20 kc, Branson Sonifier B15 ultrasonic disintegrator). Total exposure time was 7 min. The sonic extract was then centrifuged at 30000 g for 30 min at 4 °C and the supernatant was used for enzyme assays.

Enzyme assays. Spectrophotometric assays were performed on a Kontron Uvikon 820 spectrophotometer fitted with a constant-temperature housing (30 °C unless otherwise stated).

Methanol dehydrogenase (EC 1.1.99.8) was assayed using phenazine methosulphate as electron acceptor at 37 °C by the method of Bamforth & Quayle (1978) except that the pH of the Tris buffer was adjusted to 10 and 1 mM-KCN was added. Formaldehyde dehydrogenase (NAD⁺ dependent) (EC 1.2.1.1) was assayed at 37 °C by the method of Johnson & Quayle (1964) modified by using 100 mM-sodium phosphate buffer pH 8, 0.4 mM-NAD⁺, 3 mM-reduced glutathione and 16 mM formaldehyde. Formate dehydrogenase (NAD⁺ dependent) (EC 1.2.1.2) was assayed at 37 °C by the method of Johnson & Quayle (1964) modified by using 100 mM-sodium phosphate buffer pH 8, 0.4 mM-NAD⁺ and 125 mM-sodium formate.

For the assay of other enzyme activities the following methods were used without modification: glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) (DeMoss, 1955); hexulose phosphate synthase and hexulose phosphate isomerase (Cox & Zatman, 1974); fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) (van Dijken & Quayle, 1977); 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) and 6-phosphogluconate dehydratase (EC 4.2.1.12) (van Dijken & Quayle, 1977); transaldolase (EC 2.2.1.2) (Tchola & Horecker, 1966); hydroxypyruvate reductase (EC 1.1.1.81) (Large & Quayle, 1963); serine-glyoxylate aminotransferase (EC 2.6.1.45) (Blackmore & Quayle, 1970); isocitrate lyase (EC 4.1.3.1) (Dixon & Kornberg, 1959); glycerate kinase (EC 2.7.1.31) (Heptinstall & Quayle, 1970); ribulose-bisphosphate carboxylase (EC 4.1.1.39) (Bradley & Carr, 1977).

Assays were performed in duplicate against a blank containing all reactants except the substrate of the reaction. In all cases a linear relationship was obtained between protein concentration and enzyme activity. Enzyme activities are expressed as nmol min⁻¹ (mg protein)⁻¹.

Protein concentration was determined by the method of Lowry with bovine serum albumin as standard.

Chemicals. All reagents were from Sigma. Formaldehyde was prepared by heating 0.5 g paraformaldehyde in 5 ml distilled water at 100 °C for 18 h in a sealed tube.

RESULTS AND DISCUSSION

Activities of enzymes involved in methanol metabolism

Specific activities in cells grown either on methanol or on succinate were compared (Table 1). Methanol was oxidized by methanol dehydrogenase. Subsequent oxidation of formaldehyde and formate was catalysed by the corresponding dehydrogenases with NAD⁺ as cofactor. Reduced glutathione was not required for formaldehyde dehydrogenase activity. Increased specific activities of all three enzymes were observed for cells grown on methanol compared to succinate. RuMP cycle enzymes (listed in Table 1) were detected only at very low values compared to those reported for bacteria using this pathway (Zatman, 1981; Roitsch & Stolp, 1985); these values did not increase during growth on methanol. In contrast, the enzymes of the serine pathway occurred at high specific activities in cells grown with methanol (Table 1). The activities substantially decreased when growth was on succinate. As isocitrate lyase (icl) activity was not detected, it appears that strain XG uses the icl⁻ variant of the serine cycle for assimilation of methanol.

The growth of strain XG (and mutants derived from it: see below) was studied on various carbon sources (Table 2). Strain XG grew on methylamine, and slow growth could be obtained on formate. Thus oxidation of formate gives energy and reducing power that can be used to convert formate (via formyltetrahydrofolate) to 5,10-methylene tetrahydrofolate, which then could enter the serine pathway; a possible role of the Calvin cycle can also be proposed, as a low,

Table 1. Specific activities of enzymes involved in methanol metabolism by *Corynebacterium sp. XG*

Enzyme*	Specific activity† [nmol min ⁻¹ (mg protein) ⁻¹] of cells grown on:	
	Methanol	Succinate
I. Methanol dehydrogenase	608	50
Formaldehyde dehydrogenase (NAD ⁺)	11.5	6.5
Formate dehydrogenase (NAD ⁺)	60	23.5
II. Glucose-6-phosphate dehydrogenase (NAD ⁺)	3.3	3.3
(NADP ⁺)	1.6	3.3
6-Phosphogluconate dehydrogenase (NAD ⁺)	1.6	3.3
(NADP ⁺)	ND	ND
Hexulose phosphate synthase	1.6	1.6
Fructose-1,6-bisphosphate aldolase	28.5	58.5
2-Keto-3-deoxy-6-phosphogluconate aldolase	ND	ND
Transaldolase	21.5	30
III. Hydroxypyruvate reductase	1270	290
Serine-glyoxylate aminotransferase	487	40
Glycerate kinase	80	26.5
Isocitrate lyase	ND	ND

* I, enzymes of methanol dissimilation; II, enzymes of the RuMP cycle; III, enzymes of the serine pathway.

† All results are the mean of duplicate experiments. Variation about the mean was not greater than $\pm 10\%$. ND, not detected.

Table 2. Growth properties and enzyme specific activities of strain XG and its mutants

Carbon source	Growth of:							
	Wild-type XG	Mutants						
		M2	M5	M3	M4			
Methanol	+	-	-	-	-			
Formate or methylamine	+	+	+	-	-			
Formate + serine	+	+	+	+	+			
Enzyme	Specific activities [nmol min ⁻¹ (mg protein) ⁻¹]*							
	Strain XG grown on:				Mutants grown on M + S			
	M	S	M + S	MA	M2	M5	M3	M4
Methanol dehydrogenase	608	50	231	398	ND	ND	57	119
Formaldehyde dehydrogenase	11.6	6.6	10	12.8	10.5	13.3	6.6	9.6
Formate dehydrogenase	60	23	56.5	7.5	50	66.5	6.5	8
Hydroxypyruvate reductase	1270	290	660	1020	390	480	730	560
Serine-glyoxylate aminotransferase	487	40	138	265	38	48	103	99
Glycerate kinase	79	27	64	86	45	55	56	15

*Carbon substrates: M, methanol; S, succinate; MA, methylamine; M + S, methanol + succinate. Doubling times for strain XG were 4 h in S, 5.5 h in M, approximately 13 h for MA; similar values were obtained with the mutants. All results tabulated are the means of duplicate experiments. Variation about the mean was not greater than $\pm 10\%$. ND, Not detected.

but detectable ribulose biphosphate carboxylase specific activity [$3 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] could be detected (see Large, 1981; Attwood & Quayle, 1984).

While a few reports in the literature (see review by Anthony, 1982; Hazeu *et al.*, 1983; Reed & Dugan, 1987; Dijkhuizen *et al.*, 1988) describe the ability of Gram-positive bacteria (*Bacillus*, *Arthrobacter*, *Mycobacterium*) to grow on reduced C_1 compounds, the metabolic pathway(s) used have not been elucidated in these organisms. Several Gram-positive bacteria that use RuMP pathway have however been studied (e.g. Hanson, 1980; Hazeu *et al.*, 1983). A strain of *Mycobacterium* able to assimilate methanol by the autotrophic pathway has been reported by Loginova & Trotsenko (1979), but to our knowledge no report exists concerning bacteria of the genus *Corynebacterium* that assimilate methanol using the serine pathway. Strain XG would be the first such example.

Regulation of expression of the serine pathway enzymes

The above results indicated that the synthesis of several enzymes was induced (or depressed) during growth on methanol. In order to investigate this regulation further, methanol⁻succinate⁺ mutants were isolated after NTG treatment; as strain XG was quite resistant to mutagens, relatively few mutants were obtained and only four were studied. These independently isolated mutants appear to comprise two distinct groups, viz. strains M2 and M5, and strains M3 and M4.

As none of the mutants grew on methanol, growth was performed on succinate plus methanol (for induction of enzyme synthesis). There was no evidence of biphasic growth and cells were harvested at the same optical density. Specific activities were then compared to those of the parental strain grown under similar conditions; it should be noted that the comparison is not exact, as methanol could be metabolized during growth of the wild-type with concomitant variation of concentration.

No methanol dehydrogenase activity was detected in strains M2 and M5, as expected from their inability to grow on methanol and their ability to grow on formate or on methylamine (metabolized through formaldehyde) (Table 2).

More complex phenotypes were displayed by strains M3 and M4. Neither grew on formate or methylamine but both could grow on formate plus serine (though not on methanol plus serine), indicating that assimilation was impaired. In addition, the 'induced' levels of several enzymes were severely affected. We did not detect a total loss of any enzyme activity, which would indicate the inactivation of a gene; however, it should be noted that in our assays with crude extracts a basal level could be due to non-specific reactions interfering with the assay. One possible explanation for this pleiotropic phenotype is that the mutation affects a regulatory mechanism.

Several conclusions may be drawn concerning the induction of synthesis of the enzymes. (i) Formate dehydrogenase was induced only by methanol, and not by methylamine, which could be oxidized by another pathway (Anthony, 1982); this may explain the higher doubling time on methylamine (Table 2). This regulation is impaired in mutants M3 and M4. (ii) Methylamine and methanol induced formaldehyde dehydrogenase and methanol dehydrogenase (regulation was again apparently impaired in mutants M3 and M4). (iii) A common derivative of methanol and methylamine was the inducer of serine-glyoxylate aminotransferase and possibly of hydroxypyruvate reductase. The regulation thus appears to be complex.

There were few data in the literature until recently on regulation of gene expression in methylotrophs (for more detail see review by Anthony, 1982; Allen *et al.*, 1984), whose genetics is poorly developed. Recently the use of recombinant DNA techniques with *Methylobacterium* (formerly *Pseudomonas extorquens*) strain AM1 allowed Nunn & Lidstrom (1986*a, b*) to identify 10 complementation groups of mutations involved in the methanol oxidation step.

In order to study the serine pathway in strain XG at a molecular level, the possibility of genetic transfer was examined. No indigenous low-molecular-mass plasmid was found in this bacterium. Transformation of protoplasts using a derivative of a plasmid isolated in a non-methylotrophic *Corynebacterium* (J. C. Patte, unpublished data) was also unsuccessful.

Heterologous complementation of other methylotrophs using *Corynebacterium XG* DNA will be tested in order to obtain further information on the genetic organization of the corresponding genes and the regulation of their expression.

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