Expression of the structural *mox* genes in *Paracoccus denitrificans* follows wild-type regulation in mutants with a deletion in *mxoY*, the gene encoding the signal sensor

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During growth on the C₁ substrates methanol or methylamine, *Paracoccus denitrificans* is able to activate the expression of the genes encoding methanol dehydrogenase. In a previous paper the isolation of an operon containing two regulatory genes, *mxoYX* (formerly known as *moxYX*) and a third gene *mxoZ* (formerly known as *moxZ*) was described. MxoY and MxoX were shown to have homology with the signal sensors and the response regulators, respectively. Here we describe the isolation and characterization of mutants with marked and unmarked mutations in *mxoZ*, *mxoY* and *mxoX*. Expression of the structural *mox* genes was analysed by measuring the expression of a *mxoF(moxF)–lacZ* transcriptional fusion in the presence of *mxoZ*, *mxoY*, *mxoX* or combinations of these genes. Mutants that were unable to express *mxoX* were impaired for growth on methanol, did not synthesize MDH and could not express a *mxoF–lacZ* transcriptional fusion. This indicates that the response regulator MxoX is essential for expression of the structural *mox* genes. Mutants that had a deletion in *mxoY* or both *mxoY* and *mxoZ* were able to grow on methanol and were able to regulate the expression of the *mxoF–lacZ* fusion just like the wild-type. These findings indicate that *mxoY*⁺ and *mxoZ*⁺ are not essential for normal C₁ regulation. In addition the results suggest that, at least in the absence of the signal sensor MxoY, MxoX can be activated via a different, but parallel, signal transduction pathway.

Keywords: *Paracoccus denitrificans*, *mxo*, methanol oxidation, two-component regulation, parallel signal transduction

INTRODUCTION

Paracoccus denitrificans is a Gram-negative bacterium that is able to grow on methanol (Cox & Quayle, 1975), which is oxidized by the periplasmic methanol dehydrogenase (MDH). This enzyme contains two different subunits in an $\alpha_2\beta_2$ configuration and two molecules of the cofactor pyrroloquinoline quinone (PQQ) and at least one Ca²⁺ ion (Richardson & Anthony, 1992; White *et al.*, 1993). The oxidation of methanol in *P. denitrificans* is strictly regulated. No MDH was synthesized in cells grown heterotrophically or in cells grown on a mixture of a

heterotrophic substrate and methanol (De Vries *et al.*, 1988). Derepression, resulting in synthesis of low amounts of MDH, was observed in cells grown heterotrophically under carbon limitation in the chemostat. Methanol was not required for induction. High amounts of MDH were found in cells grown on either methanol, methylamine or choline. Formaldehyde is formed during metabolism of these substrates, and a product induction mechanism involved in activation of MDH synthesis was therefore hypothesized. Apart from the regulation of MDH synthesis, an inconsistency was observed in the relation between the amount of MDH that was synthesized and the enzyme activity measured *in vitro* (De Vries *et al.*, 1988).

Recently, we published details of the isolation and the nucleotide sequence of a 10·7 kb chromosomal fragment

Abbreviations: MDH, methanol dehydrogenase; PQQ, pyrroloquinoline quinone; RR, response regulator(s); SS, signal sensor(s).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics*	Source/reference
<i>P. denitrificans</i>		
Pd1222	Wild-type, Rif ^r , Spec ^r , enhanced conjugation frequencies	De Vries <i>et al.</i> (1989)
Pd0721	Pd1222, <i>mxz</i> ::Km ^r	Harms <i>et al.</i> (1993)
Pd0741	Pd1222, Δ <i>mxz</i>	Harms <i>et al.</i> (1993)
Pd0821	Pd1222, <i>mxzY</i> ::Km ^r	Harms <i>et al.</i> (1993)
Pd0841	Pd1222, Δ <i>mxzY</i>	This study
Pd9213	Pd1222, Δ <i>mxzYZ</i>	This study
Pd0921	Pd1222, <i>mxzX</i> ::Km ^r	Harms <i>et al.</i> (1993)
<i>E. coli</i>		
TG1	<i>supE hsdΔ5 thi Δ(lac-proAB) F'(traD36 proAB⁺ lacI^q lacZΔM15)</i>	Sambrook <i>et al.</i> (1989)
S17-1	Sm ^r <i>pro</i> r ⁻ m ⁺ , RP4-2 integrated (Tc::Mu) (Km::Tn7)	Simon <i>et al.</i> (1983)
Plasmid		
pRVS1	<i>oriV</i> (ColE1) Ap ^r <i>oriT</i> Sm ^r (Tn1831) Tn5 <i>placZ</i>	van Spanning <i>et al.</i> (1991b)
pMP190	<i>lacZ</i> without promoter, IncQ	Spaink <i>et al.</i> (1987)
pEG400	IncP Sm ^r Spec ^r pUC mcs <i>lacZ'</i>	Gerhus <i>et al.</i> (1990)
pCP112	Promoter region, pMP190 derivative	Harms <i>et al.</i> (1993)
pNH30/pMA11	5' <i>mxzY</i> , <i>mxzZ</i> , 5' <i>mxzF</i> , pMP190/pEG400 derivative	Harms <i>et al.</i> (1993)
pNH44	<i>mxzZYX</i> , 5' <i>mxzF</i> , pUC13 derivative	This study
pNH52/pWR112	<i>mxzZYX</i> , pEG400/pMP190 derivative	Harms <i>et al.</i> (1993)
pNH56/pWR124	<i>mxzZY</i> , pEG400/pMP190 derivative	Harms <i>et al.</i> (1993)
pHY4/pWR122	<i>mxzY</i> , pEG400/pMP190 derivative	This study
pHY3/pWR126	<i>mxzYX</i> , pEG400/pMP190 derivative	This study
pHY5/pWR115	<i>mxzX</i> , pEG400/pMP190 derivative	This study
pRTd0841	Δ <i>mxzY</i> , pRVS1 derivative	This study
pRTd9213	Δ <i>mxzZY</i> , pRVS1 derivative	This study

* Ap, Ampicillin; Km, kanamycin; Sm, streptomycin; Spec, spectinomycin; Rif, rifampicin; Tn5*p*, Km^r promoter of Tn5; IncQ, incompatibility group Q; mcs, multiple cloning site.

of *P. denitrificans* that is involved in methanol oxidation (De Vries *et al.*, 1988; Harms, 1993; Harms *et al.*, 1987, 1993; van Spanning *et al.*, 1991a). This fragment contains eight *mxz* genes and the 5' region of a ninth gene in the gene order *mxzXYZFJGIRS* (formerly known as *moxFJGIRS*; Lidstrom *et al.*, 1994). *mxzF* and *mxzI* are the structural genes, which encode the α and β subunits of MDH, respectively. *mxzG* codes for cytochrome c_{551i} , which is the electron acceptor for MDH. *mxzJ*, *mxzR* and *mxzS* encode proteins with unknown functions. *mxzY* and *mxzX* are regulatory genes and are transcribed together with *mxzZ* in the opposite direction to that of the other six genes. The nucleotide sequences of *mxzY* and *mxzX* reveal that their gene products have homology with the proteins of the two-component regulatory systems. MxY belongs to the class of signal sensors and MxX to the family of the response regulators. MxZ has no homology with other proteins sequenced thus far and its function is unknown.

In this paper we describe the isolation of two mutants with deletions in *mxzY* or in both *mxzY* and *mxzZ*. These mutants exhibit a wild-type phenotype with respect to methanol oxidation, which indicates that MxY is not essential for this oxidation. The results suggests that, at least in the absence of MxY, MxX can be activated via

another, parallel, signal-transduction pathway. Some of these data have been presented at the 7th International Symposium on Microbial Growth on C₁ Compounds (Harms, 1993).

METHODS

Bacterial strains, plasmids, media and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *P. denitrificans* were routinely grown either on brain heart infusion agar (BHIA)(GIBCO) or in mineral salts medium as described previously (Harms *et al.*, 1985). Filter-sterilized carbon sources were added to a final concentration of 100 mM methylamine, 100 mM methanol or 10 mM glucose. *Escherichia coli* strains were grown on BHIA or on M9 medium containing 0.4% glucose. Antibiotics were added, when necessary, to final concentrations of 40 μ g rifampicin ml⁻¹, 25 μ g kanamycin ml⁻¹, 25 μ g streptomycin ml⁻¹, 100 μ g ampicillin ml⁻¹, or 10 μ g tetracycline ml⁻¹. Strains were grown at 35 °C. Solid media contained 1.6% (w/v) agar.

Recombinant DNA techniques. DNA was manipulated essentially as described by Maniatis *et al.* (1982). Plasmid DNA was isolated by using Qiagen tip 20 (Diagen). DNA restriction fragments were purified from agarose gels using GeneClean (Bio 101). Southern hybridizations were done with GeneScreen Plus (Dupont) as specified by the manufacturer. Chromosomal DNA was isolated as described previously (Harms *et al.*, 1987).

Construction of mutants

Pd0841. The 5.5 kb *Pvu*II fragment containing *mxaX*, *mxaY*, *mxaZ* and the 5' region of *mxaF* (Harms *et al.*, 1993) was cloned in the *Sma*I site of pUC13. The resulting clone, pNH44, was digested with *Xho*I, and the *Xho*I fragment was isolated and cloned in the *Sa*I site of pUC13. The resulting clone was digested with *Hinc*II and re-ligated without the *Hinc*II fragments. The clone thus obtained, containing an in-frame deletion in *mxaY*, was digested with *Eco*RI, the sticky ends were filled, and the clone was subsequently digested with *Sph*I. The fragment was isolated and cloned in the *Sma*I and *Sph*I sites of the suicide vector pRVS1. The resulting clone, pRTd0841, was used to generate a deletion in *mxaY* by homologous recombination.

Pd9213. Plasmid pNH44 was digested with *Sma*I and subsequently re-ligated without the *Sma*I fragment. This clone, containing a large deletion in *mxaY* and *mxaZ*, was digested with *Eco*RI, the *Eco*RI fragment was isolated and cloned in the *Eco*RI site of the suicide vector pRVS1. The resulting clone, pRTd9213, was used for generating a deletion in the genome of *P. denitrificans* by homologous recombination.

Conjugations and gene replacements. Conjugations were performed as described by De Vries *et al.* (1989). Matings were either performed with *E. coli* strain S17-1 or triparentally using pRK2020 as a helper plasmid. Gene replacement techniques were done essentially as described earlier (van Spanning *et al.*, 1990, 1991b).

Gel electrophoresis. SDS-PAGE was performed on 13% (w/v), 1.5 mm thick slab gels prepared by the method of Laemmli (1970). Samples were incubated as described by Ras *et al.* (1991). Western blots were performed essentially as described by Harms *et al.* (1985).

Enzyme assays. β -Galactosidase activity was measured as described by Miller (1972), with the minor modification that incubation with toluene was prolonged to 90 min.

RESULTS

Isolation of regulatory *mox* mutants of *P. denitrificans*

P. denitrificans deletion mutants were isolated by using the method previously described (van Spanning *et al.*, 1991b). Strain Pd0821 was mated with *Escherichia coli* S17-1 harbouring pRTd0841. After selection for a double recombination event, a strain was isolated that contained a 939 bp unmarked and in-frame deletion in the *mxaY* gene. As result of this mutation the mutated *mxaY* gene lacks the part that encodes one of the membrane-spanning regions and the conserved histidine that is thought to be the site of autophosphorylation. The resulting mutant was designated Pd0841. Strain Pd0721 was mated with *E. coli* S17-1 harbouring pRTd9213. A double recombination event resulted in a deletion in the *mxaZ* and *mxaY* genes of *P. denitrificans*. The reading frame at the N-terminus of MxaZ was correctly continued into the C-terminus of MxaY. This mutation resulted in loss of 75% of *mxaZ* at the 3'-terminus and 91% of *mxaY* at the 5'-terminus; this mutant was designated Pd9213. Other regulatory *mox* mutants were isolated previously (Harms *et al.*, 1993), and now six different regulatory *mox* mutants are available containing lesions in *mxaZ*, *mxaY* or *mxaX*, or in combinations of these genes. The correctness of each mutation was demonstrated by Southern analysis (results

Fig 1. Physical map of the *mxaXYZF* region of *P. denitrificans*. Open bars indicate ORFs. Arrows indicate the direction of transcription. The black box represents a signal sequence. The hatched boxes indicate the membrane-spanning regions in MxaY. The conserved histidine in MxaY is indicated by an asterisk. (a) Location of the mutations in the *mxaZ*, *mxaY* and *mxaX* genes. (b) DNA fragments as cloned in both pEG400 and pMP190. For the pMP190 derivatives, the direction towards the *lacZ* gene is indicated by an arrow.

not shown). The locations of these mutations are shown in Fig. 1(a).

Characterization of regulatory *mox* mutants

Recently, some of the characteristics of regulatory *mox* mutants have been published (Harms *et al.*, 1993). For a thorough understanding of the effect of these mutations and of those described here, plasmids containing either *mxaZ*, *mxaY*, *mxaX* or combinations of these genes (see Fig. 1b), were introduced into all of the regulatory *mox* mutants. Mutant strains, either with or without the plasmids, were analysed for their ability to grow on methanol and their ability to synthesize MDH. Transcription of *mxaZ*, *mxaY*, *mxaX* or their mutant genes was under control of the *mxaZ* promoter (Harms *et al.*, 1993). The insertion mutants Pd0721, Pd0821 and Pd0921 were unable to grow on methanol (Table 2, column 3). They could be complemented for their ability to grow on methanol by all plasmids containing *mxaX*, both in the absence and the presence of additional *mxaZ*, *mxaY*, or both (Table 2, columns 6, 8 and 9). This indicates that an insertion of a kanamycin-resistance marker has an effect on the expression of the genes located downstream. In the deletion mutants, in which expression of *mxaX* is not affected, growth on methanol was found. Mutant Pd0841, which contains a deletion in *mxaY*, was able to grow on methanol just as the wild-type strain was. This proves that *mxaY* is not essential for methanol oxidation. Pd9213, which contains a deletion in both *mxaY* and *mxaZ*, has a

Table 2. Growth of *P. denitrificans* regulatory *mox* mutants on solid media containing methanol

+++ , Growth same as the wild-type; ++ , slightly less growth than the wild-type; + , considerably less growth than the wild-type; – , no growth.

Strain	Relevant characteristics	Plasmid (and relevant characteristics)							
		None	pMA11 (MxaZ)	pNH56 (MxaZ/Y)	pNH52 (MxaZ/Y/X)	pHY4 (MxaY)	pHY3 (MxaY/X)	pHY5 (MxaX)	MDH synthesis
Pd1222	Wild-type	+++	+++	+++	+++	+++	+++	+++	+++
Pd0721	Z::Km	–	–	–	+++	–	+++	+++	–
Pd0741	ΔZ	+	+	++	+++	+	+++	+++	+
Pd0821	Y::Km	–	ND	ND	ND	–	+++	+++	–
Pd0841	ΔY	+++	ND	ND	ND	+++	+++	ND	+++
Pd9213	ΔZY	+++	ND	+++	+++	+++	+++	+++	+++
Pd0921	X::Km	–	ND	ND	ND	ND	+++	+++	–

ND, Not done.

Table 3. Promoter activities of the *mxaF* promoter of *P. denitrificans*

Induction of β -galactosidase by methylamine (MeNH₂) or glucose (Glc) is indicated in Miller units (Miller, 1972). Values represent means of at least three different independent experiments, with each point assayed in duplicate.

Strain	Relevant characteristics	Plasmid (and relevant characteristics)															
		pCP112		pNH30 (MxaZ)		pWR124 (MxaZ/Y)		pWR112 (MxaZ/Y/X)		pWR122 (MxaY)		pWR126 (MxaY/X)		pWR115 (MxaX)			
		MeNH ₂	Glc	MeNH ₂	Glc	MeNH ₂	Glc	MeNH ₂	Glc	MeNH ₂	Glc	MeNH ₂	Glc	MeNH ₂	Glc	MeNH ₂	Glc
Pd1222	Wild-type	2250	30	1700	30	3200	60	2150	290	6350	3	2150	400	2150	450		
Pd0721	MxaZ::Km	90	10	90	10	140	< 1	1850	350	130	< 1	1900	260	1550	470		
Pd0741	ΔMxaZ	750	10	750	20	1200	< 1	1750	300	4100	< 1	1750	260	3050	470		
Pd0821	MxaY::Km	70	50	70	30	60	30	2600	150	80	30	1950	110	2650	420		
Pd0841	ΔMxaY	2200	20	2050	30	1800	30	2400	200	6800	20	1750	200	2400	430		
Pd9213	ΔMxaZ/Y	2500	70	2200	60	1800	120	2550	190	6900	50	1600	220	2250	370		
Pd0921	MxaX::Km	30	20	50	20	50	20	2350	200	60	20	2050	200	1800	400		

phenotype identical to that of Pd0841 and the wild-type. MxaZ is therefore also not essential for growth on methanol. However, mutant Pd0741, which has a deletion in *mxaZ*, had reduced growth on methanol. This mutant could not simply be complemented by the addition of *mxaZ* in trans. Addition of *mxaY*, present on pHY4, did not restore growth of Pd0741 to wild-type levels either. Addition of both *mxaY* and *mxaZ*, present on pNH56, complemented the mutation but not completely, whereas MxaX complemented this mutation completely. The decreased growth on methanol in Pd0741 is therefore most probably caused by downstream effects of the deletion on the expression of *mxaX*.

The ability to synthesize MDH was determined after growth on methylamine. Cells grown on this substrate were found to contain MDH (De Vries *et al.*, 1988). The protein was detected by using antisera raised against MDH. As indicated in Table 2 (column 10), MDH was absent in Pd0721, Pd0821 and Pd0921. MDH was

synthesized to wild-type levels in Pd0841 and Pd9213, but in Pd0741 a reduced amount of MDH was found. These data are in accordance with the growth data mentioned above.

Effect of *mox* mutations on expression of *mxaF-lacZ* gene fusions

To analyse the mutants for their ability to express *mxaF*, a *mxaF-lacZ* gene fusion was used. This gene fusion resided on various plasmids that contained in addition either *mxaZ*, *mxaY*, *mxaX* or combinations of these genes (see Fig. 1b). The plasmids were transferred to *P. denitrificans* wild-type and *mox* regulatory mutants, and β -galactosidase activities were measured in cells grown on methylamine or on glucose.

In general, the levels of β -galactosidase activities correlated with the synthesis of MDH as found by Western blot analysis (see Tables 2 and 3). Little or no activity was

found in the insertion mutants Pd0721, Pd0821 and Pd0921. In Pd0741, which contains a deletion in *mxz*, reduced amounts of β -galactosidase were found while in Pd0841, mutated in *mxY*, and Pd9213, mutated in both *mxZ* and *mxY*, wild-type levels of β -galactosidase were detected. Addition of extra copies of *mxX*, present on pWR112, pWR126 or pWR115, resulted in detection of β -galactosidase activities in all mutants; β -galactosidase was even detected in cells grown on glucose. In wild-type cells grown on glucose, MDH synthesis was reported to be repressed (De Vries *et al.*, 1988). Expression of the *mxAF-lacZ* fusion in cells harbouring pWR112 grown on glucose has been reported previously (Harms *et al.*, 1993). The data presented here indicate that this phenomenon is independent of the presence of extra copies of *mxY* or *mxZY* in addition to *mxX* (pWR126 or pWR112).

DISCUSSION

In a previous study we reported the isolation of three genes, *mxZ*, *mxY* and *mxX*, that were postulated to be involved in the regulation of MDH synthesis (Harms *et al.*, 1993). The gene products of *mxY* and *mxX* were found to belong to the class of two-component regulatory systems. MxY has homology with the signal sensors (SS), and MxX has homology with the response regulators (RR). On the basis of this sequence similarity we proposed that MxY and MxX function as a two-component regulatory system in a linear signal-transduction pathway, and that MxZ plays a role in this regulatory process. To provide evidence for this model we isolated several regulatory *mox* mutants and tested their ability to grow on methanol, and to synthesize MDH and express a *mxAF-lacZ* transcriptional fusion.

As expected, a mutation in *mxX*, or mutations with downstream effects on the expression of *mxX*, led to a methanol-negative phenotype. However, mutants Pd0841 and Pd9213 (which have deletions in *mxY* and *mxYZ*, respectively), were able to grow as well as the wild-type on methanol, and MDH synthesis was unimpaired. In these strains expression of the *mxAF-lacZ* fusion was comparable to that of the wild-type and was completely regulated. The most obvious conclusion from these data might be that MxY is not the sensor in the signal transduction pathway that is involved in activation of the *mxAF* promoter. However, strains that harbour pWR122, a plasmid that contains only *mxY*, were found to express the *mxAF-lacZ* fusion at elevated levels. This suggests that MxY affects *mxAF* expression. In strains lacking MxY, a parallel signal-transfer system must be able to activate MxX. From the literature it is known that in a few instances RR-dependent gene expression can take place in the absence of the cognate SS. Several explanations for this observation have been put forward. The RR might be phosphorylated by a nonspecific SS. This hypothesis is called 'cross-talk' and is used to explain the non-regulated expression of the target genes when the RR is over-expressed. Another explanation is that the RR is phosphorylated by low-molecular-mass phosphodonors such as acetyl phosphate, carbamyl phosphate or phosphoamidate (McCleary *et al.*, 1993). A third ex-

planation is that the RR is phosphorylated by a second specific SS. Two different signals trigger two different SSs to phosphorylate one RR. This physiologically relevant regulation is called cross-regulation and an example is found in the Pho regulon (Wanner & Riesenbergs, 1992). A fourth explanation is found in the Nar system. One signal is detected by two different SSs both of which can phosphorylate a RR (Rabin & Stewart, 1993). The Mx system seems to be very like this system, since one signal, probably formaldehyde, triggers two parallel signal-transduction pathways to activate MxX.

The data obtained here show that the signal-transduction pathway that functions in the expression of *mxAF* in *P. denitrificans* is not a simple linear one. The presence of more than one pathway for activating MxX provides the cell with the ability to react very precisely to changes in the concentration of the signal molecule. We proposed previously (Harms *et al.*, 1993) that the signal that is sensed by MxY is formaldehyde, the product of the oxidation reaction that is catalysed by MDH. Since formaldehyde is a toxic compound, it is very important for the cell to regulate its formation very accurately. Studies are in progress to isolate mutants in the putative alternative signal-transduction pathway that is involved in regulation of methanol oxidation.

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