Investigation of thermolabile variants of the methanol and methylamine dehydrogenases of *Methylophilus methylotrophus* and the effect of an 11.5 kbp region of the chromosome on the stability of these enzymes

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The thermal stabilities *in vitro* of the methylamine and methanol dehydrogenases of *Methylophilus methylotrophus* varied, depending on the conditions in which the organism was grown. Methylamine dehydrogenase activity was more stable in extracts of cells grown on methylamine plus NH_4^+ or on methanol plus methylamine than when methylamine provided the sole carbon and nitrogen source. In contrast, the methanol dehydrogenase activity in extracts of methylamine-grown cells was more stable than that from cells grown on medium containing methanol with either NH_4^+ or methylamine as the nitrogen source. The stability of thermolabile forms of the dehydrogenases present in extracts of mutants which have temperature-sensitive defects in the oxidation of C_1 compounds also varied with the growth conditions. When cosmid pAD833, which complemented these temperature-sensitive mutants, was present during growth of the wild-type strain on methylamine, the thermal stabilities of both methylamine and methanol dehydrogenases increased. However, the effects of subclones of pAD833 were complex and our results indicate that the DNA region carried on this cosmid encodes gene products which can both increase and decrease the *in vitro* thermal stability of enzymes involved in C_1 metabolism.

Introduction

Methanol dehydrogenase (EC 1.1.99.8), a pyrrolo-quinoline-quinone-dependent periplasmic protein composed of two alpha and two beta subunits, catalyses the first step in the oxidation of methanol by Gram-negative methylotrophs (Anthony, 1988). Methylamine oxidation is catalysed by methylamine dehydrogenase (EC 1.4.99.3), which is another periplasmic quinoprotein composed of two alpha and two beta subunits (Anthony, 1988); the prosthetic group of this enzyme is tryptophan tryptophylquinone (McIntire *et al.*, 1991).

We have recently isolated two temperature-sensitive mutants of *Methylophilus methylotrophus* with defects in the oxidation of C_1 compounds (Hutchinson & Goodwin, 1993). One (mutant NH-7) was unable to grow on either methanol or methylamine at the restrictive temperature (42 °C); it grew slowly on methanol at 30 °C, and the

cells were more fragile than those of the wild-type strain. Preliminary studies indicated that this mutant makes thermolabile forms of at least three proteins involved in C_1 oxidation, namely methanol and methylamine dehydrogenases and cytochrome c_L . These unstable forms could arise if NH-7 is defective in a gene required for the translocation, processing or assembly of these periplasmic proteins.

The other mutant, NH-4, also had a thermolabile methylamine dehydrogenase, but in this strain methanol dehydrogenase was normal. The mutant was unable to grow on methylamine as sole carbon and nitrogen source at 42 °C but, surprisingly, it could grow at this temperature when methylamine provided the sole carbon source and NH_4^+ was an alternative nitrogen source, and on medium containing methylamine as sole nitrogen source and methanol as an alternative carbon source. The methylamine oxidation system of cells grown under these conditions was not thermolabile, in contrast to that in cells grown on methylamine at 30 °C.

In this paper we present further information about the thermal denaturation of the methanol and methylamine dehydrogenases of the wild-type and mutant strains

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and we demonstrate that an 11.5 kbp region of *M. methylotrophus* DNA has complex effects on the stability of these enzymes in the wild-type strain.

Methods

Growth of bacteria. The bacterial strains and cosmids used are shown in Table 1 of the preceding paper (Hutchinson & Goodwin, 1993). Bacteria were grown in S2 salts medium as described by Southgate & Goodwin (1989) and Dawson & Goodwin (1990). Methanol and/or methylamine hydrochloride were added to give final concentrations of 0.4% (v/v) and 0.4% (w/v) respectively.

Whole-cell oxidation studies. The abilities of whole cells to oxidize methanol and methylamine were determined as described by Dawson *et al.* (1990). For each strain the assays were done using at least two concentrations of cells and each experiment was repeated with three different batches of cells. Results were reproducible to within $\pm 5\%$.

Preparation of cell extracts and enzyme assays. Cell extracts were prepared as described by Southgate & Goodwin (1989). Methanol dehydrogenase was assayed polarographically by the method of Dunstan *et al.* (1972), as modified by Tatra & Goodwin (1985) and Southgate & Goodwin (1989). Methylamine dehydrogenase was also assayed polarographically, using a method based on that of Eady & Large (1968) as modified by Dawson *et al.* (1990). In both assays phenazine ethosulphate was the electron acceptor. For the inactivation experiments extracts were incubated at 50 °C for 1 h; samples were removed every 10 min and assayed at 50 °C. Each enzyme assay was repeated using at least two concentrations of extract to check that the enzyme was limiting. Each experiment was repeated with at least two different batches of cells. Results were reproducible to within $\pm 5\%$.

Results

Oxidation of methylamine by strains grown on different combinations of carbon and nitrogen sources

The unexpected growth properties of mutant NH-4 prompted us to investigate in more detail both the methylamine oxidation system and the methylamine dehydrogenase activity of the wild-type and mutant strains grown at 30 °C on different carbon and nitrogen sources (Table 1). Whole cells of the wild-type grown on methylamine (as sole carbon and nitrogen source), methanol plus methylamine, or methylamine plus NH_4^+ , oxidized methylamine at similar rates and the assay temperature (30 °C or 42 °C) had little effect on activity. In contrast, the specific activity of methylamine dehydrogenase assayed at 30 °C was tenfold lower in cells grown on methanol plus methylamine than in cells using methylamine as the sole carbon source. Furthermore, the assay temperature did affect the activity of methylamine dehydrogenase: the enzyme from cells grown on methanol plus methylamine or on methylamine plus NH_4^+ was two- to threefold more active at 42 °C than at 30 °C, and a further increase in activity was observed when the assay was done at 50 °C. Wild-type cells grown

on methanol plus NH_4^+ did not oxidize methylamine at a detectable rate, nor did they contain measurable methylamine dehydrogenase activity.

The methylamine oxidation activity of whole cells of mutant NH-4 grown on methylamine (as sole carbon and nitrogen source) and assayed at 30 °C was slightly higher than that observed in wild-type cells (Table 1); however, as described previously (Hutchinson & Goodwin, 1993), the ability of the mutant cells to oxidize methylamine was reduced almost fourfold when the assay was done at 42 °C, indicating that this activity was thermolabile. In contrast, the methylamine oxidation activity in mutant NH-4 grown on methanol plus methylamine or on methylamine plus NH_4^+ was 1.5- to 2-fold greater than in wild-type cells when assayed at 30 °C and it was not thermolabile, confirming results previously obtained using cells grown at 42 °C (Hutchinson & Goodwin, 1993). The thermolability of the methylamine oxidation activity of mutant NH-7, unlike that of NH-4, did not depend on the combination of carbon and nitrogen sources in the growth medium.

Assays of methylamine dehydrogenase activity in the mutants grown under different conditions also yielded unexpected results. Firstly, methylamine dehydrogenase activity was detected at 30 °C in extracts of the mutants grown on methanol plus NH_4^+ whereas it was never detected in wild-type cells grown under these conditions. Furthermore, when NH-7 was grown under conditions where methylamine provided the carbon and/or nitrogen source the specific activity of methylamine dehydrogenase at 30 °C was between three- and tenfold greater than in extracts of wild-type cells grown under the same conditions; however, the enzyme was thermolabile, the activity decreasing by at least 50% when it was assayed at 42 °C.

The effect of the assay temperature on the methylamine dehydrogenase of mutant NH-4 varied, depending on the initial growth conditions. As shown previously, the enzyme in extracts of methylamine-grown cells was about fourfold less active at 42 °C than at 30 °C (Hutchinson & Goodwin, 1993). When cells were grown at 30 °C on methylamine plus NH_4^+ the methylamine dehydrogenase activity of NH-4 assayed at 30 °C was very low compared with the other strains, although it decreased by less than 50% at 42 °C. In contrast, in extracts of NH-4 grown on methanol plus methylamine the specific activity was three times greater at 42 °C than at 30 °C. However, our previous results indicated that the specific activity of methylamine dehydrogenase in extracts of mutant NH-4 grown on methanol plus methylamine at 42 °C was low and that the activity was similar at 30 °C and 42 °C (Hutchinson & Goodwin, 1993). Thus the temperature of growth, as well as the growth substrates, influenced the specific activity of the

Table 1. Oxidation of methylamine by wild-type and mutant strains grown at 30 °C on different carbon and nitrogen sources

Methylamine oxidation by whole cells is expressed as nmol oxygen used min^{-1} (mg total cell protein) $^{-1}$ and the specific activity of methylamine dehydrogenase as nmol oxygen used min^{-1} (mg protein) $^{-1}$. Values are the means of three independent experiments. ND, Not detectable; –, not done.

Carbon/nitrogen source(s)	Oxidation of methylamine by whole cells at (°C)		Specific activity of methylamine dehydrogenase at (°C)		
	30	42	30	42	50
<i>Wild-type</i>					
Methylamine	172	141	122	171	196
Methanol/methylamine	146	149	10	29	71
Methylamine/ NH_4^+	195	192	107	256	364
Methanol/ NH_4^+	ND	ND	ND	ND	–
<i>Wild-type(pAD833)</i>					
Methylamine	–	–	104	–	602
<i>Wild-type(pAD833-1)</i>					
Methylamine	–	–	230	–	271
<i>Wild-type(pAD833-2)</i>					
Methylamine	–	–	170	–	161
<i>NH-4</i>					
Methylamine	231	64	714	145	140
Methanol/methylamine	366	350	55	179	156
Methylamine/ NH_4^+	329	457	9	5	5
Methanol/ NH_4^+	ND	ND	10	ND	–
<i>NH-4(pAD833)</i>					
Methylamine	–	–	401	–	1109
<i>NH-4(pAD833-1)</i>					
Methylamine	–	–	520	–	612
<i>NH-4(pAD833-2)</i>					
Methylamine	–	–	482	–	542
<i>NH-7</i>					
Methylamine	147	53	1316	667	200
Methanol/methylamine	145	66	106	34	5
Methylamine/ NH_4^+	141	37	350	103	72
Methanol/ NH_4^+	5	ND	22	ND	–
<i>NH-7(pAD833)</i>					
Methylamine	–	–	729	–	1225
<i>NH-7(pAD833-1)</i>					
Methylamine	–	–	264	–	221
<i>NH-7(pAD833-2)</i>					
Methylamine	–	–	973	–	51

mutant methylamine dehydrogenase and its response to alterations in assay temperature.

Inactivation of methylamine dehydrogenase in extracts of wild-type and mutant cells

The effect of temperature on the methylamine dehydrogenase activities in wild-type and mutant strains grown at 30 °C on (a) methylamine, (b) methanol plus methylamine, and (c) methylamine plus NH_4^+ was further investigated by incubating extracts at 50 °C, removing samples every 10 min and assaying for activity at 50 °C.

The initial activities of the wild-type enzyme in extracts of cells grown in each of the three conditions was greater at 50 °C than at 30 °C (Table 1) and this effect was considerably more pronounced in cells grown on methanol plus methylamine or on methylamine plus NH_4^+ than when methylamine provided the sole carbon and nitrogen source. Furthermore, when cells were grown on methylamine plus NH_4^+ or on methanol plus methylamine the activities increased during the 1 h incubation at 50 °C (Fig. 1). However, the methylamine dehydrogenase of cells grown on methylamine as sole carbon and nitrogen source was partially inactivated during the 1 h incubation

Fig. 1. Inactivation at 50 °C of the methylamine dehydrogenases of wild-type and mutant strains of *M. methylotrophus* grown at 30 °C on different carbon and nitrogen sources: (a) methylamine, (b) methanol plus methylamine, and (c) methylamine plus NH_4^+ . Extracts, prepared as described in Methods, were incubated at 50 °C; samples were removed every 10 min and assayed for methylamine dehydrogenase activity at 50 °C. Initial activities are shown in Table 1. ■, Wild-type; ▼, mutant NH-4; ●, mutant NH-7.

at 50 °C. The original activity was regained when the temperature of assay was decreased to 30 °C, suggesting that the denaturation was reversible.

As expected, the methylamine dehydrogenase of mutant NH-4 was more thermolabile than the wild-type enzyme, but the activity of the enzyme from mutant cells grown on methanol plus methylamine or on methylamine plus NH_4^+ was lost less rapidly than that from methylamine-grown cells (Fig. 1). Eighty percent of the original activity was regained when extracts which had been incubated for 1 h at 50 °C were assayed at 30 °C, indicating that the loss of activity was partially reversible. In contrast, the methylamine dehydrogenase of mutant NH-7 grown on methanol plus methylamine or on methylamine plus NH_4^+ was inactivated completely within 10 min; the enzyme from methylamine-grown cells was more stable, but still lost 90% of its activity over 40 min. The activity of the methylamine dehydrogenase of NH-7 was not regained when samples were assayed at 30 °C.

Oxidation of methanol by strains grown on different combinations of carbon and nitrogen sources

The methanol oxidation activity assayed at 30 °C in the wild-type was higher in cells grown at 30 °C in the presence of methanol than when methylamine provided the sole carbon source (Table 2). The specific activity of methanol dehydrogenase was also several-fold higher, but there was no direct correlation with the ability of whole cells to oxidize methanol.

The effect of assay temperature on the ability of wild-type cells to oxidize methanol depended on the nitrogen

source in the growth medium. In cells grown in the presence of NH_4^+ the methanol oxidation activity was slightly greater at 42 °C than at 30 °C. However, when methylamine provided the sole nitrogen source for growth the methanol oxidation activity at 42 °C was 30–50% lower than at 30 °C. Similar results were obtained with mutant NH-4, but the methanol oxidation activity of mutant NH-7 assayed at 30 °C was lower than in the wild-type and was undetectable when the mutant was grown on methylamine plus NH_4^+ . Furthermore, when methanol oxidation activity of NH-7 was assayed at 42 °C the activity fell, sometimes to undetectable levels. The specific activity of methanol dehydrogenase in extracts of NH-7 was always lower when assayed at 42 °C than at 30 °C, but the decrease was least in cells grown on methylamine plus NH_4^+ .

Inactivation of methanol dehydrogenase

The thermostability of methanol dehydrogenase in extracts of wild-type and mutant strains grown at 30 °C on methylamine (as sole carbon and nitrogen source), on methanol plus NH_4^+ , and on methanol plus methylamine, was also investigated. In extracts of wild-type cells the assay temperature had little effect on the initial activity (Table 2). However, a time course of inactivation of methanol dehydrogenase at 50 °C indicated that the enzyme from cells grown in the presence of methanol was less stable than that from cells which used methylamine as sole carbon source (Fig. 2). The methanol dehydrogenase activity of mutant NH-7 was much more thermolabile than the wild-type enzyme; no activity was detected at 50 °C in methylamine-grown cells and the

Table 2. Oxidation of methanol by wild-type and mutant strains growth at 30 °C on different carbon and nitrogen sources

Methanol oxidation by whole cells is expressed as nmol oxygen used min^{-1} (mg total cell protein) $^{-1}$ and the specific activity of methanol dehydrogenase as nmol oxygen used min^{-1} (mg protein) $^{-1}$. Values are the means of at least three independent experiments. ND, Not detectable; –, not done.

Carbon/nitrogen source(s)	Oxidation of methanol by whole cells at (°C)		Specific activity of methanol dehydrogenase at (°C)		
	30	42	30	42	50
<i>Wild-type</i>					
Methylamine	276	181	124	167	196
Methanol/methylamine	456	343	743	701	702
Methylamine/ NH_4^+	30	36	154	269	–
Methanol/ NH_4^+	426	490	1069	1133	1184
<i>Wild-type(pAD833)</i>					
Methylamine	–	–	118	–	196
<i>Wild-type(pAD833-1)</i>					
Methylamine	–	–	123	–	160
<i>Wild-type(pAD833-2)</i>					
Methylamine	–	–	624	–	1224
<i>NH-4</i>					
Methylamine	333	257	116	156	140
Methanol/methylamine	544	423	776	730	730
Methylamine/ NH_4^+	58	108	107	164	–
Methanol/ NH_4^+	495	541	1333	1241	1173
<i>NH-4(pAD833)</i>					
Methylamine	–	–	110	–	208
<i>NH-4(pAD833-1)</i>					
Methylamine	–	–	132	–	175
<i>NH-4(pAD833-2)</i>					
Methylamine	–	–	917	–	1680
<i>NH-7</i>					
Methylamine	97	14	12	ND	ND
Methanol/methylamine	77	20	208	66	10
Methylamine/ NH_4^+	ND	ND	41	32	–
Methanol/ NH_4^+	17	ND	442	164	156
<i>NH-7(pAD833)</i>					
Methylamine	–	–	120	–	182
<i>NH-7(pAD833-1)</i>					
Methylamine	–	–	630	–	840
<i>NH-7(pAD833-2)</i>					
Methylamine	–	–	169	–	20

enzyme from cells grown on methanol plus methylamine or on methanol plus NH_4^+ lost most of its activity during the first 10 min of incubation at 50 °C (Fig. 2).

Effect of recombinant cosmids which complement mutants NH-4 and NH-7 on the stability of methylamine and methanol dehydrogenases

We have previously shown that cosmid pAD833, which was isolated from a *Sau3A* clone bank of *M. methylotrophus* DNA, complemented both NH-4 and NH-7

(Hutchinson & Goodwin, 1993). Subclone pAD833-1 complemented NH-7 and subclone pAD833-2 complemented NH-4.

The activity and stability *in vitro* of the methylamine dehydrogenase of the mutant and wild-type strains carrying pAD833 and its subclones was investigated in detail. The specific activity of this enzyme assayed at 30 °C in extracts of the wild-type and wild-type(pAD833) grown on methylamine was similar (Table 1). However, when assayed at 50 °C the enzyme in wild-type extracts increased in activity by about 60%, whereas that from cells grown in the presence of the cosmid increased about

Fig. 2. Inactivation at 50 °C of the methanol dehydrogenases of wild-type and mutant strains of *M. methylotrophus* grown at 30 °C on different carbon and nitrogen sources: (a) methylamine, (b) methanol plus NH_4^+ , and (c) methanol plus methylamine. Extracts, prepared as described in Methods, were incubated at 50 °C; samples were removed every 10 min and assayed for methylamine dehydrogenase activity at 50 °C. Initial activities are shown in Table 2. ■, Wild-type; ▼, mutant NH-4; ●, mutant NH-7.

Fig. 3. Effect of cosmid pAD833 and its subclones on the inactivation of the methylamine dehydrogenases of wild-type and mutant strains. Bacteria were grown at 30 °C on medium containing methylamine as the sole carbon and nitrogen source. Extracts, prepared as described in Methods, were incubated at 50 °C; samples were removed every 10 min and assayed for methylamine dehydrogenase activity at 50 °C. Initial activities are shown in Table 1. (a) ■, Wild-type(pAD833); ■, wild-type(pAD833-1); □, wild-type(pAD833-2). (b) ▼, Mutant NH-4(pAD833); ▼, mutant NH-4(pAD833-1); ▽, mutant NH-4(pAD833-2). (c) ●, mutant NH-7(pAD833); ●, mutant NH-7(pAD833-1); ○, mutant NH-7(pAD833-2). The dashed lines indicate the response of the respective parent strain which did not carry a cosmid (taken from Fig. 1).

sixfold. The activities of the methylamine dehydrogenases of the complemented mutants were also higher at 50 °C than at 30 °C, whereas the enzymes from the parent strains were thermolabile. Thus it is likely that a gene product(s) encoded by pAD833 plays a role in the stimulation of activity when the assay temperature is increased. The presence of pAD833 during growth also increased the thermal stability of the wild-type and

mutant methylamine dehydrogenases during a 60 min incubation at 50 °C (Fig. 3).

The increased activity and thermal stability of methylamine dehydrogenase in extracts of cells carrying pAD833 is presumably the result of a gene dosage effect, but results obtained using subclones of this cosmid indicate that it is unlikely to be due simply to the increased level of the product of one specific gene (Table

Fig. 4. Effect of cosmid pAD833 and its subclones on the inactivation of the methanol dehydrogenases of wild-type and mutant strains. Bacteria were grown at 30 °C on medium containing methylamine as sole carbon and nitrogen source. Extracts, prepared as described in Methods, were incubated at 50 °C; samples were removed every 10 min and assayed for methanol dehydrogenase activity at 50 °C. Initial activities are shown in Table 2. (a) $\blacksquare$, Wild-type(pAD833-1); $\blacksquare$, wild-type(pAD833-2). (b) $\blacktriangledown$, Mutant NH-4(pAD833); $\blacktriangledown$, mutant NH-4(pAD833-1); $\triangledown$, mutant NH-4(pAD833-2). (c) $\bullet$, mutant NH-7(pAD833); $\bullet$, mutant NH-7(pAD833-1); $\circ$, mutant NH-7(pAD833-2). The dashed lines indicate the response of the respective parent strain which did not carry a cosmid (taken from Fig. 2).

1, Fig. 3). The specific activities of the methylamine dehydrogenases assayed at 50 °C in extracts of the wild-type and mutant NH-4 carrying pAD833-1 were higher than in the parent strains which did not carry the cosmids, but the effect was not so pronounced as with pAD833 (Table 1). Furthermore, the activity was only slightly greater at 50 °C than at 30 °C. The methylamine dehydrogenase activity at 50 °C in mutant NH-4 complemented by pAD833-2 was similar to that in NH-4(pAD833-1), but in the wild-type carrying pAD833-2 the specific activity was slightly less than that in the parent strain which did not carry the cosmid.

The two subclones of pAD833 had unexpected effects on the thermal stability of methylamine dehydrogenase (Fig. 3). When extracts of either the wild-type or mutant NH-4 carrying subclone pAD833-2 were incubated at 50 °C over a 1 h period, the methylamine dehydrogenase activity fell over the first 20 min by about 30%, then recovered over the next 40 min. However, the effect of subclone pAD833-1 was different in the two strains. In extracts of wild-type(pAD833-1), activity fell by about 20% over the first 40 min, then recovered to the original level over the next 20 min. The enzyme present in extracts of mutant NH-4(pAD833-1) was more stable than that from extracts of mutant cells which did not contain this subclone, but it lost nearly 60% of its activity in the first 40 min of incubation at 50 °C and then recovered slightly in the next 20 min.

The influence of the two subclones on the methylamine dehydrogenase of mutant NH-7 was also different (Fig. 3). Cosmid pAD833-1 complemented mutant NH-7 and

the specific activity of methylamine dehydrogenase assayed at 30 °C or at 50 °C was similar to that in extracts of wild-type(pAD833-1), and about fivefold less than in extracts of NH-7(pAD833). The effect of incubation at 50 °C over a 1 h period on the methylamine dehydrogenase activity in extracts of mutant cells which carried this subclone was similar to that in the wild-type(pAD833-1). In contrast, in extracts of NH-7(pAD833-2) the initial activity of methylamine dehydrogenase was about fourfold higher than in NH-7(pAD833-1) and this decreased by about 50% over the first 20 min of incubation at 50 °C and then remained fairly constant.

When cosmid pAD833 was introduced into NH-7 the mutant regained its ability to grow on methanol as well as on methylamine at 42 °C; the specific activity of methanol dehydrogenase in extracts of the complemented mutant grown on methylamine was similar to that in the wild-type, and the enzyme was completely stable during incubation at 50 °C for 1 h (Table 2, Fig. 4). The presence of this cosmid during growth of the wild-type (or mutant NH-4) on methylamine at 30 °C also increased the stability of the wild-type enzyme (Fig. 4). Mutant NH-7 was also complemented by cosmid pAD833-1 and the methanol dehydrogenase activity in mutant and wild-type strains grown in the presence of this subclone was completely stable during incubation at 50 °C. However, when subclone pAD833-2 was present during growth the methanol dehydrogenase activity of the wild-type and mutant strains was much less stable than normal (Fig. 4), although the initial activities in

extracts of both the wild-type and mutant NH-4 were enhanced several-fold compared with the parent strains.

Discussion

Our results confirm the observations of Dawson *et al.* (1990) that methylamine is not oxidized by wild-type *M. methylotrophus* grown in the absence of methylamine and that methylamine oxidation and methylamine dehydrogenase activities are induced or derepressed in cells grown on media containing methylamine as sole carbon and/or nitrogen source. We have also confirmed that there is no obvious correlation between the activities of (a) methylamine oxidation and methylamine dehydrogenase and (b) methanol oxidation and methanol dehydrogenase, and our results are consistent with the suggestion that it is unlikely that either dehydrogenase catalyses the rate-limiting step in the oxidation of its particular substrate. In addition, we have demonstrated that the effect of temperature on the *in vitro* activities of both the oxidation systems and the dehydrogenases of the wild-type and mutant NH-4 vary with the growth conditions, as do the thermal stabilities of the dehydrogenases. The thermolability of the methylamine dehydrogenase activity of mutant NH-7 also varied with the growth conditions, but in contrast with the other two strains, the enzyme was most stable in cells using methylamine as sole carbon and nitrogen source. Furthermore, the loss of activity of the enzyme in mutant NH-7 was irreversible, whereas the inactivation of the enzymes from the wild-type and mutant NH-4 was reversible. Thus, although the mutations in both NH-4 and NH-7 resulted in an alteration in the conformation of methylamine dehydrogenase (Hutchinson & Goodwin, 1993), the effects on the thermal stability of this enzyme were different.

Our results imply that *M. methylotrophus* must be able to synthesize one or more factors which affect the response of components of the C_1 oxidation pathways to temperature, and that their production varies with the growth conditions. Further evidence for this is provided by the experiments using cosmid pAD833 and its subclones. The methylamine dehydrogenase in extracts of wild-type(pAD833) grown on methylamine was more stable than the enzyme from cells grown in the absence of this cosmid (Fig. 3) and this decreased rate of inactivation was presumably due to the effect of increased concentration of one or more of the gene products encoded by plasmid pAD833. However, there was still a slight loss of activity during incubation at 50 °C for 1 h, in comparison with the 40–60% increase in activity of the enzyme from cells grown on methanol plus methylamine or on methylamine plus NH_4^+ in the absence of the cosmid. Further work is required to determine

whether cells grown on these substrates have very high levels of expression of the relevant chromosomal genes which are carried on pAD833, or whether additional gene products are involved.

The effects of the presence of the two subclones of pAD833 during growth indicate that the parent cosmid encodes at least two different gene products which affect the stability of methylamine dehydrogenase *in vitro*. In extracts of wild-type cells grown in the presence of either subclone, the enzyme activity during incubation at 50 °C fell and then recovered, although the time course of recovery was different (Fig. 3). Thus these gene products probably help to promote refolding and/or reassembly of the mature protein rather than preventing its denaturation. It is possible that they can act together, since in extracts of cells grown in the presence of pAD833, there was little loss of activity during incubation at 50 °C. However, we cannot exclude the possibility that the subcloning has inactivated either a gene encoding a protein which prevents denaturation *in vitro* or a gene essential for the correct folding and/or assembly of methylamine dehydrogenase *in vivo*, loss of which would lead to the formation of a more thermolabile enzyme.

The thermostability of the methanol dehydrogenase of wild-type cells also depended on the substrates used for growth, but in this case the enzyme from cells grown on methylamine (as sole carbon and nitrogen source) was more stable during incubation at 50 °C for 1 h than that from cells provided with methanol plus methylamine (Fig. 2). The presence of cosmid pAD833 or pAD833-1 during growth resulted in the production of a more stable enzyme (Fig. 4). However, the methanol dehydrogenase in extracts of cells carrying pAD833-2 was more thermolabile than normal. This contrasts with the loss and then recovery of the methylamine dehydrogenase activity in extracts of cells carrying pAD833-2 during the 1 h incubation at 50 °C. pAD833-2 may encode a protein which enhances the thermal denaturation of methanol dehydrogenase *in vitro*; alternatively the maturation/assembly of methanol dehydrogenase could be affected, such that a less stable form of the enzyme is produced.

Our results therefore indicate that the factors determining the thermostability of methanol and methylamine dehydrogenases in extracts of *M. methylotrophus* are complex. We have identified a region of the *M. methylotrophus* chromosome which encodes gene products that both prevent and promote loss of activity *in vitro* in response to a rise in temperature. Further work is needed to characterize the relevant genes and to determine whether they encode polypeptides similar to any of the previously described molecular chaperones. It will also be interesting to ascertain if alterations in the level of expression of these genes contribute to the

differences observed in *in vitro* in the activities and thermal stabilities of these enzymes in cells grown on different combinations of growth substrates.

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