***gdhB*, a gene encoding a second quinoprotein glucose dehydrogenase in *Pantoea citrea*, is required for pink disease of pineapple**

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The pink disease of pineapple, caused by the bacterium *Pantoea citrea*, is characterized by a dark coloration on fruit slices after canning. A glucose dehydrogenase (Gdh) encoded by the *gdhA* gene has been implicated in the colour formation activity of *P. citrea*. In this paper it has been shown that *P. citrea* contains a second, homologous *gdh* gene and its product, GdhB, represents the main source of Gdh activity in this organism. Unlike *gdhA*, *gdhB* is constitutively expressed during the exponential phase of growth and is induced in stationary phase. A previously isolated chemical mutant, CMC6, which is deficient in Gdh activity and pink disease formation, failed to express *gdhB* during the stationary phase of growth. The CMC6 mutant can be complemented by a 54 bp DNA fragment located upstream of *gdhA*. This fragment, which contains an operator-like 11 bp inverted repeat, strongly enhances the expression of *gdhA*, probably by titrating away a negative effector of its expression. These results illustrate the complex interplay operating between the two *gdh* genes and emphasize the role of glucose metabolism in the pathway leading to pink disease.

Keywords: *Pantoea*, pink disease, glucose dehydrogenase

INTRODUCTION

Pink disease of pineapple is characterized by a dark coloration of the pineapple flesh when the fruit is canned (Rohrbach, 1989). The fruits, however, remain superficially asymptomatic in the field, making it difficult to discriminate between diseased and healthy fruits. Recently, a bacterium isolated in the Philippines was shown to be responsible for pink disease and identified as *Pantoea citrea* (Cha *et al.*, 1997b). This bacterium belongs to the *Enterobacteriaceae* and is mainly found in fruits and soil samples (Sonoyama *et al.*, 1988; Kageyama *et al.*, 1992). The genes of *P. citrea* involved in pathogenesis are currently being identified and characterized (Cha *et al.*, 1997a; Pujol & Kado, 1998). We previously generated a nitrosoguanidine-mediated mutant of *P. citrea* called CMC6, which was unable to

induce the dark coloration characteristic of pink disease. This mutant could be complemented by a 3.9 kb genomic DNA fragment containing a gene encoding a glucose dehydrogenase (Gdh) (Cha *et al.*, 1997a). This gene, designated *gdhA*, is homologous to various bacterial Gdh genes. Its product, GdhA, belongs to the quinoprotein-glucose dehydrogenase family, whose members are usually associated with the cytoplasmic membrane, require pyrroloquinoline quinone (PQQ) as a cofactor (Duine *et al.*, 1979), oxidize D-glucose to D-gluconate in the periplasm and can also oxidize aldoses and disaccharides in *Pseudomonas stutzeri* LMD26-38 (Cleton-Jansen *et al.*, 1988) and *Acinetobacter calcoaceticus* (Cleton-Jansen *et al.*, 1989), respectively. PQQ-dependent Gdhs are synthesized in a wide variety of bacterial species, some of which are able to synthesize PQQ, such as *A. calcoaceticus* (Duine *et al.*, 1979), *Pseudomonas fluorescens* (Ameyama *et al.*, 1981), *Klebsellia aerogenes* (Neijssel *et al.*, 1983), *Gluconobacter oxydans* (Buchert & Viikari, 1988) and *Erwinia herbicola* (Liu *et al.*, 1992), while others such as *Escherichia coli* (Hommes *et al.*, 1984), *Klebsiella pneumoniae* (Hommes *et al.*, 1989) and *Acinetobacter lwoffii* (Van Schie *et al.*, 1984) synthesize only the

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Abbreviations: CRP, cAMP receptor protein; Gdh, glucose dehydrogenase; PQQ, pyrroloquinoline quinone.

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apoenzyme whose activity is dependent on an exogenous source of PQQ (Van Schie *et al.*, 1985).

The role of Gdh in glucose metabolism differs between various organisms. Some organisms use a modified Entner–Doudoroff metabolic pathway to oxidize glucose to gluconate and 2-ketogluconate, which in turn are phosphorylated into 6-phosphogluconate and 2-keto-6-phosphogluconate, respectively (Conway, 1992). In *E. coli*, the enzymes of the Entner–Doudoroff pathway are induced either by glucose and PQQ (Fliege *et al.*, 1992) or by gluconate (reviewed by Fraenkel, 1996). A number of *Acetobacter*, *Erwinia*, *Gluconobacter* and *Pantoea* species can oxidize gluconate further into 2-ketogluconate and then into 2,5-diketogluconate by a membrane-bound gluconate dehydrogenase and a 2-ketogluconate dehydrogenase, respectively (Matsushita *et al.*, 1982; Shinagawa *et al.*, 1984; Sonoyama *et al.*, 1988; Yum *et al.*, 1997). Likewise, *P. citrea* forms 2,5-diketogluconate as the end product of glucose oxidation (C. Pujol & C. Kado, unpublished).

We found that elimination of the *gdhA* gene, which was previously thought to be responsible for pink disease (Cha *et al.*, 1997a), did not dramatically reduce Gdh activity and pink colour formation, suggesting that another *gdh* gene may be involved. This was verified by genetic analyses which revealed the presence of a second *gdh* gene, *gdhB*, whose product, GdhB, is shown here to be the primary enzyme involved in Gdh activity linked to pink disease development.

METHODS

Bacterial strains, media, growth conditions and antibiotics.

Bacterial strains used are listed in Table 1. *E. coli* strains were grown at 37 °C in LB or M9 media (Sambrook *et al.*, 1989) and *P. citrea* strains at 30 °C in LB medium containing rifampicin, Mannitol/Glutamate/Yeast (MGY) medium or pineapple juice (Cha *et al.*, 1997a). Media were solidified with 1.5% Bacto-agar (Difco). *E. coli* and *P. citrea* were transformed by electroporation as described previously (Cha *et al.*, 1997a). Transformants were selected on LB agar containing 100 µg ampicillin ml⁻¹, 40 µg X-Gal ml⁻¹ (Denville) and 0.1 mM IPTG (Gibco-BRL). Antibiotics (Sigma) were used as follows (µg ml⁻¹): rifampicin (Rif), 100; chloramphenicol (Cm), 25 and 20; tetracycline (Tc), 15 and 10; ampicillin (Ap), 250 and 100 for *P. citrea* and *E. coli*, respectively.

Plasmids and DNA manipulations. Cloning vectors and plasmids used are listed in Table 1. Plasmid screening was carried out by alkaline lysis (Birnboim, 1983). Plasmid DNAs were prepared using the Qiagen plasmid kit. Total DNA was purified as described by te Riele *et al.* (1986). Purified DNAs were cleaved with restriction endonucleases as specified by the suppliers (Boehringer Mannheim or New England Biolabs). Ligations were performed with T4 DNA ligase (Boehringer Mannheim) and nested deletions were constructed by using exonuclease III and S1 nuclease (Boehringer Mannheim) according to standard protocols (Sambrook *et al.*, 1989).

DNA hybridization and sequencing. Southern blot hybridizations were performed as described by Sambrook *et al.* (1989) and were carried out at 65 °C with Rapid-Hyb Buffer (Amersham). Probes were prepared by nick-translation (Boehringer Mannheim) using [α -³²P]dCTP (DuPont-NEN Products). dsDNA sequences were determined by the dideoxy

chain-termination method (Sanger *et al.*, 1977) using the Sequenase version 2.0 kit (USB) and [α -³²S]dATP (DuPont-NEN Products), as described previously (Pujol & Kado, 1998). Sequence compilation and comparative analyses were made with the Genetics Computer Group program version 9.0 (University of Wisconsin, Madison).

Pink disease test and enzyme assay. The pink disease test was performed in pineapple juice as described previously (Cha *et al.*, 1997a). Briefly, *P. citrea* was grown in pineapple juice for 3 d at 30 °C. The culture was then autoclaved for 5 min at 121 °C and the cells were removed by centrifugation (10000 g, 5 min). The absorbance of the clarified supernatant was determined spectrophotometrically at 420 nm.

Gdh activity *in vivo* was detected on MGY agar containing 2% glucose and eosin yellow/methylene blue (Cha *et al.*, 1997a). *In vitro* activity was measured as follows: cells were cultured in 20 ml LB medium for 72 h at 30 °C, collected by centrifugation (5000 g, 10 min), resuspended in 2 ml lysis buffer (0.1 M Tris/HCl, pH 7.0, 10 mM EDTA, 0.1% PMSF, 5 mg lysozyme ml⁻¹) and incubated for 30 min at 37 °C. The entire mixture was then lysed by sonication using a MS50 sonicator (Heat Systems-Ultrasonics). Cell debris were removed by centrifugation (8000 g, 10 min). Total protein content was determined with a Protein (Bradford) assay kit (Bio-Rad). Samples of 100 µl of cell extracts were mixed with assay buffer [0.1 M Tris/HCl, pH 7.2, 0.1 mg Wurster's Blue ml⁻¹ (N,N,N',N'-tetramethyl-*p*-phenylenediamine; Sigma), 50 mM glucose]. Gdh activity was measured spectrophotometrically at 610 nm. Calculations of Gdh activity were based on the molar extinction coefficient for Wurster's Blue of 12400 M⁻¹ cm⁻¹ at 610 nm (Dokter *et al.*, 1986).

Identification of *gdhB* – insertional mutation of *gdhA* and *gdhB*.

For *gdhA*, an internal 1750 bp *HpaI* fragment was removed from a 3875 bp DNA fragment containing the *gdhA* gene previously cloned in pUCD5723 (Cha *et al.*, 1997a), resulting in plasmid pUCD5020. A 2168 bp *HaeIII*–*XbaI* fragment carrying the chloramphenicol acetyl transferase gene (*cat*) of plasmid pACYC184 was treated with the Klenow fragment of *E. coli* DNA polymerase I and cloned into the unique *HpaI* site of pUCD5020, resulting in plasmid pUCD5021. The 3645 bp *AatIII*–*HindIII* fragment of pUCD5021, containing the *cat* gene and partial sequences of *gdhA*, was cloned into the *Sall* site of the integrative plasmid pUCD5700 after treatment with the Klenow enzyme, generating plasmid pUCD5022 in *E. coli* S17-1 λ pir. *P. citrea* 1056R cells were transformed with pUCD5022 and plated on LB agar plates containing Rif and Cm. Selection of insertion mutants was based on Cm^r and Ap^r. Total DNA from an overnight culture of the putative mutants was purified, digested with *Bam*HI and *Eco*RI, analysed by agarose gel electrophoresis and transferred onto a Hybond-N+ Nylon membrane (Amersham). The DNA was then hybridized with a ³²P-labelled probe containing the *Bam*HI–*Eco*RI fragment of pUCD5723 carrying *gdhA* (Cha *et al.*, 1997a). One mutant, designated 1058RC, was selected. Inactivation of *gdhA* in strain CMC6 was carried out in the same way. One Rif^r Cm^r Ap^r strain (1062RC) was selected and the disruption of *gdhA* was verified by Southern blot hybridization.

To identify a putative second *gdh* gene, we used the genomic DNA of strain 1058RC (Δ *gdhA::cat*) as template in PCR reactions using two 18-mer degenerate oligonucleotides representing 48 different sequences: pqq2 5'-CTGCCAGTDAAC-GAYGGYCGYCTG-3' and pqq4 5'-TTCRTARGTCATYG-GRGTHGCCTG-3', where R represents any purine, Y represents any pyrimidine, D represents T, A or G, and H represents A, T or C. Amplification cycles (5 cycles of 94 °C

Table 1. Bacterial strains and plasmids used

Strain	Genotype or phenotype	Source or reference
<i>Escherichia coli</i>		
DH5 α	<i>hsdR17</i> ($r_k^- m_k^+$) $\Delta(lacZYA-argF)$ U169 $\phi 80dlacZ\Delta M15$ <i>supE44 thi-1 recA1 endA1 gyrA96 relA1</i>	Sambrook <i>et al.</i> (1989)
S17-1 λ <i>pir</i>	<i>thi pro hsdR^- hsdM^+ recA</i> RP4 2-Tc::Mu Km::Tn7 λ <i>pir</i> ; Sm r Tp r	Simon <i>et al.</i> (1983)
<i>Pantoea citrea</i>		
1056R	Wild-type, Rif r	Cha <i>et al.</i> (1997b)
1058RC	$\Delta gdhA::cat$; Rif r Cm r	This study
1060RT	<i>gdhB::tet</i> ; Rif r Tc r	This study
1061RCT	$\Delta gdhA::cat$ <i>gdhB::tet</i> ; Rif r Tc r Cm r	This study
CMC6	Chemical mutant; Gdh $^-$, pink $^-$; Rif r	Cha <i>et al.</i> (1997b)
1062RC	Derivative of CMC6; $\Delta gdhA::cat$; Rif r Cm r	This study
1063RT	Derivative of CMC6; <i>gdhB::tet</i> ; Rif r Tc r	This study
Plasmids		
pBluescript SKII(–)	ColE1 origin; Ap r	Stratagene
pACYC184	p15A origin; Tc r Cm r	New England Biolabs
pBR322	Tc r Ap r	New England Biolabs
pUCD5700	R6K origin Mob RP4; Ap r	J.-S. Cha, unpublished
pUCD5723	Derivative of pBR322; <i>gdhA</i> (3875 bp); Ap r Tc r	Cha <i>et al.</i> (1997a)
pUCD5020	Derivative of pUCD5723; <i>gdhA</i> Δ <i>HpaI</i> (2125 bp); Ap r Tc r	This study
pUCD5021	Derivative of pUCD5020; <i>gdhA</i> Δ <i>HpaI::cat</i> (3448 bp); Cm r Ap r Tc r	This study
pUCD5022	Derivative of pUCD5700; <i>gdhA</i> Δ <i>HpaI::cat</i> (3448 bp); Cm r Ap r	This study
pUCD5026	Derivative of pUCD5723; Δ <i>BglII-NcoI</i> (2865 bp); Ap r Tc r	This study
pUCD5027	Derivative of pBluescript SKII(–); <i>BglII-NcoI</i> from pUCD5723 (1010 bp); Ap r	This study
pUCD5035	Derivative of pUCD5027 (846 bp); Ap r	This study
pUCD5046	Derivative of pUCD5027 (790 bp); Ap r	This study
pUCD5058	Derivative of pUCD5027 (203 bp); Ap r	This study
pUCD5059	Derivative of pUCD5027 (117 bp); Ap r	This study
pUCD5061	Derivative of pBluescript SKII(–); 54 bp upstream of <i>gdhA</i> ; Ap r	This study
pUCD5062	As for pUCD5061 but with inverse orientation of the 54 bp fragment; Ap r	This study
pUCD5063	Derivative of pBluescript SKII(–); partial <i>gdhB</i> (1387 bp); Ap r	This study
pUCD5080	Derivative of pUCD5063 (2727 bp); Ap r Tc r	This study
pUCD6702	Derivative of pUCD5700; partial <i>gdhB</i> (2727 bp); Ap r Tc r	This study
pUCD6712	Derivative of pBluescript SKII(–); <i>gdhA</i> (2504 bp); Ap r	This study
pUCD6715	Derivative of pBluescript SKII(–); <i>gdhB</i> (2703 bp); Ap r	This study

for 1 min, 42 °C for 1 min and 72 °C for 1 min, then 25 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min) were carried out with *Taq* polymerase (Promega). The amplified fragment was cloned into pBluescript SKII(–) resulting in plasmid pUCD5063. A 1340 bp *HindIII*–*StyI* fragment from pBR322, containing the tetracycline resistance gene (*tet*), was cloned into the unique *BstEII* site of pUCD5063 after treatment with Klenow polymerase, resulting in plasmid pUCD5080. A 2752 bp fragment containing partial sequences of *gdhB* and the *tet* gene was amplified using *Pfu* DNA polymerase (Stratagene) and primers *gdhPCR1* 5'-TTCGTAAGATCTC-GGGGTTGCCTGACCAACCC-3' and *gdhPCR2* 5'-TTAAC-GAGATCTGCCTGATTGCGGTGGATGC-3'. The underlined nucleotides correspond to *BglII* restriction sites. The amplified fragment was digested with *BglII* and cloned into the unique *BglII* site of the integrative vector pUCD5700, to produce plasmid pUCD6702 in *E. coli* S17-1 λ *pir*. *P. citrea* 1056R competent cells were transformed with pUCD6702 and plated on LB agar plates containing Rif and 15 μ g Tc ml $^{-1}$. Insertion mutants were selected for Tc r and Ap r . Mutants were checked by Southern blot hybridization using the 2.8 kb

XbaI–*HindIII* fragment of pUCD5080 as probe. One mutant, designated 1060RT, containing an insertion in *gdhB* was selected. *gdhB* was also inactivated using the same approach in strain CMC6, resulting in strain 1063RT. A double *gdhA* $^-$ *gdhB* $^-$ mutant was obtained by transforming *P. citrea* 1058RC with plasmid pUCD6702. Mutants were selected for Cm r , Tc r and Ap r and checked by Southern blot hybridization as described above. One mutant strain, 1061RCT, was selected.

Cloning of *gdhA* and *gdhB*. *gdhA* and *gdhB* were cloned by PCR, using *Pfu* DNA polymerase (Stratagene). Primers *gdhAEco1* 5'-TGTGAATTCATTGTAAACACTTGGTGT-AAACGG-3' (nt 1306–1337) and *gdhAEco2* 5'-CGTA-GAATTCAAATGCTTTAGAAAGGCC-3' (nt 3806–3774), and template plasmid pUCD5723 were used to amplify a 2501 bp DNA fragment containing *gdhA*. Primers *gdhBEco1* 5'-GAAGCGAATTCCTCCACTCGGAACATA-3' (nt 50–77) and *gdhBEco2* 5'-ATTCTGAATTCTGCCGCGAATCTAT-GG-3' (nt 2753–2730), and genomic DNA of 1056R were used to amplify a 2703 bp DNA fragment containing *gdhB*. The underlined nucleotides correspond to *EcoRI* restriction sites. The primers used allowed the amplification of the putative

natural expression signals of each Gdh. The amplified fragments were cloned into the *EcoRI* site of pBluescript SKII(–), leading to plasmids pUCD6712 and pUCD6715 containing the *gdhA* and *gdhB* genes, respectively.

The 54 bp DNA fragment containing the 11 bp inverted repeat upstream of *gdhA* was amplified by PCR using *Taq* polymerase, plasmid pUCD5723 as the template and primers HR3 5'-AGCTCATTGTAAACACTGG-3' (nt 1305–1324) and HR4 5'-AAAGTAAAACAGAATACTTAGTG-3' (nt 1359–1337). The amplified 54 bp DNA fragment was cloned in both orientations into the *EcoRV* site of pBluescript SKII(–) to yield plasmids pUCD5061 and pUCD5062.

RNA extraction and Northern blot hybridization analysis. RNA was isolated and purified from cells grown in MGY medium with or without 2% glucose during both exponential (4.5 h) and stationary phases (14 h) of growth (Pujic, 1997). RNA electrophoresis was performed in 0.8% SeaKem GTG agarose (FMC) in 10 mM sodium phosphate, pH 7.0, 10 mM sodium acetate. RNA was then transferred onto a Nytran 0.2 µm membrane (Schleicher & Schuell). Hybridization was performed using primers *gdhA*RNA 5'-GCTGATGAGTC-GCCCTGAACAGATTG-3' and *gdhB*RNA 5'-TCTGAGG-ATCTGTTGCCGTATTAAAG-3' as probes to detect *gdhA*- and *gdhB*-specific-transcripts, respectively (Pujic *et al.*, 1998). Probes were 5'-end-labelled using T4 DNA kinase (Pharmacia) and [γ -³²P]dATP (DuPont-NEN Products).

RESULTS

P. citrea contains a second *gdh* gene

CMC6, a nitrosoguanidine mutant unable to induce pink disease, can be complemented by a DNA fragment containing the *gdhA* gene that encodes a Gdh protein

(Cha *et al.*, 1997a). This observation suggested that *gdhA* had been inactivated in CMC6, in agreement with the finding that CMC6 was deficient for Gdh activity (Cha *et al.*, 1997a). To verify that the *gdh*-deficient phenotype of CMC6 was due to the loss of *gdhA* function, we constructed a strain in which the *gdhA* gene was inactivated by insertion of a chloramphenicol resistance (*cat*) gene. This mutant strain (1058RC) was tested for Gdh activity *in vivo* using a plate assay in which the activity is detected by the formation of a halo around colonies grown in presence of glucose and methylene blue/eosin yellow. Unexpectedly, strain 1058RC was found to still possess the enzymic activity at wild-type levels (Fig. 1) and was still able to induce the pink coloration of pineapple juice, albeit with 10–15% less colour intensity than that of the wild-type strain (Table 2). Furthermore, when the *in vitro* Gdh activity was measured in crude extracts, no significant reduction was observed compared to the wild-type strain. As shown in Table 2, the Gdh activity of the wild-type strain 1056R is low when cells are grown in MGY but increases eightfold when glucose is added to the growth medium. Similar basal and induced levels are detected with the *gdhA*[–] strain 1058RC. These results indicate that, under the conditions used here, *gdhA* contributes only marginally to the total Gdh activity expressed by *P. citrea* and, hence, to pink disease. These results also suggest that (a) *P. citrea* 1056R contains at least another active *gdh* gene and (b) the mutation in strain CMC6 did not affect *gdhA* itself. Instead, we hypothesized that the CMC6 mutation affected the other putative *gdh* gene(s) or their expression.

To verify this hypothesis, we first tried to identify and clone other *gdh* genes in *P. citrea*. We selected conserved sequence motifs produced from the alignment of Gdhs from *E. coli*, *A. calcoaceticus* and *G. oxydans* to design the degenerate PCR primers pqq2 and pqq4, which were then used to amplify other potential *gdh* loci as described in Methods. When the genomic DNA of strain 1058RC was used as template, a single, specific 1.4 kb DNA fragment was amplified. This DNA fragment was cloned in pBluescript SKII(–), resulting in plasmid pUCD5063, and its nucleotide sequence was determined. The 1387 bp fragment was 73% similar at the nucleotide level to that of *gdhA* (nt 881–2268). When used as a probe in Southern blot hybridization experiments, the amplified fragment hybridized to a specific 4.3 kb *EcoRI*–*Bam*HI fragment derived from the genomic DNA of strain 1056R, as opposed to the 3.3 kb *EcoRI*–*Bam*HI fragment containing *gdhA* (data not shown). *P. citrea* therefore contains a second *gdh* gene.

This new putative *gdh* gene was called *gdhB* and was inactivated by the insertion of a tetracycline resistance (*tet*) cassette, as described in Methods, resulting in strain 1060RT. To clone the entire *gdhB* gene, the genomic DNA of strain 1060RT was digested with *EcoRI*, ligated into the *EcoRI* site of pBluescript SKII(–) and transformed into *E. coli* DH5α cells with selection for tetracycline resistance. Among the transformants, a plasmid containing a 9.5 kb DNA insert corresponded

Fig. 1. *In vivo* Gdh activity detected on solid MGY medium supplemented with 2% glucose. After growth for 2 d at 30 °C, colonies were overlaid with 10 ml 0.5% agar containing methylene blue (65 µg ml^{–1}) and eosin yellow (0.4 mg ml^{–1}). A dark (purple) halo around colonies indicates Gdh activity. 1056R, wild-type *P. citrea*; 1058RC, single mutant Δ *gdhA::cat*; 1060RT, single mutant *gdhB::tet*; 1061RCT, double mutant Δ *gdhA::cat gdhB::tet*.

Table 2. Colour-forming and Gdh *in vitro* activities of *gdhA* and *gdhB* mutants

Colour-forming activity was measured at 420 nm after 3 d growth in pineapple juice. Gdh *in vitro* specific activities were measured after 10 s with crude extracts of cells grown for 3 d in MGY medium without (–) or with (+) 2% glucose. Gdh specific activity is expressed in pmol Wurster's Blue reduced ($\mu\text{g protein}^{-1} \text{ s}^{-1}$). Each value corresponds to the mean of three measurements $\pm$ SD.

Strain	Genotype	Colour-forming activity (%)	Gdh specific activity	
			– Glucose	+ Glucose
1056R	Wild-type	100	70 $\pm$ 7	562 $\pm$ 13
1058RC	$\Delta\text{gdhA}::\text{cat}$	90 $\pm$ 6	68 $\pm$ 7	576 $\pm$ 13
1060RT	$\text{gdhB}::\text{tet}$	7 $\pm$ 4	73 $\pm$ 5	93 $\pm$ 5
1061RCT	$\Delta\text{gdhA}::\text{cat } \text{gdhB}::\text{tet}$	1 $\pm$ 1	78 $\pm$ 4	70 $\pm$ 7

to an *EcoRI* DNA fragment harbouring *gdhB* inactivated by the *tet* cassette. This plasmid, designated pUCD6711, was used to determine the complete nucleotide sequence of *gdhB*.

Sequence analysis of *gdhB* and its corresponding product

Sequence analysis of a 2767 bp fragment from pUCD6711 revealed an ORF of 2370 nt (nt 263–2633) that would encode a putative GdhB protein of 790 aa with a predicted molecular mass of 85.6 kDa. Its amino acid sequence is 75% similar to that of GdhA and 72, 63 and 62% similar to Gdhs of *E. coli*, *A. calcoaceticus* and *G. oxydans*, respectively. A putative PQQ-binding site is located near the C terminus of GdhB (nt 2444–2578). The hydrophobic profile of GdhB, determined by the method of Kyte & Doolittle (1982), showed four potential membrane-spanning domains near the N terminus (nt 284–424, 440–490, 510–574 and 611–667), suggesting that GdhB might be anchored to the cytoplasmic membrane. The *gdhB* gene is preceded by a putative –10 box (TATAAA) 30 nt upstream of the ORF (nt 233–238) but no consensus sequence for a –35 box could be detected. Two 12 bp inverted repeats (IR) are also located immediately upstream of *gdhB* (IR1, nt 96–123, and IR2, nt 111–151). These IRs show no significant homology with sequences deposited in the databases but may be involved in the transcriptional/translational regulation of *gdhB*. Finally, *gdhB* is followed by a potential ρ -independent transcriptional terminator 58 bases downstream of the translational stop codon (nt 2694–2729).

GdhB is functional *in vivo* and *in vitro*, and is mainly responsible for pink colour formation

To determine if GdhB is functional in *P. citrea* and to study its involvement in pink disease, we compared the properties of strain 1060RT (*gdhB::tet*) with that of the wild-type strain 1056R. Each strain was tested for *in vivo* Gdh activity and for its ability to colour pineapple juice. As shown in Fig. 1, no halo typifying Gdh activity was produced by 1060RT, while the wild-type strain

1056R produced a profuse halo. Accordingly, 1060RT was unable to colour pineapple juice significantly (Table 2). Under the conditions tested here, it appears that *gdhB* is functional *in vivo* and is contributing to most of the Gdh activity, which results in the intense coloration of pineapple juice. To confirm this, we measured the Gdh activity associated with the mutant and wild-type strains *in vitro*. As shown in Table 2, inactivation of *gdhB* alone (1060RT) results in 90% loss of Gdh activity when cells are grown in presence of glucose. It is worth noting that the double mutant strain (1061RCT), which is not impaired in its growing abilities in pineapple juice or LB medium, shows virtually no Gdh activity (a non-specific background of 12% is observed), demonstrating that (a) *gdhA* contributes in a minor way to the overall cellular Gdh activity (Table 2) and (b) *P. citrea* probably contains only two active Gdhs.

gdhB expression is induced by glucose in stationary phase

The data presented in Table 2 suggest that the expression of *gdhB* is induced by glucose. The measurement of the transcription of *gdhB* would verify this prediction. Total RNA of 1056R cells grown in MGY medium with or without glucose (2%) was extracted during exponential and stationary phases of growth and examined by Northern blot hybridization analyses. As shown in Fig. 2(a), a specific transcript corresponding to *gdhB* was observed in all cases. The size of this transcript (~ 2.4 kb) is in good agreement with an initiation occurring at the promoter region upstream of *gdhB* and termination occurring at the ρ -independent terminator located downstream of the coding sequence. Quantitative estimates of Fig. 2(a) show that the amount of *gdhB* mRNA is two to threefold higher in cells during the exponential phase of growth (4.5 h) than in cells in stationary phase (14 h). However, while no significant difference in the amount of *gdhB* transcripts is detected upon addition of glucose during the exponential phase, a three to fourfold stimulation of *gdhB* transcription is observed during stationary phase (Fig. 2a). These results corroborate the *in vitro* measurements of Gdh activity, which, to mimic conditions used for pink assays, are

Fig. 2. Transcript analysis of *gdhB* in wild-type strain 1056R (a) and strain CMC6 (b). Total RNAs (30 µg per lane) from cells grown in MGY medium without (–) or with (+) 2% glucose during exponential (E) or stationary phase (S). The size marker used was the RNA Millenium Marker (Ambion).

performed well inside the stationary phase of growth. They also indicate that expression of *gdhB* is likely to be under growth-phase-dependent regulation. Under similar conditions, *gdhA* mRNA transcript levels remained undetectable (see below).

The mutation in strain CMC6 affects *gdhB* transcription during stationary phase

Strain CMC6 is deficient in Gdh activity and is unable to produce the pink colour (Cha *et al.*, 1997a). Since pink disease requires *gdhB*, a mutation affecting this gene might be expected to be present in the mutant strain. To monitor the status of this gene, we analysed the transcription of *gdhB* in strain CMC6 by Northern blot hybridization. As shown in Fig. 2(b), *gdhB* transcripts were produced abundantly during the exponential phase

Fig. 3. Sequence comparison between the 11 bp IR upstream *gdhA* and the CRP-binding site in the *E. coli* galactose operon. IRs are indicated by arrows and nucleotides in bold letters. Identical nucleotides are shown in capital letters.

of growth. However, in sharp contrast to the wild-type strain, no *gdhB*-specific mRNA was detected during the stationary phase of growth (Fig. 2b). These results indicate that strain CMC6 is affected in the stationary-phase-specific expression of *gdhB*. This behaviour could be explained by a mutation affecting the promoter region of *gdhB*, where potential regulatory proteins are expected to bind. However, sequencing of a region of 500 bp located immediately upstream of the GdhB ORF did not reveal any mutation in strain CMC6 (data not shown).

Strain CMC6 can be complemented by a non-coding DNA sequence located upstream of *gdhA*

In a previous study, plasmid pUCD5723, which carries a 3.9 kb genomic DNA fragment that includes *gdhA*, was identified by its ability to complement the mutation in strain CMC6 (Cha *et al.*, 1997a). This complementation could be due either to the overexpression of GdhA in a way that would compensate for the lack of GdhB or to other DNA sequences present in this fragment. To investigate if the former possibility fully accounts for the complementation, we cloned a 2.5 kb fragment containing only the *gdhA* gene with its promoter region (nt 1301–3802) into pBluescript SKII(–) (plasmid pUCD6712) and measured the level of expression afforded by this construct in the Gdh-deficient double mutant strain 1061RCT. As expected, pUCD6712 restored the ability of strain 1061RCT to produce haloes on MGY plates containing glucose and methylene blue/eosin yellow, but only to one-third of their normal size. In the same assay, *gdhB* cloned into pBluescript SKII(–) (plasmid pUCD6715) fully restored the ability of strain 1061RCT to produce haloes (data not shown). These results indicate that overexpression of GdhA might only partially account for the complementation of strain CMC6 by pUCD5723. We therefore decided to search for sequences in the 3.9 kb genomic fragment that could promote complementation independently of GdhA. For this, we created plasmid pUCD5020 by deleting a 1750 bp DNA fragment internal to *gdhA*. To our surprise, pUCD5020 was still able to complement strain CMC6 as judged by the production of haloes and by the coloration of pineapple juice (data not shown). Thus, the original 3.9 kb DNA fragment contained additional sequences responsible for complementation, besides *gdhA*. Convenient restriction enzyme sites reduced the size of the complementing DNA to a 1010 bp *Bgl*III–*Nco*I fragment located upstream of *gdhA* (pUCD5027). Strain CMC6 containing pUCD5027 was

Table 3. Complementation of strain CMC6

Colour-forming and Gdh *in vitro* activities were measured as described in the legend to Table 2 in MGY +2% glucose. Gdh specific activity is expressed in pmol Wurster's Blue reduced ($\mu\text{g protein}^{-1} \text{ s}^{-1}$). Each value corresponds to the mean of three measurements $\pm$ SD.

Strain	Plasmid	Colour-forming activity (%)	Gdh specific activity
1056R	None	100	562 $\pm$ 13
CMC6	None	6 $\pm$ 1	45 $\pm$ 2
CMC6	pBluescript SKII(–)	14 $\pm$ 2	149 $\pm$ 3
CMC6	pUCD5061	68 $\pm$ 7	181 $\pm$ 3
CMC6	pUCD5062	63 $\pm$ 7	125 $\pm$ 5

able to colour pineapple juice to 55–60% of the wild-type level (data not shown). To further reduce the size of the complementing DNA fragment, the 1010 bp fragment was subjected to exonuclease III and S1 nuclease digestion. The complementing sequence is located within a 36 bp fragment and contains an 11 bp IR located 38 bp upstream of the *gdhA* start codon. Interestingly, this 11 bp IR is homologous to sequences which bind the cAMP receptor protein (CRP) of the galactose operon of *E. coli* (Musso *et al.*, 1977) (Fig. 3), suggesting that a CRP homologue may be operating as a *gdhA* control in *P. citrea*. No significant homology was found between this 11 bp IR and the two IRs located upstream of *gdhB*.

To verify that the 11 bp IR was responsible for the complementation of strain CMC6, a 54 bp DNA fragment containing this IR was amplified by PCR and cloned into the *EcoRV* site of pBluescript SKII(–). Both orientations of the insert were obtained, resulting in plasmids pUCD5061 and pUCD5062. These plasmids were tested for their ability to restore the pink colour activity and Gdh activity of CMC6 *in vivo* and *in vitro*. As shown by their ability to produce a halo on MGY plates containing glucose and methylene blue/eosin yellow, both plasmids restored the ability of strain CMC6 to oxidize glucose *in vivo* (data not shown). Additionally, both pUCD5061 and pUCD5062 complemented strain CMC6 for Gdh and colour-forming activities (Table 3). The intensity of the colour was 63–68% of that produced by the wild-type strain and *in vitro* Gdh activity was 22–32% of that of the wild-type level (Table 3). It therefore appears that the initial complementation of CMC6 by pUCD5723 was due to the cumulative effects of GdhA overexpression and the action of the 11 bp IR.

The CRP-like operator restores transcription of *gdhA* in strain CMC6

To understand further the complementation of strain CMC6 by the 11 bp IR, we decided to analyse the transcription of *gdhA* and *gdhB* in strain CMC6

Fig. 4. Transcript analysis of *gdhA* (a) and *gdhB* (b) in strain CMC6 in the absence (–) or presence (+) of pUCD5061. Total RNAs (30 μg per lane) from cells grown in MGY medium without (–) or with (+) 2% glucose during exponential phase. The size marker used was the RNA Millenium Marker (Ambion).

containing pUCD5061 by Northern blot hybridization. In *P. citrea* harbouring only two active Gdhs, complementation can be due to restoration of either *gdhA* or *gdhB* expression (or both). Results showed that, while no *gdhA*-specific mRNAs were detected in CMC6, transcripts corresponding to *gdhA* were detected during exponential growth in the presence of pUCD5061 (Fig. 4a). *gdhB* transcripts were still present irrespective of the presence or the absence of pUCD5061 during exponential phase (Fig. 4b). Despite several attempts, we were unable to obtain mRNAs from strain CMC6 containing pUCD5061 during the stationary phase of growth. CMC6 cells containing pUCD5061 grew more slowly in exponential phase in the presence of glucose than CMC6 cells without the plasmid. This analysis therefore suggests that the 54 bp DNA fragment containing the putative operator allows expression of *gdhA*, probably by titrating away a putative negative regulator. In agreement with this, we found that complementation of the CMC6 phenotype was strictly dependent on the presence of a functional *gdhA* gene and was independent of the presence of *gdhB*: a *gdhA*[–] mutant of CMC6 (1062RC) was not complemented by pUCD5061 while a

gdhB⁻ mutant (1063RT) containing this plasmid showed Gdh activity *in vivo* and was able to generate pink colour (data not shown).

DISCUSSION

P. citrea was found to possess two *gdh* genes, whose products are involved in the formation of the dark colour associated with the pink disease of pineapple. The second gene, *gdhB*, encodes a 790 aa Gdh which is 75% similar to the previously described GdhA protein (Cha *et al.*, 1997a). GdhB contains four hydrophobic regions at the N terminus, all or some of which might be potential membrane-spanning domains. In agreement with the presence of a putative PQQ-binding site, oxidation of glucose by GdhB requires PQQ as a cofactor. Indeed, complementation of an *E. coli* *gdh*-deficient strain by *gdhB*, when glucose is the sole carbon source, is only achieved if PQQ is present (data not shown). Moreover, PQQ seems to be endogenously synthesized in *P. citrea*, since *in vivo* and *in vitro* Gdh activities can be measured without adding exogenous PQQ in the reaction assays. In agreement with this, two independent chemical mutants, CMF46 and CMK8 (Cha *et al.*, 1997b), which were unable to colour pineapple juice and to oxidize glucose *in vivo*, were restored in their ability to produce Gdh activity when PQQ was provided in the growth medium. Other cofactors such as NAD, NADP or NADPH could not substitute for PQQ in these experiments (data not shown). GdhB is mainly involved in the oxidation of glucose, since inactivation of *gdhB* (mutant 1060RT) destroys the major Gdh activity both *in vivo* and *in vitro* and also decreases dramatically the pink-colour-forming activity of *P. citrea*. This demonstrates that *gdhB* is required for pink disease and reinforces the notion that oxidation of glucose is the first step leading to colour formation. In agreement with this, the ability of *P. citrea* strains CMC6 and 1061RCT to colour pineapple juice is restored upon addition of gluconate, the product of oxidation of glucose (Cha *et al.*, 1997a; C. Pujol & C. Kado, unpublished).

Measurement of Gdh activity in crude extracts showed that GdhB activity is stimulated eightfold by the presence of glucose in the culture medium (Table 2). This induction by glucose was apparent during the stationary phase of growth and is in agreement with our finding that transcription of *gdhB* is increased by a factor of 3–4 during stationary phase in the presence of glucose. These results suggest that *gdhB* is under the control of glucose-inducible, stationary-phase-specific regulation.

Our results also show that *gdhA*, which was originally thought to be responsible for pink-colour-forming activity, only plays a minor role in disease development. GdhA is functional *in vivo* in *E. coli* and in *P. citrea* when carried on a high-copy-number vector. However, when compared to *gdhB* carried on the same vector, GdhA activity was considerably lower. Complementation of strain 1061RCT (*gdhA*⁻ *gdhB*⁻) with *gdhA* only partially restored pink-colour-forming activity;

however, when complemented with *gdhB*, this double mutant strain was restored to a level nearly equivalent to that of the wild-type strain (data not shown). Using two other assays, halo formation and *in vitro* measurement of Gdh activity, no detectable activity could be ascribed to *gdhA* (Fig. 1, Table 2). This lack of activity can probably be attributed to the low expression of the gene rather than to an intrinsically low activity of the protein. No *gdhA* mRNA could be detected in Northern blot analyses under the conditions tested here. It is possible that, under circumstances when *gdhA* is expressed, GdhA activity is more specific for certain sugars as has been shown with Gdhs from *P. stutzeri* (Cleton-Jansen *et al.*, 1988), *A. calcoaceticus* (Cleton-Jansen *et al.*, 1989) and *G. oxydans* (Cleton-Jansen *et al.*, 1991). However, no Gdh activity could be detected in presence of fructose, sucrose, maltose, lactose or arabinose (data not shown). Even if GdhA is able to oxidize some other substrate, this process is not involved in the pink-colour-forming activity of *P. citrea*.

Our results show that strain CMC6, isolated as being unable to induce pink colour formation (Cha *et al.*, 1997b), is specifically affected in expression of *gdhB* during the stationary phase of growth: no *gdhB* mRNA could be detected in Northern blot experiments after CMC6 cells entered stationary phase (Fig. 2b). Interestingly, the expression of *gdhB* during the exponential phase of growth was not affected. This suggests that the expression of GdhB during exponential phase is not relevant to pink disease formation. Although the CMC6 mutation was not identified in this study, the results suggest it affects the stationary-phase-specific expression of *gdhB*. Since no mutation was found in the 500 bp region upstream of *gdhB* encompassing the putative regulatory region, we presently favour the hypothesis that CMC6 is affected in a specific regulator required for *gdhB* expression. This notion has been recently reinforced by our isolation of two independent mutants showing identical phenotypes to CMC6, which are inactivated in genes encoding proteins showing homology to regulators of the XylS-AraC family (C. Pujol & C. Kado, unpublished). However, we cannot completely rule out the possibility that *gdhB* mRNAs are being rapidly degraded in CMC6 cells during stationary phase.

Strain CMC6 can be, at least partially, complemented by a 54 bp DNA fragment (pUCD5061) derived from the non-coding region upstream of *gdhA*. This 54 bp element, which does not carry any mutation in strain CMC6 compared to the wild-type strain, restored 55–67% of the colour-forming activity of CMC6 cells when carried on a high-copy-number plasmid (Table 3). Interestingly, this fragment contains an 11 bp inverted repeat homologous to that of the CRP-binding site in the *gal* operon of *E. coli* (Musso *et al.*, 1977; Weickert & Adhya, 1993), suggesting that it corresponds to a binding site for a repressor down-regulating *gdhA* expression. We propose that the observed complementation of strain CMC6 by the 54 bp fragment cloned on a high-copy-number vector is due to the titration of this putative

repressor by binding to this fragment and reducing its effective concentration, thus allowing expression of *gdhA*. This repressor titration hypothesis is supported by the fact that *gdhA*-specific mRNAs are produced during exponential phase in CMC6 cells containing the 54 bp fragment, while the CMC6 strain produces no *gdhA* mRNA (Fig. 4a). Furthermore, complementation of strain CMC6 by the 54 bp fragment requires a functional *gdhA* gene but not a *gdhB* gene (data not shown). No effect on pink coloration was observed when pUCD5061 was introduced into the wild-type strain (data not shown).

The presence of two *gdh* genes, whose regulation remains to be further elucidated in *P. citrea*, reflects a redundant and complex mechanism for glucose utilization by this organism. Clearly, *gdhB* appears to be the main locus involved in pink disease. Although the redundancy of *gdh* genes is not unusual, their regulation based on both glucose concentration and growth phase in a reciprocal manner seems novel and reflects a highly evolved component of plant-microbe interaction.

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Effects of gene disruptions in the nisin gene cluster of *Lactococcus lactis* on nisin production and producer immunity

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The lantibiotic nisin is produced by several strains of *Lactococcus lactis* subsp. *lactis*. The chromosomally located gene cluster *nisABTCIPRKFE*G is required for biosynthesis, development of immunity, and regulation of gene expression. In-frame deletions in the *nisB* and *nisT* genes, and disruption of *nisC* by plasmid integration, eliminated nisin production and resulted in a strongly reduced level of immunity of the strains. The transcription of two nisin operons was inactivated in these mutant strains, but could be restored by addition of small amounts of nisin to growing cultures. The immunity levels of the mutants were also raised by adding nisin to growing cultures, albeit not to wild-type level. A strain with an in-frame deletion in the *nisI* gene was still able to produce active nisin, but the production and immunity levels were markedly lower. By measuring immunity levels of the knock-out strains and determining mRNA levels, it is concluded that NisI has an important function for nisin immunity and must cooperate with *nisFE*G-encoded proteins to provide a high level of immunity. Maximal immunity could not be obtained in the mutant strains, probably because the wild-type transcription levels from *nisA* and *nisF* promoters are not reached when essential *nis* genes are disrupted. Using Southern hybridization with a consensus promoter probe, no other DNA sequences similar to the *nisA* and *nisF* promoters could be detected, indicating that these two elements are probably the only ones in the chromosome regulated by nisin and are thus the only ones involved in the regulation of producer immunity.

Keywords: *Lactococcus lactis*, nisin, producer immunity, in-frame deletions

INTRODUCTION

The antimicrobial peptide nisin belongs to the family of lantibiotics and is produced by several strains of *Lactococcus lactis* (Hurst, 1981). It is used as a natural preservative in the food industry because it inhibits the growth of food-spoilage bacteria (Delves-Broughton *et al.*, 1996). Nisin is ribosomally synthesized as a precursor peptide that undergoes post-translational modifications, i.e. dehydration of serine and threonine residues and formation of five intramolecular thioether ring structures called (β -methyl)lanthionine residues (Gross & Morell, 1971). The eleven genes required for nisin

synthesis are located in a gene cluster on the nisin-sucrose transposon Tn5276 (Buchman *et al.*, 1988; Kaletta & Entian, 1989; Steen *et al.*, 1991; Rauch & de Vos, 1992; Engelke *et al.*, 1992; van der Meer *et al.*, 1993; Kuipers *et al.*, 1993; Engelke *et al.*, 1994; Immonen *et al.*, 1995; de Vos *et al.*, 1995a, b; Siegers & Entian, 1995). The organization of these genes is shown in Fig. 1. The *nisA* gene encodes the 57 aa precursor peptide; *nisB* and *nisC* probably encode membrane-associated proteins that are involved in the posttranslational modification of nisin (Engelke *et al.*, 1992; Kuipers *et al.*, 1993; Siegers *et al.*, 1996). *nisT* encodes a protein that shares significant homology with ATP-dependent translocator proteins, and recently it has been shown that NisT is involved in the translocation of the fully modified precursor nisin across the cytoplasmic membrane (Qiao

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Fig. 1. Organization of nisin biosynthetic, regulatory and immunity genes. Regulated promoters are indicated by P* and constitutive promoters by P. An inverted repeat is indicated by IR.

& Saris, 1996). *nisl* encodes a lipoprotein that is involved in immunity (Kuipers *et al.*, 1993; Qiao *et al.*, 1995) and *nisFEG* encode putative transporter proteins that probably, together with *nisl*, accomplish full nisin immunity of the producer strain (Siegers & Entian, 1995; Dodd *et al.*, 1996). *nisP* encodes an extracellular subtilisin-like protease involved in precursor processing (van der Meer *et al.*, 1993; Qiao *et al.*, 1996). *nisR* and *nisK* encode a response regulator (van der Meer *et al.*, 1993) and a sensor of the histidine protein kinase family (de Vos *et al.*, 1995a; Immonen *et al.*, 1995; Siegers & Entian, 1995), respectively, that belong to the class of two-component regulatory systems (Stock *et al.*, 1990). It has been shown that both genes are involved in the regulation of nisin biosynthesis (van der Meer *et al.*, 1993; Engelke *et al.*, 1994). Recently, it has been demonstrated that fully modified nisin is the extracellular input signal for the sensor NisK (Kuipers *et al.*, 1995). Small amounts of fully modified nisin can activate the transcription of its own structural gene, the transcription of the downstream genes, and transcription of *nisFEG*, via this two-component regulatory system (Kuipers *et al.*, 1995; de Ruyter *et al.*, 1996; Ra *et al.*, 1996).

In this paper we describe disruptions in several genes of the nisin gene cluster, of which all except one were made in-frame to avoid polar effects on downstream genes. The effects of these disruptions on the transcription of nisin genes, nisin production and producer immunity were analysed. Remarkably, several levels of immunity could be distinguished in the knock-out strains relative to the wild-type strain, depending on the production levels of NisI and NisFEG, but the wild-type level could never be reached.

METHODS

Bacterial strains, plasmids and media. *L. lactis* strain NZ9700 (Kuipers *et al.*, 1993) is a nisin A producing transconjugant, which was obtained from a mating between *L. lactis* MG1614 (Gasson, 1983) and the nisin A producer *L. lactis* NIZO R5 (Rauch & de Vos, 1992). NZ9800 is a derivative of NZ9700, with a 4 bp deletion in the pronisin-encoding part of the *nisA* gene, which is unable to produce nisin (Kuipers *et al.*, 1993). *L. lactis* strains were grown in M17 broth (Difco) supplemented

with 0.5% (w/v) glucose or sucrose at 30 °C without aeration. *Escherichia coli* strain MC1061 (Casadaban *et al.*, 1980) was used as a host strain for cloning experiments; it was grown in Tryptone Yeast (TY) medium (Sambrook *et al.*, 1989) at 37 °C. Antibiotics were used in the following concentrations: ampicillin, 50 µg ml⁻¹; erythromycin, 2.5 µg ml⁻¹; chloramphenicol, 10 µg ml⁻¹.

Construction of plasmids. The construction of the integrative plasmid pNZ9135, used for the disruption of the *nisB* gene by gene replacement, has been described previously (Kuipers *et al.*, 1995). The gene replacement results in a Δ *nisB* gene, in which the codons for amino acid residues 474–535 were specifically deleted. To construct the integrative plasmid pNZ9143 for disruption of the *nisT* gene, a 3.9 kb *SstI*–*AccI* fragment containing the *nisT* gene and flanking regions was cloned into a *SstI*–*AccI*-digested pUC19 vector, which had an additional erythromycin resistance marker. The *nisT* gene was changed by introducing a 231 bp in-frame deletion in the middle of the gene; this was accomplished by removing an internal *SpeI* fragment, resulting in the removal of the codons for amino acid residues 318–395. To construct the single-crossover integrative plasmid pNZ9134 (Δ *nisC*), an internal 753 bp *NdeI*–*NcoI* (filled in with Klenow) fragment of *nisC* was cloned into a *NdeI*–*SmaI*-digested pUC19 vector, which had an additional erythromycin resistance marker from pE194 (Leenhouts *et al.*, 1991). To construct the integrative plasmid pNZ9147, a 4.7 kbp *SpeI*–*BclI* fragment containing the *nisl* gene and flanking regions was cloned into a *XbaI*–*BamHI*-digested pUC19 vector, which had an additional erythromycin resistance marker. The *nisl* gene was almost completely removed by introducing a 399 bp in-frame deletion, resulting in removal of the codons for amino acid residues 57–190 of NisI. This was accomplished by removing an internal *HpaI* (partial digest)–*AvaI* (filled in with Klenow) fragment from the gene.

DNA, RNA and protein techniques. Plasmid and chromosomal DNA of *L. lactis* were isolated as described previously (Vos *et al.*, 1989). *L. lactis* cells were transformed by electroporation (Holo & Nes, 1989). Plasmid isolations from *E. coli* cells, and transformations of *E. coli* strains, were carried out according to established procedures (Sambrook *et al.*, 1989). Restriction enzymes, T4 DNA ligase and other DNA-modifying enzymes were purchased from Gibco-BRL Life Technologies, New England Biolabs, Pharmacia or Promega and used as recommended by the manufacturers. Cloning procedures, radio-labelling of DNA fragments, agarose-gel electrophoresis and Southern blot hybridizations were carried out according to established procedures (Sambrook *et al.*, 1989). PCRs were performed using the conditions described before (Kuipers *et*

Table 1. Primers used in this study

Primer	Sequence	Location
1	5'-GAGTCCGAACCGAGTAC-3'	<i>nisB</i>
2	5'-GAGTTGAAATGTCTCGTG-3'	<i>nisB</i>
3	5'-GGAACATGGAACATTGGG-3'	<i>nisT</i>
4	5'-CATATGTTGAAAGTTTGCTAAC-3'	<i>nisC</i>
5	5'-GAGAAGATATTTAATACTTATTGTGG-3'	<i>nisI</i>
6	5'-ATGCTTCAGTAAGAGATTTTCCTC-3'	<i>nisI</i>
7	5'-GTTTTGATGCGTCGCAACTTCATAG-3'	<i>nisR</i>
NIS121	5'-CTGAITAIATTCTGAAIITGTGTT-3'	Promoter fragment of <i>nisA</i> and <i>nisF</i>

al., 1991). DNA sequencing was performed by the dideoxy-nucleotide chain-termination method (Sanger *et al.*, 1977). Oligonucleotides, used as primers in sequencing reactions and for PCR, were purchased from Pharmacia. Primers used in this study are listed in Table 1. RNA isolation, Northern blotting and subsequent hybridization with radiolabelled probes were performed as described previously (Ra *et al.*, 1995, 1996). The methods for nisin immunity assays for *L. lactis* (Kuipers *et al.*, 1993) and nisin induction experiments (Kuipers *et al.*, 1995) have also been described before. Pre-induction was performed by adding nisin A (0.01 µg ml⁻¹) to the freshly diluted culture. The nisin produced was quantified using an agar diffusion test with *Micrococcus luteus* as indicator. Dilution series of the samples were assayed in order to determine the minimal inhibitory concentration (MIC). This MIC value was used to compare percentage differences, which gives a more reliable estimation than values based on arithmetic measures of diffusion of nisin through agar. Western analyses were done with the KH1422 NisI-specific antiserum as described previously (Qiao *et al.*, 1995).

Introduction of in-frame deletions in chromosomal nisin genes by gene replacement. The plasmids pNZ9135 (Δ *nisB*), pNZ9143 (Δ *nisT*) and pNZ9147 (Δ *nisI*), all pUC19 derivatives that cannot replicate in *L. lactis*, were used to transform the nisin A producing *L. lactis* NZ9700. Erythromycin-resistant (Em^r) colonies were obtained that were the result of the integration of the plasmid caused by a recombination event involving one of the flanking regions on the plasmid and the corresponding regions on the chromosome. After subculturing for 100–200 generations in the absence of erythromycin, Em^s colonies were obtained as a result of a second recombination event with the flanking region on the other side of the disrupted region relative to the first recombination event. The expected disruptions in the chromosomal genes were confirmed by PCR analysis, Southern analysis and sequence analysis.

RESULTS

In-frame deletions in *nisB*, *nisT* and *nisI* and single-crossover disruption of *nisC*

After electroporation of NZ9700 with pNZ9135 to disrupt the *nisB* gene, one of several Em^r colonies with the plasmid integrated in the correct location of the chromosome as judged by Southern analysis was grown without erythromycin. After plating, several Em^s colonies were obtained in which a second recombination

event had occurred resulting in excision of the plasmid from the chromosome. Southern blot hybridization, PCR analysis with primers 1 and 2 (Table 1) and sequence analysis confirmed the expected in-frame disruption of the *nisB* gene on the chromosome in one of the colonies. The resulting strain was named NZ9735. Using a similar approach with other integrative plasmids, in-frame deletions were also obtained in *nisT* (NZ9743) and *nisI* (NZ9747). Attempts to obtain a double-crossover integration to disrupt the *nisC* gene on the chromosome of *L. lactis* NZ9700 were unsuccessful. After changing the strategy to obtain single-crossover integrants, Southern blot analysis of a picked Em^r colony revealed that four copies of pNZ9134 had integrated in the same spot of the *nisC* gene on the chromosome. The integrated plasmids could not be excised by culturing without erythromycin.

Effects of in-frame deletions and plasmid integration on nisin production and immunity

The effects of the various gene disruptions in the nisin gene cluster on nisin production were studied. Supernatants of overnight cultures were tested in a bioassay, and TCA-precipitated supernatants were analysed by SDS-PAGE for the production of nisin or nisin precursor (Table 2). Nisin production was blocked in all cases with one exception, i.e. strain NZ9747, carrying the in-frame deletion in the *nisI* gene, which was still able to produce nisin, although the production level was reduced to approximately 20–40% of that of the wild-type NZ9700. This confirms that intact *nisB*, *nisC* and *nisT* genes are essential for the production of active nisin in the supernatant and shows for the first time that intact *nisI* is not essential.

A disruption in the *nisI* gene resulted in an immunity level that was still approximately 10–30% of the maximum immunity level of the wild-type nisin-producing strain. Disruptions in *nisB*, *nisT* and *nisC* resulted in very low immunity levels, comparable to the immunity level of the Δ *nisA* strain NZ9800 (Kuipers *et al.*, 1993), which is more than 100 times lower than that of the wild-type nisin producer NZ9700, but still much higher than that of strain MG1614, carrying no nisin

Table 2. Effects of gene disruptions on nisin production, immunity and transcription of *nisFEG*

The amount of nisin used for pre-induction of the cultures was 0.01 µg ml⁻¹. Nisin production levels: + + +, 100%; + +, 20–40%; –, no production. Immunity levels: + + + +, 100%; + + +, 30–60%; + +, 10–30%; +, 5–10%; –, <1%. *nisFEG* transcript levels: + + + +, 100%; + + +, 50–70%; + +, 10–30%; –, <1%.

Strain	Extracellular nisin production	Immunity		Transcription of <i>nisFEG</i>	
		No induction	Pre-induction with nisin	No induction	Pre-induction with nisin
MG1614	–	–	–	–	–
NZ9700	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
NZ9800 <i>ΔnisA</i>	–	–	+ +	–	+ +
NZ9735 <i>ΔnisB</i>	–	–	+ +	–	+ +
NZ9743 <i>ΔnisT</i>	–	–	+ +	–	+ +
NZ9734 <i>ΔnisC</i>	–	–	+	–	+ +
NZ9747 <i>ΔnisI</i>	+ +	+ +	+ +	+ + +	+ + +

Fig. 2. Northern blot using total RNA from various *L. lactis* strains with the structural *nisA* gene as a probe. Lanes: 1, NZ9700; 2 and 3, MG1614; 4 and 9, NZ9800 (*ΔnisA*); 5 and 10, NZ9735 (*ΔnisB*); 6 and 11, 9743 (*ΔnisT*); 7 and 12, NZ9734 (*ΔnisC*); 8 and 13, NZ9747 (*ΔnisI*). The RNA in lanes 4–7 was isolated from cells that had been pre-induced with 0.01 µg nisin A ml⁻¹.

Fig. 3. Western blotting analysis of cells of *L. lactis* strains. A polyclonal NisI-specific antiserum was used to recognize the NisI protein. Lanes: 1 and 2, NZ9800 (*ΔnisA*); 3 and 4, NZ9735 (*ΔnisB*); 5 and 6, NZ9743 (*ΔnisT*); 7 and 8, NZ9734 (*ΔnisC*); 9 and 10, NZ9747 (*ΔnisI*); 11, NZ9700; 12, MG1614. The cells of the samples in lanes 2, 4, 6 and 8 were pre-induced with 0.01 µg nisin A ml⁻¹.

genes. However, when cells were pre-induced with 0.01 µg nisin A ml⁻¹, the immunity levels were elevated to approximately 20% of the wild-type level (Table 2). Although no production of nisin was found in the extracellular medium after induction, it is possible that an intracellular, partly modified precursor nisin is produced by strains NZ9735 (*ΔnisB*), NZ9734 (*ΔnisC*) and NZ9743 (*ΔnisT*). However, we were not able to detect any accumulation of intracellular nisin precursor with antibodies against the nisin leader peptide. The presence of a partly modified nisin precursor secreted by nisin-induced cells of strains NZ9735 or NZ9734 could also not be detected.

Effects of gene disruptions and nisin induction on transcription of nisin immunity genes

Strains NZ9735, NZ9743, NZ9734 and NZ9747, with disruptions in the *nisB*, *nisT*, *nisC* and *nisI* genes, respectively, were checked for the presence of a *nisA* and a *nisFEG* transcript by Northern hybridizations. In all cases, except in NZ9747, *nisA* and *nisFEG* transcription was absent (Fig. 2, Table 2). Addition of nisin to the

growing cultures restored the transcription of *nisA* and *nisFEG* in all knock-out strains (Fig. 2, Table 2). In accordance with this, Western blotting analysis with NisI-specific antiserum showed that without induction, NisI is not produced in the mutant strains, whereas induced cells produce NisI, except strain NZ9747 (*ΔnisI*) and strain NZ9734 (*ΔnisC*) (Fig. 3). In strain NZ9734 four plasmids had integrated into the *nisC* gene and this is likely to hinder transcriptional readthrough of the *nisC* to the *nisI* gene from the induced *nisA* promoter. Interestingly, this strain was also less immune than the other knock-out strains, even after induction.

Analysis of the number of promoters similar to the *nisA* and *nisF* promoters

In order to evaluate whether nisin induction could potentially initiate the transcription of genes other than *nisABTCIPRKFEFG*, a Southern analysis was performed with *EcoRI*/*EcoRV*-digested chromosomal DNA of a nisin-producing strain using a degenerate oligonucleotide probe which hybridizes to the conserved regions of the two known nisin promoters. The expected size of the fragments with these promoters is 3 and

Fig. 4. Southern blot analysis of *L. lactis* strain MG1614, which does not contain the nisin operons (lane 1), and the nisin-producing strain NZ9700 (lane 2). A degenerate probe (NIS121) recognizing the homologous sequences of the *nisA* and *nisF* promoters was used.

1.3 kb. The Southern blot result (Fig. 4) did not visualize any other bands than the expected ones, indicating that a nisin producer has no additional promoters in the chromosome with high homology to the *nisA* or *nisF* promoters.

DISCUSSION

Gene disruptions with polar effects have previously been reported for the *nisA*, *nisB*, *nisT*, *nisC*, *nisI*, *nisP*, *nisR*, *nisF* and *nisE* genes (Kuipers *et al.*, 1993; Siegers & Entian, 1995; Siegers *et al.*, 1996; Dodd *et al.*, 1996; Ra *et al.*, 1996; Qiao *et al.*, 1996). However, correlating a phenotype to a specific locus using a mutant strain that contains a polar mutation, especially if the mutation is in a large operon like the biosynthetic operon expressed from the *nisA* promoter, is not without risk. To avoid possible ambiguities, in-frame deletions were constructed in the *nisB*, *nisT* and *nisI* genes. Attempts to obtain an in-frame deletion in the *nisC* gene were unsuccessful and resulted in a strain with this gene disrupted by plasmid integration. Nisin production had ceased in the strains with the in-frame mutations in *nisB* and *nisT* and could not be restored by addition of subinhibitory amounts of nisin. Transcription of the genes downstream of the mutated *nisB* or *nisT* genes was deduced to take place, because after nisin induction the product of the downstream *nisI* gene was detected in Western analysis using a NisI-specific antiserum. The fact that all nisin genes were transcribed after nisin induction and still no nisin was produced by the NZ9735

and NZ9743 strains conclusively demonstrates that NisB and NisT are essential for the biosynthesis of nisin. The putative structure of NisT and the recent results of Siegers *et al.* (1996) and Qiao & Saris (1996) suggest that NisT is involved in transport of precursor nisin and NisB in dehydration of the nisin precursor.

Analysis of the NZ9734 strain with the disrupted *nisC* gene showed that the plasmid insertion in *nisC* resulted in polar effects as judged from the fact that no NisI protein could be detected after nisin induction. Because no modified nisin precursor was secreted by strain NZ9734 upon nisin induction, as would be expected if NisC was not essential for the biosynthesis of nisin, it can be concluded that NisC is essential for nisin maturation.

Nisin induction of the knock-out strains (Δ *nisB*, Δ *nisT*, Δ *nisC* and Δ *nisI*) did not result in full restoration of transcription of the nisin operons. A lower transcription level of the nisin operons is likely to explain the lower immunity level of induced mutant strains compared to nisin-producing strains (Dodd *et al.*, 1996). A wild-type level of transcription of the nisin operons might require gradually increasing nisin concentrations as in a nisin producer and such fine-tuning may not occur when nisin is externally added at a certain time point to mutant strains. The increase in immunity levels of the nisin mutant strains by nisin induction could also be a result of induction of nisin-inducible genes other than *nisABTCIPRKFE*G. If such genes did exist, their promoter would most likely share sequence similarity with the nisin-inducible promoters in front of *nisA* and *nisF* (de Ruyter *et al.*, 1996; Ra *et al.*, 1996). The Southern blot analysis of DNA of a nisin-producing strain using a degenerate probe recognizing the conserved sequences of the *nisA* and *nisF* promoters (de Ruyter *et al.*, 1996; Ra *et al.*, 1996) suggests that a nisin-producing strain does not contain additional nisin-inducible promoters. Therefore, the increase in the level of nisin immunity of the nisin mutant strains observed after nisin induction is likely to be the sole result of the increased transcription of the two known nisin-inducible operons, i.e. *nisABTCIP* and *nisFE*G.

The NZ9747 strain with a deletion in the *nisI* gene did not produce the NisI protein but could still produce nisin. The lack of the NisI immunity protein affected the growth of the strain. It did not grow to as high cell densities as the parental strain and the maximum amount of nisin produced was also lower: approximately 20% of the highest amount that could be produced by the wild-type strain. The cells of strain NZ9747 could tolerate the amount of nisin that they produced themselves, probably due to the expression of the *nisFE*G genes, but the sensitivity to externally added nisin was approximately five times higher than in the wild-type. The nisin immunity level of strain NZ9747 showed that without the NisI lipoprotein the NisF, NisE and NisG immunity proteins can protect the cells to approximately 20% of the wild-type level. Duan *et al.* (1996) described from *L. lactis* a plasmid-encoded nisin

resistance determinant consisting of only the *nisRKFE*G genes. The presence of this nisin resistance plasmid resulted in an immunity level of approximately 20% of a wild-type nisin producer. The nisin immunity level of strain NZ9747 corresponds well to the nisin resistance level of the nisin resistance plasmid containing the *nisRKFE*G genes. From these results some conclusions can be drawn concerning the question whether NisI cooperates with the NisFEG polypeptides or whether they represent separate immunity systems. Expressed to wild-type and higher levels without other *nis*-encoded genes NisI gives only 1–4% of the wild-type immunity level (Kuipers *et al.*, 1993; Qiao *et al.*, 1995). If NisI represents a non-cooperating immunity system, then the question arises why the immunity level of NZ9747 is only 20% of the wild-type level and is not in the range of >95% as would be expected if NisI formed an independent immunity system. Therefore, NisI clearly cooperates with some of the transposon-encoded polypeptides, probably with the NisFEG polypeptides. The observed 80% reduction of nisin immunity in the NisI-deficient strain can be partly explained by assuming that the efficiency of the immunity proteins is also influenced by the presence of functional complexes with other *nis*-encoded membrane proteins, e.g. NisB, NisP or NisT. This has been suggested by previous results of Kuipers *et al.* (1993) showing that expression of *nisI* resulted in at most 1–4% of wild-type immunity, whereas when *nisI* was expressed together with the *nisABTC* genes the immunity level was higher, ranging between 8 and 20% of the wild-type level. Thus, full nisin immunity seems to require nisin production and fully induced *nisI* and *nisFEG* genes. This notion is further supported by recent studies showing that the production of antisense-*nisEG* or antisense-*nisG* RNA severely reduced the immunity levels in the *L. lactis* strain tested (Immonen & Saris, 1998).

The killing activity of nisin requires pore formation in the target cell. It has been speculated that NisI as a lipoprotein could destabilize this pore formation (Entian & de Vos 1996; Saris *et al.*, 1996) or assist the putative transport function of NisFEG. Recently Qiao (1996) has shown by circular dichroism (CD) spectroscopy and biomolecular interaction analysis (BIA) that purified NisI does indeed have physical interactions with nisin. On the basis of sequence homology the *nisFEG*-encoded proteins belong to the family of ABC transporters (Siegers & Entian, 1995), which strongly suggests that nisin immunity is dependent on nisin translocation. Our present view of nisin immunity comprises cooperative interactions of NisI with the putative NisFEG complex, in which translocation of nisin from the membrane to the cell exterior by NisFEG activity is mediated or facilitated by NisI interactions with nisin.

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Role of multiple gene copies in particulate methane monooxygenase activity in the methane-oxidizing bacterium *Methylococcus capsulatus* Bath

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Genes for the subunits of particulate methane monooxygenase, PmoABC, have been sequenced from the γ -proteobacterial methanotroph *Methylococcus capsulatus* Bath. *M. capsulatus* Bath contains two complete copies of *pmoCAB*, as well as a third copy of *pmoC*. The two *pmoCAB* regions were almost identical at the nucleotide sequence level, differing in only 13 positions in 3183 bp. At the amino acid level, each translated gene product contained only one differing residue in each copy. However, the *pmoC3* sequence was more divergent from the two other *pmoC* copies at both the far N-terminus and far C-terminus. Chromosomal insertion mutations were generated in all seven genes. Null mutants could not be obtained for *pmoC3*, suggesting that it may play an essential role in growth on methane. Null mutants were obtained for *pmoC1*, *pmoC2*, *pmoA1*, *pmoA2*, *pmoB1* and *pmoB2*. All of these mutants grew on methane, demonstrating that both gene copies were functional. Copy 1 mutants showed about two-thirds of the wild-type whole-cell methane oxidation rate, while copy 2 mutants showed only about one-third of the wild-type rate, indicating that both gene copies were necessary for wild-type particulate methane monooxygenase activity. It was not possible to obtain double null mutants that were defective in both *pmo* copies, which may indicate that some expression of pMMO is important for growth.

Keywords: methane monooxygenase, methanotroph, duplicate genes, *pmo*

INTRODUCTION

Methanotrophs are a group of Proteobacteria that utilize methane as a sole source of carbon and energy (Hanson *et al.*, 1990). The initial transformation involves the conversion of methane to methanol by methane monooxygenase (MMO). Two types of MMO are known, a cytoplasmic or soluble form (sMMO) and a membrane-bound or particulate form (pMMO). All known meth-

anotrophs contain the pMMO, but the sMMO is found in only a few methanotrophs, as a second enzyme (Hanson *et al.*, 1990; Koh *et al.*, 1993; Nakajima *et al.*, 1992). In those strains that contain both enzymes, sMMO is expressed only under copper-limitation (Prior & Dalton, 1985a; Smith & Dalton, 1989). The sMMO is an NADH-linked iron enzyme with a broad substrate specificity (Colby & Dalton, 1976; Fox *et al.*, 1989; Green & Dalton, 1989). The genes for the subunits of sMMO have been cloned and sequenced from *Methylococcus capsulatus* Bath (Stainthorpe *et al.*, 1990), *Methylosinus trichosporium* OB3b (Cardy *et al.*, 1991) and *Methylocystis* sp. strain M (McDonald *et al.*, 1997). Insertion mutants in sMMO genes in *Methylosinus trichosporium* OB3b still express pMMO normally (Martin & Murrell, 1995). Recent studies have suggested that in *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b, transcription of the sMMO

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Abbreviations: pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase; NMS, nitrate mineral salts medium; NMSF, NMS medium containing iron and formate.

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genes is negatively regulated by copper (Nielsen *et al.*, 1997).

The pMMO is a copper enzyme with a relatively narrow substrate specificity, and it contains at least three subunits, of approximately 45, 26 and 23 kDa (Smith & Dalton, 1989; Nguyen *et al.*, 1994, 1998; Zahn & DiSpirito, 1996). The 26 kDa subunit apparently contains the active site, as it binds the suicide inhibitor acetylene (Prior & Dalton, 1985b; DiSpirito *et al.*, 1992). Hybridization studies have suggested that methanotrophs contain multiple copies of genes encoding the 45 and 26 kDa subunits (*pmoB1* and *pmoA1*, respectively) (Semrau *et al.*, 1995). One copy of the genes encoding these two subunits was cloned and sequenced, and the genes were found to be adjacent to each other in the order *pmoA1*–*pmoB1* (Semrau *et al.*, 1995). In addition, a gene encoding the 23 kDa subunit (*pmoC1*) has been shown to be present upstream of *pmoA1* (Nguyen *et al.*, 1996). *pmoA1* and *pmoB1* showed substantial similarity to genes encoding the analogous subunits of a related enzyme, ammonia monooxygenase (Semrau *et al.*, 1995), which are also present in multiple copies in nitrifying bacteria (McTavish *et al.*, 1993; Norton *et al.*, 1996). In the nitrifiers, the genes are also organized in a cluster in the order *amoCAB* with an additional, single copy of *amoC* (Klotz *et al.*, 1997; Sayavedra-Soto *et al.*, 1998). In *Nitrosomonas europaea*, an insertion mutant defective in one of the *amoA* genes showed slower growth than the wild-type, while a mutant containing an insertion in the other *amoA* gene showed normal growth (Hommes *et al.*, 1998). However, mutants are not available in the other *amo* genes. A 3.3 kb transcript covering *pmoA*, *pmoB* and *pmoC* as well as smaller transcripts are detectable in *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b expressing pMMO, but it is not known which of the sets of genes is responsible for these transcripts (Nielsen *et al.*, 1997). Likewise, analogous transcripts have been detected in nitrifying bacteria, and it has been shown that the *amoA* mutant showing slower growth also has decreased amounts of these transcripts (Hommes *et al.*, 1998; Sayavedra-Soto *et al.*, 1998).

In this paper, we present the entire sequence of both *pmoCAB* gene clusters and a third copy of *pmoC* from *Methylococcus capsulatus* (Bath), as well as the phenotypes of insertion mutants generated in each of the seven genes.

METHODS

Bacterial strains, plasmids and growth conditions. *Escherichia coli* strains DH5 α , DH5 α MCR (Bethesda Research Laboratories), Inv α F' (Invitrogen) and S17-1 (Simon *et al.*, 1983) were used in this study (Table 1). They were grown in LB medium in the presence of appropriate antibiotics as described by Sambrook *et al.* (1989). *Methylococcus capsulatus* Bath was grown on nitrate mineral salts medium (NMS) (Whittenbury & Dalton, 1981) in batch culture at 42 °C with or without copper added as CuSO₄·5H₂O (10 μ M final concentration), with a 1:1 (v/v) CH₄/air ratio in the head space, or on agar plates with the same medium. In some cases

NMS without copper, with 0.5 mM ferrous sulfate and 1 mM formate (NMSF) was used to grow bacteria with sMMO expressed. All *M. capsulatus* Bath mutants were grown from frozen cultures and tested by diagnostic PCR after growth to ensure that the insertion mutations were correct.

DNA manipulations. Plasmid isolation, *E. coli* transformation, restriction enzyme digestion, ligation, blunting ends with T4 DNA polymerase, or filling in ends with Klenow enzyme were carried out as described by Sambrook *et al.* (1989). The chromosomal DNA of *M. capsulatus* Bath grown in batch culture was isolated by the procedure of Marmur (1961). For the insertion mutants, chromosomal DNA was isolated from cells grown on agarose plates. In this case, cells were washed from the agarose surface using 3 ml TEN (50 mM Tris/EDTA + 150 mM NaCl) and the liquid was collected in 25 ml Correx tubes. Tubes were spun for 5 min at 10000 g and 4 °C and the supernatant poured off. The pellet was resuspended by adding 3 ml TEN with 4 mg lysozyme ml⁻¹ and incubated at 37 °C for 1 h. Next, 3 ml TEN with 4% (w/v) SDS was added to the tubes and incubated in a 45–50 °C water bath until the solution was clear (approximately 30 min). DNA was extracted using phenol and precipitated using ethanol by standard procedures (Sambrook *et al.*, 1989).

DNA sequencing. DNA sequencing was carried out by the Caltech Sequencing Facility and the University of Washington Sequencing Facility, with an Applied Biosystems automated sequencer, from both strands.

DNA–DNA hybridization. DNA–DNA hybridizations were carried out with dried agarose gels as described by Meinkoth & Wahl (1984) at 68 °C for DNA fragment probes or 42 °C for oligonucleotide primer probes. For hybridizations, 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate) was used; for washes, 0.5 $\times$ SSC was used. Dried gels are not appropriate for DNA fragments less than 0.8 kb, and for these gels, nucleic acids were transferred to a nylon membrane (HybondN⁺) by capillary blotting and treated according to the vendor's recommendation. For DNA labelling, a random-primed DNA labelling kit (Amersham) or direct nucleic acid labelling and detection systems (Amersham) were used for radioactive or non-radioactive labelling, respectively. T4 polynucleotide kinase (Boehringer Mannheim) was used for oligonucleotide labelling.

PCR. PCR was performed in 50 μ l reaction mixtures in 0.5 ml microcentrifuge tubes. Reactions were carried out in a Perkin-Elmer GeneAmp PCR System 9600 thermal cycler. Thirty cycles of 92 °C for 1 min; 55 °C for 1 min; 72 °C for 1 min were performed followed by a final extension of 5 min at 72 °C to amplify fragments up to 3 kb. To amplify fragments more than 3 kb, the program recommended by Boehringer Mannheim for the Expand PCR System was used: 2 min at 92 °C, 10 cycles of 92 °C for 15 s, 57 °C for 30 s, 68 °C for 4 min and 15 cycles with elongation times of 20 s, with a final step of 72 °C for 7 min. *Taq* polymerase (Boehringer Mannheim) was used. Primers used were as follows. For the Km-cassette (accession no. X06404): KBF1 (5'-CGATAGATTGTCGCACCTG-3') and KBR2 (5'-CACTTTCTGGCTGG-ATG-3'). For amplification of *pmo* genes (accession no. L40804): CSS1F (5'-CAAACAATGGCAGCAACAAC-3') 118 to 138 bp, CSS2F (5'-CCTGTGGGTGCGGTGGTAC-3') 222 to 241 bp, CSSK4F (5'-GGCACTGGCCGTCATGGGCAC-3') 799 to 820 bp, CSS9F (5'-GCCACACCTTCTGGTTC-ATGG-3') 727 to 747 bp, CSS9R (5'-GCCTTCGTCCACG-GCTTC-3') 875 to 893 bp, CSS10R (5'-AGATGAACGCC-GCAAAAC-3') 601 to 618 bp, CSS16R (5'-GTGAGCCAC-TTCTTGTCACG-3') 172 to 190 bp, CSS17R (5'-GAGCT-

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant trait(s)	Source or reference
<i>E. coli</i>		
DH5 α	(ϕ 80 <i>dlacZ</i> Δ M15) Δ (<i>lacZY-argF</i>)U169 <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (r_k^- m_k^+) <i>supE44</i> <i>gyrA96</i> <i>relA</i>	Bethesda Research Laboratories
DH5 α MCR	DH5 α <i>mcrA</i> Δ (<i>mmr-hsdRMS-msrBC</i>)	Bethesda Research Laboratories
Inv α F'	(ϕ 80 <i>lacZ</i> Δ M15) Δ (<i>lacZYA-argF</i>) <i>deoR</i> ⁺ F' <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (r_k^- m_k^+) <i>supE44</i> <i>gyrA96</i> <i>relA</i>	Invitrogen
S17-1	<i>thi pro recA hsdR</i> RP4-2- <i>tet</i> ::Mu <i>aphA</i> ::Tn7	Simon <i>et al.</i> (1983)
<i>M. capsulatus</i> Bath		
T401	Uncharacterized Tn5 chromosomal insertion	This study
MAK5	<i>pmoA1</i> ::Km ^r	This study
MAK16	<i>pmoA2</i> ::Km ^r	This study
MBK1-1	<i>pmoB1</i> ::Km ^r	This study
MBK206	<i>pmoB2</i> ::Km ^r	This study
MCK60	<i>pmoC1</i> ::Km ^r	This study
MCK62	<i>pmoC2</i> ::Km ^r	This study
MBS2	<i>pmoB1</i> ::Sm ^r	This study
MBS202	<i>pmoB2</i> ::Sm ^r	This study
Plasmids		
pUC18/19Ap ^r	<i>lacZ</i>	Yanisch-Perron <i>et al.</i> (1985)
pCR II	Ap ^r Km ^r <i>lacZ</i> α	Invitrogen
pCR2.1	Ap ^r Km ^r <i>lacZ</i> α	Invitrogen
pUC4K	Ap ^r Km ^r	Vieira & Messing (1982)
pAYC61	Ap ^r Tc ^r	Chistoserdov <i>et al.</i> (1994)
pBR325	Ap ^r Cm ^r Tc ^r	Bolivar (1978)
pBR325 Ω	pBR325:: Ω Sm ^r /Sp ^r	C. Murrell, University of Warwick, UK
pSUP2021	Ap ^r Km ^r	Simon <i>et al.</i> (1983)
pRK2013	Km ^r	Figurski & Helinski (1979)
pAMC100	pUC19 with 2.9 kb <i>KpnI</i> insert	Semrau <i>et al.</i> (1995)
pAMC101	pUC19 with 1.8 kb <i>KpnI</i> PstI insert	Semrau <i>et al.</i> (1995)
pAMC102	pUC19 with 2.5 kb <i>EcoRI</i> insert	Semrau <i>et al.</i> (1995)
pAMC103	pUC19 with 1.0 kb <i>EcoR</i> insert	This study

GCACCACCAATGGTTG-3') 135 to 155 bp, CSS19R (5'-GTACCACCGCACCCTCAGG-3') 222 to 241 bp, ASS1F (5'-CTGGGACTTCTGGTCGGACTG-3') 1202 to 1223 bp. Copy 2 specific (accession no. U94337): ESSR (5'-CCTGACGTCTAAAATCCAGC-3') 3242 to 3262 bp. For amplification of *pmoC3* (accession no. AF091320): C3F1 (5'-GGCATCAATCCAACACC-3') 1 to 17 bp, C3R1 (5'-GCGGACGGTGGCTCCCTGG-3') 855 to 874 bp.

All sequence generated by PCR was confirmed from a second, separate amplification reaction.

Computer analysis. Translation and analyses of DNA and DNA-derived polypeptide sequences were carried out using Genetic Computer Group (Wisconsin) programs and the National Center for Biotechnology Information BLAST server using the BLAST algorithm (Altschul *et al.*, 1990, 1997).

Construction of insertion mutants affected in pMMO genes and a control strain. The Km^r cassette from pUC4K and the

Ω Sm^r/Sp^r cassette from pBR325 Ω were used as selective inactivating markers. pBR325 Ω was constructed in the laboratory of J. C. Murrell (University of Warwick, UK) using the omega fragment from pHP45-omega (Prentki & Kirsh, 1984). The constructs with mutated genes were subcloned into the suicide vector pAYC61 (Chistoserdov *et al.*, 1994) and introduced into *M. capsulatus* Bath by conjugation. *E. coli* S17-1 was used as donor. Matings were conducted as described by Stoltyar *et al.* (1995). Donor and recipient cells (ratio 1/5–1/10) were mixed and resuspended in NMS medium, then cells were pelleted, resuspended by pipetting in 30–40 μ l NMS and plated as a spot on NMS agar. Plates were incubated at 37 °C for 48 h under methane/air, and then cells were transferred to selective plates. Mutants of *M. capsulatus* Bath were selected on plates with 50 μ g Km ml⁻¹ or 100 μ g Sm and Sp ml⁻¹ and screened on plates with 75 μ g Ap ml⁻¹ to test for vector absence. Ap-sensitive clones were examined by hybridization to DNA blots with gene-specific and vector-specific

probes to verify recombinational events, as well as by PCR amplification using primers for diagnostic regions.

A control strain was generated for comparative phenotypic studies in which a Tn5 (Km^r gene) was inserted into a random chromosomal location by using pSUP2021 and pRK2013 (as a helper plasmid). This strain shows normal growth on methane, and is able to grow on methane in the presence of kanamycin.

sMMO assay. sMMO activity was detected on plates using the naphthalene plate assay described by Graham *et al.* (1992). Maximum sMMO activity on plates was achieved when cells were grown on NMSF medium.

Whole-cell methane oxidation assay. Rates of whole-cell methane-dependent O₂ uptake were determined polarographically using an oxygen electrode chamber at 42 °C, as described by McNerney & O'Connor (1980). Cells were washed and resuspended in 50 mM HEPES buffer pH 7.0.

RESULTS

Sequencing of *pmoC1*

A 2.9 kb *KpnI* fragment of *M. capsulatus* Bath DNA had been previously cloned (pAMC100), and sequencing of the 3' region revealed *pmoA1* and part of *pmoB1* (Fig. 1; Semrau *et al.*, 1995). A 1.8 kb *KpnI*-*PstI* fragment containing most of *pmoA1* and 1 kb of the upstream region was subcloned into pUC19 to generate pAMC101 (Fig. 1), and the insert was sequenced. One ORF was found upstream of *pmoA1*. A region near the N-terminus starting 10 residues after the first methionine revealed an almost perfect match to an amino acid sequence (AAAEAPLKDKKWLTF) that had been determined by Edman degradation for the 23 kDa pMMO subunit (Nguyen *et al.*, 1998). The only discrepancy was that the underlined lysine was predicted by the nucleotide sequence to be a leucine. This ORF was designated *pmoC1* and is predicted to encode a polypeptide of 260 amino acids with a molecular mass of 29 000 Da, assuming it starts at the position determined by Edman degradation. A hydropathy analysis of *pmoC1* predicts a minimum of three and a maximum of six membrane-spanning regions with no classical leader sequence at the N-terminus. A search of both protein and DNA databases revealed significant identity with *amoC* sequences from nitrifiers (Klotz *et al.*, 1997; Sayavedra-Soto *et al.*, 1998) (44–54% identity at the amino acid level).

Sequencing and analysis of the second copy of *pmo* genes

Hybridization experiments had suggested that part of the second copy of *pmoB* was located on a 1.0 kb *EcoRI* fragment (Semrau *et al.*, 1995). This fragment was cloned into pUC19 to generate pAMC103 (Fig. 1), and the insert was sequenced. This sequence showed high identity to the *pmoB1* sequence, and an ORF was present whose predicted product showed high identity to PmoB1. All attempts to clone the upstream *KpnI* and *EcoRI* fragments were unsuccessful, even with low-

copy-number vectors. It has been suggested that these gene products may be toxic in alternative hosts (Semrau *et al.*, 1995). Hybridization analysis of chromosomal DNA digested with different restriction enzymes and probed with *pmoA1*, *B1* and *C1* sequences demonstrated that the segment of chromosomal DNA upstream of *pmoB2* contained *pmoA* and *pmoC* sequences similar to the genes previously characterized. Therefore, the sequence of this region was obtained by PCR, using Km-insertion strains to target the second copy.

To obtain the missing sequences for the second copy of *pmoAB*, strain MBK206 containing a Km insertion in the *KpnI* site of *pmoB2* (Fig. 1; and see below) was used for amplification from the chromosome. The primers targeted the 5' end of *pmoA2* (ASSF1) and the Km-resistance cassette (KBF1), to amplify a fragment overlapping the unsequenced region. The use of the Km-resistance insertion mutant ensured that the amplified fragment was from copy 2, not copy 1. A PCR product with the expected size of 1.9 kb was obtained. This DNA fragment was cloned into the Invitrogen vector pCR2.1 and sequenced. As expected, it contained most of *pmoA2* and the 5' portion of *pmoB2*.

For the region containing *pmoC2*, probing experiments had shown that *pmoC2* and the 5' end of *pmoA2* were present on a 1.4 kb *Sall* fragment (data not shown). Assuming that *pmoC2* was similar in size to *pmoC1*, this *Sall* fragment should contain all of *pmoC2* and 0.4 kb of DNA 5' to *pmoC2*. Attempts to clone this *Sall* fragment were unsuccessful. Therefore, an inverse PCR procedure was used that involved the insertion mutant MCK62, which contains a Km-resistance gene in the *BbsI* site of *pmoC2* (Fig. 1; see below for details of mutant construction). First, the appropriate size fraction of *Sall*-digested MCK62 DNA was isolated from a gel. This pool of DNA was then ligated, which joined the *Sall* fragments into a circle. This DNA was then digested with *HindIII*, which cuts within the Km-resistance gene but not within the *pmoC2* region. This procedure separated *pmoC2* and its upstream region into two inverted parts flanked by portions of the Km-resistance cassette. It was then possible to amplify the entire region using appropriate primers targeted to the ends of the Km-resistance cassette (KBF1 and KBR2). A fragment of the correct size (2 kb) was obtained, and it was sequenced directly as a PCR product. Based on the known sequence for the first copy of *pmoCA* and for the Km-resistance cassette, we identified *pmoC2*, the *pmoC2*-*pmoA2* intergenic region and 170 bp of *pmoA*. The sequence of the 5' end of *pmoC2* and the region immediately upstream of the translational start site was confirmed by direct PCR from chromosomal DNA, using appropriate primers. These data completed the sequence of all three subunits of the second copy of the *pmo* genes.

We have also amplified 3.5 kb and 3.1 kb fragments containing the entire *pmoCAB2* gene cluster. The 3.5 kb fragment containing all three genes plus the known upstream region was amplified using a primer specific to

Fig. 1. Physical and genetic map of the fragments containing the *pmo* genes of *M. capsulatus* Bath. Triangles indicate sites of the *Km* (open) and *Sm* (filled) resistance genes insertions; the names of each insertion mutant are noted above each triangle. Lines above depict cloned fragments used in this study. Spheres indicate putative hairpin structures.

a region just inside the *SalI* site upstream of *pmoC2* and the ESSR1 primer complementary to the region immediately downstream of *pmoB2*. The primers used in amplifying the 3.1 kb fragment were CSS1F and ESSR1. This produced a fragment with the same 3' end as the 3.5 kb fragment, but with a shorter 5' end that begins 6 nucleotides upstream of the start codon for *pmoC2*. Therefore, both fragments contain all three ORFs, but the smaller one lacks a 0.4 kb 5' region. These fragments were the expected size, confirming that the *pmoCAB2* genes that have been sequenced are linked on the chromosome. We were able to clone the 3.1 kb fragment using the pCR2.1 vector but were unable to clone the larger fragment. In addition, we were able to clone a PCR product containing a 400 bp region upstream of the *pmoC2* translational start site. These results suggest that the difficulties in cloning these genes are related to expression of *pmoC2*.

The nucleotide sequence of the region containing the second copy of *pmoCAB* was almost identical to the first copy. Starting with the ATG identified for *pmoC1*, the sequences of *pmoC1* and *pmoC2* differ only in 3 nucleotides, which result in only one amino acid change, at residue 239, where PmoC1 contains an arginine and PmoC2 contains a serine (Fig. 2). However, the 125 bp sequence upstream of the start site for *pmoC1* showed

only 40% identity to the same region for *pmoC2*. The *pmoC*–*pmoA* intergenic region showed more divergence than the *pmoC* coding region, with differences in 5 of 164 nucleotides.

The two regions containing *pmoAB* were even more similar at the nucleotide sequence level, differing in only 5 out of 2236 nucleotides. The predicted amino acid sequence of PmoA1 differs from that of PmoA2 only at residue 173, where PmoA1 contains an asparagine while PmoA2 contains a tyrosine. Likewise, the predicted amino acid sequences of PmoB1 and PmoB2 differ only at residue 385, where PmoB1 contains a serine instead of an arginine in PmoB2. The *pmoA1*–*pmoB1* and *pmoA2*–*pmoB2* intergenic regions are identical. The sequences of the two gene copies begin to diverge 80 nucleotides downstream of the termination codon for *pmoB*, after which the identity drops to 35%. Immediately before this divergent point are conserved sequences that could form a double-hairpin structure with a calculated ΔG° of $-194.6 \text{ kJ mol}^{-1}$ ($-46.5 \text{ kcal mol}^{-1}$) for copy 1 and $-204.3 \text{ kJ mol}^{-1}$ ($-48.8 \text{ kcal mol}^{-1}$) for copy 2. This structure is a candidate for a transcriptional terminator. Because of the similarity in the sequences of the two copies, in all cases they were sequenced at least twice from different DNA preparations, and in each case the sequences agreed exactly.

PmoC3	MATTTAGGIAAIDRLLLDKKWLVFAIGIYTVFYLVWRWYEGVYGSAGLDSFAPEFETYW
PmoC2	..A..I..A..AEAP.....T..LA.....
PmoC1	..A..I..A..AEAP.....T..LA.....
	MNFLYTEIVLEIVTASILWGYLWKTRDRNLAALTPREELRRNFTHLVWLVAWAIYWGA
	
	
	SYFTEQDGTWHQITIVRDTDFTPSHIIEFYLSYPIYIITGFAAFIYAKTRLPPFAKGISLP
	
	
	YLVLVVGGPFMILPNVGLNEWGHTFWFMEELFVAPLHYGFVIFGWLALAVMGTLTQTFYSF
	
	R.
241	SHLFERD-LCPDIR 253
	AQGGLGQS..EAVDEGLIAK 260
	AQGGLGQS..EA

Fig. 2. Comparison of the three translated products of the *pmoC* genes. Periods denote residues identical to the PmoC3 sequence; a dash denotes a gap.

Construction of *pmo* gene disruptions and phenotypic characterization of mutants

For the *pmoA* mutants, Km-resistance insertions were generated in the *Sall* site of *pmoA1* in pAMC101, and were used in allelic exchange experiments to obtain insertions in the chromosome for both *pmoA1* and *pmoA2*. For the *pmoC* insertions, Km-resistance insertions were generated in the *BbsI* site of *pmoC1* in pAMC101, and were used to obtain insertions in the chromosome for both *pmoC1* and *pmoC2*. For the *pmoB* mutants, Km-resistance insertions were generated in the *KpnI* site of *pmoB2* in pAMC103, but in this case chromosomal insertions were only obtained in *pmoB2*. Therefore, a second construction was generated with a Km-resistance insertion in the *KpnI* site of *pmoB1* in pAMC102 and was used to generate chromosomal insertions in *pmoB1*. Each of these recombination events was confirmed by hybridization and/or by PCR of specific *pmo*-Km cassette fragments. The hybridization experiments also confirmed previous results (Semrau *et al.*, 1995) showing that two copies of *pmoA* and *pmoB* were present.

Mutants were isolated on plates designed either for pMMO or for sMMO expression, and tested for growth on both types of media. For pMMO expression, plates of NMS medium plus copper were used. For sMMO expression, several types of medium were tested with the wild-type, and it was determined by the naphthalene colorimetric assay that maximal sMMO expression could be achieved with NMS plates containing no added copper, but with added formate and iron (NMSF medium; see Methods). All of these mutants grew on methane on both types of media. On plates of NMSF medium, it was shown by the naphthalene colorimetric assay that the wild-type and the mutants exhibited substantial sMMO activity, which appeared similar by visual examination. On plates of NMS medium containing copper, none of the strains showed detectable sMMO activity, and were assumed to be expressing pMMO.

All of the mutants showed some genetic instability, due to recombination between the gene copies. Therefore, for phenotypic characterization, transfers were kept to a

Fig. 3. Growth curves, determined from Klett readings with a no. 6 filter, of the insertion mutants for the *pmo* genes. (a) *pmoC1*:Km (♦) and *pmoC2*:Km (○); (b) *pmoA1*:Km (♦) and *pmoA2*:Km (○); (c) *pmoB1*:Km (♦) and *pmoB2*:Km (○). Growth curves for the T401 strain (wild-type containing a Tn5 insertion) were the same as for the copy 1 mutants.

minimum and cultures were inoculated from plates that had been streaked directly from frozen stocks. To ensure that the cultures used for phenotypic characterization carried the appropriate mutation, all cultures were tested after growth for the correct insertion by diagnostic PCR.

In broth culture, the growth rate of the mutants defective in the copy 1 genes in the presence of kanamycin was not significantly different from that of a wild-type strain containing a Km-resistance marker, strain T401 (Fig. 3; Table 2). However, the growth rate of the mutants with insertions in the copy 2 genes was consistently 15–20% slower than the wild-type or the copy 1 mutants, and longer lag phases were common. Methane-dependent O₂ uptake rates were determined in mutant and wild-type strains, as a measure of pMMO activity. Mutants in the copy 1 genes had rates that were 65–85% of the wild-type rate. However, mutants in copy 2 genes showed lower rates, 37–50% of the wild-type rate (Table 2).

Attempts were made to generate double mutants in *pmoB* using both Km- and Sm-resistance cassettes. A

Table 2. Doubling time and methane oxidation rates for wild-type and *pmo* mutants

Strain	Affected gene	Doubling time (h)*	CH ₄ oxidation [nmol (mg protein) ⁻¹ min ⁻¹]
T401 (Km ^r wild-type)		7.8 ± 1.0	351
MCK60	<i>pmoC1</i>	6.6 ± 2.1	299
MCK62	<i>pmoC2</i>	9.2 ± 1.3	177
MBK1-1	<i>pmoB1</i>	8.0 ± 0.4	232
MBK206	<i>pmoB2</i>	9.3 ± 2.1	130
MAK5	<i>pmoA1</i>	5.5 ± 2.1	225
MAKF016	<i>pmoA2</i>	8.8 ± 1.2	165

* Measurements are means and standard deviations for triplicate cultures grown in the presence of kanamycin.

Fig. 4. DNA blot showing hybridization of *SalI*-digested *M. capsulatus* Bath chromosomal DNA to a *pmoC1* probe (the *KpnI*–*SmaI* fragment of pAMC101). 1, MCK62 (*pmoC2* insertion strain); 2, MCK60 (*pmoC1* insertion strain); 3, wild-type.

Sm-resistance cassette was inserted into the *MscI* site within *pmoB2* in pAMC103, and an *EcoRI* fragment with the disrupted *pmoB2* sequence was recloned into the suicide vector pAYC61. The MBK1-1 mutant carrying a Km insertion in *pmoB1* was used as a recipient, and selection was carried out on both NMS and NMSF plates. Although colonies were obtained on both types of media that were resistant to both Sm and Km, most of them showed decreased growth on plates with each transfer, and were not viable after three to four transfers. Those that were able to grow through multiple transfers were Ap-resistant, suggesting they were single-crossover recombinants containing a complete *pmoB* gene. Hybridization analysis confirmed the suspected recombination events (data not shown). When the same Sm-resistance construction was used in wild-type, double-crossover recombinants were obtained

separately in *pmoB1* and *pmoB2*, demonstrating that the Sm-resistance plasmid was functional for generating insertion mutations. These mutants were also used as recipients for constructions containing Km insertions into each *pmoB* gene, and in these cases also, no Ap-sensitive clones were obtained.

Third copy of *pmoC*

As noted above, all of the insertion mutants were analysed by hybridization with probes for the genes that were disrupted and for the Km-resistance cassette. In the case of the *pmoA* and *pmoB* mutants, these experiments produced the expected results and confirmed the presence of two copies. However, hybridization of *SalI*-digested *M. capsulatus* Bath chromosomal DNA to a *pmoC1* probe (the *KpnI*–*SmaI* fragment of pAMC101) revealed three bands of hybridization of 1.5, 2.4 and 2.7 kb (Fig. 4). Because it was known that neither *pmoC1* nor *pmoC2* contained a *SalI* site, these results suggested the presence of a third copy of *pmoC* in the genome of *M. capsulatus* Bath. This idea was supported by results from hybridization of *SalI*-digested DNA from the insertion mutants MCK60 and MCK62, which contain a Km-resistance insertion in *pmoC1* and *pmoC2*, respectively. In the *pmoC1* mutant, hybridization with the *pmoC1* probe showed that the 2.7 kb band was increased in size about 1.3 kb, as expected for the insertion, while the other two bands remained the same. In the *pmoC2* mutant, hybridization with the same probe showed that the 1.5 kb band increased to about 2.8 kb, as expected, and the other two bands were not well resolved (Fig. 4). These results suggested that the 2.4 kb band contained a third copy of *pmoC*. *SalI*-digested chromosomal DNA from the MCK60 mutant (containing an insertion in *pmoC1*) was hybridized with primers specific for *pmoC* sequence (CSS16R, CSS17R, CSSK4F, CSS9R and CSS10R) as well as the *pmoA*-specific primer ASS1F. As expected, all of the primers hybridized to the bands corresponding to copy 1 and copy 2, and most of the *pmoC*-specific primers hybridized to the 2.4 kb band containing the putative *pmoC3*. However, CSS17R and ASS1F did not hybridize

to the 2.4 kb *Sall* fragment or to any new fragments. These results suggested that a third copy of *pmoA* is not present and that significant divergence of the *pmoC3* sequence occurs in the N-terminal region. Therefore, we used an inverse PCR strategy similar to that used for the copy 2 genes to obtain the N-terminal, C-terminal and upstream regions of *pmoC3*.

Sall-digested DNA from MCK60 was ligated and redigested with *Afl*III. This DNA was used as a template for PCR with the CSS19R and CSS9F primers. A PCR product of the predicted size was obtained, cloned into pCR2.1 vector and sequenced, providing the sequence of the flanking regions. PCR primers (C3F1 and C3R1, see Methods) were then designed to amplify the whole ORF of *pmoC3* and complete the sequence. An ORF of 253 amino acids was identified with high identity to the other copies of *pmoC* in the central region but containing divergence at the N- and C-termini (Fig. 2).

The entire ORF for *pmoC3* was generated by PCR and cloned into the pCR2.1 vector. However, only plasmids containing *pmoC3* in the orientation opposite to the direction of transcription from the pCR2.1 *lac* promoter were obtained. Attempts were made to generate insertion mutations in *pmoC3*, using the *Bbs*I site for a Km^r cassette and the cloned PCR product containing the entire *pmoC3*. Matings were tested on both NMS plates with copper (for pMMO expression) and NMSF plates (for sMMO expression). However, only single-cross-over insertions were obtained, which generated an intact *pmoC3*. These mutants grew normally on methane.

DISCUSSION

The results in this report complete the sequence of two sets of *pmoCAB* genes, and provide the sequence of a third copy of *pmoC*. For the first two sets, the sequences are almost identical within the coding and intergenic regions, but they diverge outside the *pmoCAB* regions. For *pmoC3*, the sequence diverges in the N- and C-terminus of the encoded protein (Fig. 2). The genetic organization and numbers of gene copies for this *pmo* system are highly similar to those observed for *amo* genes in *Nitrosomonas europaea* (Hommes *et al.*, 1998; Sayavedra-Soto *et al.*, 1998), but other nitrifiers have three sets of *amo* genes (Norton *et al.*, 1996; Sayavedra-Soto *et al.*, 1998). The role of these multiple gene copies has not been well understood in either methanotrophs or nitrifiers. Methanotrophs such as *M. capsulatus* Bath have two alternative methane oxidation systems, the pMMO and the sMMO. Therefore, the physiological role of pMMO is different in these methanotrophs from that of ammonia monooxygenase in nitrifiers, since nitrifiers do not contain an alternative ammonia oxidation system.

In this study, double-crossover insertion mutants were isolated for each *pmo* gene found in the two sets of complete *pmoCAB* clusters, to assess the contribution of each gene copy to pMMO activity. These mutants all grew on methane in the presence of copper and in the absence of detectable sMMO expression, suggesting

that pMMO must be produced from each set of genes under these growth conditions. This conclusion is confirmed by the results of the methane oxidation rate experiments in mutants and wild-type, which showed that about a third of the whole-cell methane oxidation is due to copy 1 genes, while about two-thirds is due to copy 2 genes. The presence of the two copies of *pmo* genes apparently gives the cells excess methane oxidation capacity, since impairment of the copy 1 genes did not affect growth. However, impairment of the copy 2 genes did result in a small growth defect, suggesting that cells need the copy 2 gene expression for normal growth.

These results are similar in some respects to the results obtained so far for the *amo* system in nitrifying bacteria. In *N. europaea*, mutants have only been generated in *amoA* gene copies, so no information is available for *amoB* or *amoC* mutants. For *amoA*, mutants defective in each gene were viable, but mutants in one copy showed decreased growth while the other mutants showed normal growth (Hommes *et al.*, 1998), similar to the results we report here for *pmoA* mutants. However, in the nitrifier the whole-cell ammonia oxidation rate did not change significantly in the mutant with normal growth rate, suggesting that under these conditions the wild-type ammonia monooxygenase activity could be sustained by the other gene copy. This is in contrast to our results, in which both copies are required to maintain wild-type pMMO activity.

Since the two clusters of *pmo* genes are so similar in sequence, it seems likely that the enzymes produced from each copy have a similar function. Therefore, the role of the duplicate gene clusters may be simply to provide increased gene expression, although it appears that this higher level of pMMO activity is not necessary for maximal growth under laboratory conditions. It may be that the two copies are important for different types of growth conditions, for instance under stress conditions that might be encountered in the natural environment. It is also possible that the two copies are differentially expressed under different growth conditions, since the sequences upstream of *pmoC1* and *pmoC2* are highly divergent. Further work involving promoter identification and expression will be required to resolve these questions.

We have also examined *pmoC3*, since the role of this separate *pmoC* gene is unknown and in *M. capsulatus* Bath it has a more divergent sequence than the other two *pmoC* copies. However, we were unable to isolate a null mutant in *pmoC3*. We cannot be certain whether this failure is due to an unknown technical problem that caused double-crossover recombination to occur at extremely low frequencies, or whether it is due to the essential nature of *pmoC3* under the tested growth conditions. However, since we were able to isolate null mutants in *pmoC1* and *pmoC2* using a similar procedure and similar constructions, it is likely that *pmoC3* null mutants are not viable. Therefore, our results suggest that *pmoC3* has an essential function in methane oxidation separate from the function of *pmoC1* and

pmoC2. The role of this separate copy of *pmoC* is not yet clear, but it probably fulfils a function necessary for generating active pMMO from both *pmo* clusters. It is unlikely that this function is to provide PmoC for pMMO, since the major transcript from the two clusters contains all three genes (*pmoCAB*) and it is likely that all three are translated (Nielsen *et al.*, 1997).

Since *M. capsulatus* Bath contains an alternative methane oxidation system (sMMO), it should be possible to obtain a pMMO-negative strain under conditions in which sMMO is expressed. We developed plate growth conditions that maximized expression of sMMO, and attempted to generate a double mutant that contained double-crossover (null) mutations in both *pmoB* genes. However, all isolates always contained one wild-type *pmoB*, the result of a single-crossover recombination event. It is unlikely in this case that a technical problem occurred, because null mutants were readily obtained in each *pmoB* copy individually using both markers. Similar attempts to obtain double mutants in *pmoA* were also unsuccessful (unpublished data). It is known that *M. capsulatus* Bath can grow quite well in broth culture under conditions in which sMMO is the dominant methane oxidation enzyme, with only a small amount of pMMO present (Prior & Dalton, 1985a; Zahn & DiSpirito, 1996). Therefore, it is not clear why null mutants could not be obtained on plates. It is possible that the sMMO levels achieved on plates were too low to allow for growth, although they appeared to be substantial as judged by the colorimetric plate assay. Alternatively, it is possible that low level pMMO expression is required for growth on methane on plates even when sMMO is expressed.

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Expression of the *Oenococcus oeni* *trxA* gene is induced by hydrogen peroxide and heat shock

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Sequencing of the DNA region located upstream of the α -acetolactate synthase and decarboxylase (*alsS*–*alsD*) cluster of *Oenococcus oeni* allowed identification of an ORF, named *trxA*. This encodes a protein of 104 amino acids very similar to known thioredoxins. The protein encoded by the cloned fragment was able to complement *Escherichia coli* strains lacking a functional thioredoxin. Considering the results of protein sequence comparisons and complementation experiments, it was concluded that the *trxA* gene encodes a functional thioredoxin. Studies of *trxA* expression showed that the abundance of *trxA* mRNA was similar during all growth stages. A significant increase in *trxA* mRNA levels was observed in the presence of hydrogen peroxide in the medium or after heat shock. A single transcriptional start site was determined with total RNA isolated from cells subjected or not subjected to oxidative stress or heat shock. In each case the same promoter region was identified and shown to have a high similarity to the consensus promoter sequence of Gram-positive bacteria, as well as to that of *E. coli* and the previously mapped promoters from *O. oeni*.

Keywords: *Oenococcus oeni*, thioredoxin, oxidative stress, heat shock

INTRODUCTION

Thioredoxins have been isolated and characterized from many micro-organisms, plants and animals (Holmgren, 1985). Such proteins are involved in a wide variety of biochemical processes in living cells. Reduced thioredoxin serves as an efficient hydrogen donor for many reductase enzymes and protein disulfide isomerases (Holmgren, 1989). The active site of thioredoxin (Trp-Cys-Gly-Pro-Cys) is highly conserved and contains two redox-active cysteine residues, which form either a disulfide (oxidized form) or a dithiol (reduced form). The S–S bond of oxidized thioredoxin is reduced by NADPH and a specific enzyme called thioredoxin reductase (Moore *et al.*, 1964).

Thioredoxin appears to be an essential protein for many organisms, such as *Bacillus subtilis* (Scharf *et al.*, 1998), *Synechocystis* (Navarro & Florencio, 1996) and *Rhodobacter sphaeroides* (Pasternak *et al.*, 1997), in contrast to

Escherichia coli (Holmgren *et al.*, 1978). A variety of stresses, including heat, salt stress or ethanol treatment, strongly enhance the synthesis of thioredoxin in *B. subtilis* (Scharf *et al.*, 1998). In this species, a double control of stress induction at two different promoters has been demonstrated: the first promoter is σ^B -dependant, whilst the second is presumably σ^A -dependant and might require activation by some other unknown mechanism.

Oenococcus oeni is a lactic acid bacterium which is able to grow in wine and to perform the malolactic fermentation (Dicks *et al.*, 1995; Kunkee, 1967; Labarre *et al.*, 1996a). No information is available on thioredoxin synthesis and activity in this heterofermentative bacterium. In the course of our investigations on the stress response of *O. oeni*, we previously studied an 18 kDa small heat-shock protein (Lo18), highly induced by multiple stresses such as heat shock, acid shock, the presence of ethanol or sulfite, and in stationary phase (Guzzo *et al.*, 1997, 1998). The *hsp18* gene encoding this protein was cloned and characterized (Jobin *et al.*, 1997). After purification of the protein, it was shown that Lo18 could form oligomers and prevent citrate

The GenBank accession number for the sequence reported in this paper is XT93091.

synthase from thermal aggregation (F. Delmas, personal communication).

We report here the nucleotide sequence of a chromosomal DNA fragment encoding the thioredoxin of *O. oeni*. The size of the transcript and the transcriptional start site were determined and the expression of the gene in response to oxidative and heat stresses was investigated.

METHODS

Bacterial strains, plasmids and media. The strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) broth or LB agar at 37 °C. In the selective medium the antibiotic erythromycin was used at a concentration of 200 µg ml⁻¹. *O. oeni* strain Lo84.13 was grown at 30 °C in FT80 medium (pH 5.3) (Cavin *et al.*, 1989), modified by adding meat extract (5 g l⁻¹) instead of Casamino acids.

DNA manipulations, sequencing and analysis. Molecular techniques were carried out using standard methods (Sambrook *et al.*, 1989). Double-stranded plasmid DNA was purified with a Qiagen plasmid kit and sequenced using the dideoxy chain-termination method (Sanger *et al.*, 1977). Computer analyses of nucleotide and amino acid sequences were carried out with the BLAST (Altschul *et al.*, 1990) and CLUSTAL W programs (Higgins *et al.*, 1994).

Isolation of total RNA and Northern blot analysis. *O. oeni* total RNA was purified as previously described (Labarre *et al.*, 1996b) from control cells (grown at 30 °C), or cultivated in the

Fig. 1. *O. oeni* *trxA-alsSD* cluster. The positions of *trxA*, *alsS* and *alsD* are indicated by boxes. The direction of transcription is indicated by arrows. *Scal* restriction sites are shown. The probe used for Northern blotting is indicated by the bold line below the map.

presence of hydrogen peroxide (10 mg l⁻¹), or after addition of hydrogen peroxide (8–20 mg l⁻¹) for 30 or 60 min, or submitted to heat shock for 5–30 min. RNA (30 µg) was separated in a 1.2% (w/v) agarose/6.6% (v/v) formaldehyde gel and then transferred to Hybond-N+ membranes (Amersham). The *trxA* probe (Fig. 1) was radiolabelled with [α -³²P]dATP, using a Random Primer DNA Labelling System (Gibco-BRL). All the Northern blot analyses were repeated at least twice.

Primer extension analysis. Oligonucleotides (TRX1, 5'-CCG GAT TTG GTT GCT TCC TC-3'; TRX2, 5'-TCA GTC ACA GTC ACG CCG GA-3'; and TRX3, 5'-TTG CCG AAT TTA ACC CTC GA-3') were used to map the 5' terminus of *trxA* mRNA in *O. oeni*. Total RNA (5 µg) was mixed with 2 pmol of each primer. After a 5 min denaturation step at 100 °C, the mixture was cooled in ice for annealing. Reverse transcription was carried out as recommended by the manufacturer with the Superscript II RNase H⁻ Reverse Transcriptase (Gibco-BRL). Analysis of the extension products was performed as previously described (Jobin *et al.*, 1997).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
Strains		
<i>O. oeni</i>		
Lo84.13	MLE ⁺	Oenological Institute of Bordeaux
<i>E. coli</i>		
TG1	<i>supE hsdΔ5 thi Δ(lac-proAB) F'(traD36 proA⁺B⁺ lacI^q lacZ ΔM15)</i>	Gibson (1984)
AR1062	<i>F⁻ thr leu ara azi fhuA lacY tsx minA gal rspL xyl mtl thi hsdR</i>	D'Enfert <i>et al.</i> (1987)
BH2012	<i>F⁻ araD139 ? galU galK hsdR rpsL metE46 argH1 trxA2 ilvC::Tn5</i>	Lim <i>et al.</i> (1985)
A312	<i>metE::Tn10</i>	Russel & Model (1986)
A313	<i>trxA::kan metE::Tn10</i>	Russel & Model (1986)
Plasmids		
pGID023	Shuttle vector for <i>E. coli</i> and <i>L. plantarum</i> , derivative of pJDC9 containing pE194 replication functions; Em ^R	Hols <i>et al.</i> (1994)
pGID600	pJDC9 containing a 7 kbp <i>Sau3A</i> fragment from <i>O. oeni</i> DNA	Garmyn <i>et al.</i> (1996)
pGID608	pGID023 containing a 863 bp <i>Scal</i> fragment from pGID600	This study

RESULTS

Nucleotide sequence of *trxA*

In a previous publication, we reported the isolation of a recombinant clone (pGID600) carrying the *O. oeni alsSD* cluster (Fig. 1) and the corresponding nucleotide sequence (Garmyn *et al.*, 1996). We found an ORF in the same orientation as *alsSD* that terminated 115 bp upstream of *alsSD*. We have now determined the nucleotide sequence upstream of this region by primer-walking using pGID600 as a template. An ORF of 104 codons is preceded by a putative ribosome-binding site (AAGGAGG) complementary to the sequence 3'-UUUCCUCCA (complementary bases underlined) at the 3' end of *Leuconostoc mesenteroides* 16S rRNA (Yang & Woese, 1989). *L. mesenteroides* was taken as reference because the 3' end of *O. oeni* 16S rRNA has not been determined, and it is known that the sequence is conserved at the 3' ends of different Gram-positive bacteria (Chassy & Murphy, 1993). Furthermore, the genus *Leuconostoc* is closely related to *Oenococcus* (Dicks *et al.*, 1995). Inspection of the 3' noncoding sequence showed an inverted repeat sequence that could form a stem-and-loop structure in mRNA with a calculated ΔG_f of -68 kJ mol^{-1} . The deduced primary structure of the encoded TrxA is composed of 104 amino acids, with a predicted molecular mass of 11.5 kDa. The *O. oeni* TrxA exhibits sequence similarity to other bacterial TrxA (Fig. 2), with a particularity high sequence identity (48%) to *B. subtilis* (Chen *et al.*, 1989). The primary structure of *O. oeni* thioredoxin revealed

the presence of a typical active centre. However, the sequence between the two vicinal Cys residues was different from that found in most thioredoxins, with the Gly residue replaced by a Thr residue.

Expression in *E. coli* of the *O. oeni trxA* gene

The predicted amino acid sequence provided strong evidence that the cloned DNA contained the *trxA* gene encoding the thioredoxin of *O. oeni*. To identify the *trxA* gene product, we used an *E. coli* minicell system which allowed specific labelling of plasmid-encoded proteins (D'Enfert *et al.*, 1987). The 863 bp *ScaI* DNA fragment of pGID600 (Fig. 1) was subcloned in pGID023. Minicells were prepared from *E. coli* AR1062, which was transformed with plasmid pGID023 and pGID608. Proteins were labelled with L-[^{35}S]methionine and analysed by SDS-PAGE (Labarre *et al.*, 1996a). Minicells carrying pGID608 expressed a protein that had a molecular mass of almost 12 kDa (data not shown). This apparent molecular mass is consistent with the calculated value deduced from the nucleotide sequence.

An *in vivo* assay for thioredoxin function was used to test whether the *O. oeni* TrxA protein can substitute for *E. coli* thioredoxin. *E. coli* contains the enzyme methionine sulfoxide reductase, which uses thioredoxin as a source of reducing power and thereby enables methionine-requiring strains to utilize methionine sulfoxide for growth (Russel & Model, 1986). Two different mutant strains, BH2012 and A313, with phenotype

Fig. 2. Comparison of the predicted amino acid sequence of *O. oeni* TrxA (OOENI) with those of TrxA from *B. subtilis* (BACSU) (Chen *et al.*, 1989), *Chlamydia psittaci* (CHLPS) (Rockey *et al.*, 1996), *Mycoplasma genitalium* (MYCGE) (Fraser *et al.*, 1995), *E. coli* (ECOLI) (Lim *et al.*, 1985), *R. sphaeroides* (RHOSH) (Pille *et al.*, 1990), *Synechocystis* sp. (SYNY3) (Kaneko *et al.*, 1995) and *Clostridium litorale* (CLOLI) (Kreimer & Andreessen, 1995). Amino acids identical to those of *O. oeni* TrxA are boxed. Asterisks indicate residues that are identical in all sequences. Black boxes indicate redox-active cysteines. Numbers on the right indicate the position of the last amino acid. Percentages in parentheses indicate the identity scores between TrxA from *O. oeni* and from other bacterial species.

Fig. 3. Detection of *trxA* mRNA as a function of growth stage. RNA samples were taken from cells during exponential phase (OD_{600} 0.6 and 0.9), at the beginning of stationary phase (OD_{600} 1.2) and during stationary phase (OD_{600} 1.6).

Fig. 4. Northern blot analysis of stress-induced *trxA* expression. (a) Effect of oxidative stress. RNA samples were isolated from exponentially growing *O. oeni* cells growing in the presence (lane 1) or absence (lane C) of a sublethal dose of hydrogen peroxide (10 mg l^{-1}). In the adjacent lanes, cells in exponential phase were submitted to treatment for 30 or 60 min with various doses (8, 10, 12 and 20 mg l^{-1}) of hydrogen peroxide, as indicated. (b) Effect of heat shock. RNA samples were isolated from *O. oeni* cells grown at 30°C (lane C) or from heat-shocked cells at 42°C . For heat-shocked cells, samples were submitted to a rapid temperature shift (less than 2 min). The end of the shift was considered as the start of heat shock, and cells were withdrawn at the time points (in minutes) indicated.

$\text{Met}^- \text{TrxA}^-$ and harbouring pGID023 were unable to use methionine sulfoxide to satisfy their methionine requirement. The control strain A312 ($\text{Met}^- \text{TrxA}^+$) harbouring pGID023, and both strains BH2012 and A313, carrying the recombinant plasmid pGID608 promoting *O. oeni* TrxA synthesis, grew equally well on minimal plates supplemented with L-methionine sulfoxide or L-methionine. This indicated that *O. oeni* thioredoxin serves as a cofactor for *E. coli* methionine sulfoxide reductase *in vivo*.

Transcription analysis of the *trxA* gene

Total RNA was isolated and used to prepare Northern blots from samples taken during exponential phase (OD_{600} 0.6 and 0.9), at the beginning of stationary phase (OD_{600} 1.2) and during stationary phase (OD_{600} 1.6). These samples were hybridized with the *trxA* probe which corresponds to the 550 kb fragment containing part of the *trxA* gene. Autoradiograms (Fig. 3) showed a single 0.7 kb transcript hybridizing with the probe. *trxA*

Fig. 5. Mapping of the 5' end of *trxA* mRNA by primer extension. Equal amounts of RNA isolated before (lane 1) and 60 min after (lane 2) the addition of hydrogen peroxide (10 mg l^{-1}), and 15 min after heat shock (lane 3) were hybridized with primers complementary to the 5' region of *trxA* (see Methods). The extension products obtained with primer TRX3 for each RNA sample were electrophoresed in parallel with the sequencing products (g, a, t and c) obtained with the same labelled primer on a sequencing gel. The arrow indicates the position of the deduced start site.

mRNA was detected at a significant level and was estimated to be at roughly the same level during every growth stage, except in late-exponential phase where the transcript appeared at a higher level.

To investigate the effect of oxidative stress, *O. oeni* cells were cultivated in the presence of a sublethal dose of hydrogen peroxide (10 mg l^{-1}). When the exponential phase was reached, cells were withdrawn and total RNA prepared as described in Methods. Under these growth conditions, a higher level of *trxA* mRNA was detected in comparison to the control (Fig. 4a, compare lanes 1 and C). Moreover, treatment of exponential cells with hydrogen peroxide at various concentrations (8 – 20 mg l^{-1}) for 30 or 60 min significantly enhanced the amount of mRNA detected. The maximum rate of induction was achieved after 60 min of incubation in the presence of hydrogen peroxide at every concentration tested. No significant increase in the signal was observed at longer incubation times.

In order to examine the effect of temperature increase on *trxA* expression, Northern blot experiments were performed with total RNA extracted from cells submitted to heat shock. The amount of *trxA* messenger was enhanced 5 min after temperature shift from 30°C to 42°C and reached a high level of induction after 30 min of heat shock (Fig. 4b). This temperature induction suggested that the product of *O. oeni trxA* is a heat-shock protein.

To map the transcription initiation site of the *trxA* gene, nested oligonucleotides TRX1, TRX2 and TRX3 (see Methods) were used in a primer extension assay. RNA samples were extracted from *O. oeni* cells in exponential phase as controls, after addition of hydrogen peroxide (10 mg l⁻¹ for 60 min) and after heat shock (42 °C for 15 min). In all culture conditions, a single signal was obtained with oligonucleotides TRX1, TRX2 and TRX3 (Fig. 5), allowing the location of the 5' end of *trxA* mRNA 17 nucleotides upstream of the initiation codon. This transcription start point allowed the identification of the promoter for *trxA* as TTGCAT-17 bp-TACAAT. This sequence shows close similarity to the consensus found in the housekeeping promoter of Gram-positive bacteria as well as in *E. coli* (Graves & Rabinowitz, 1986) and other *O. oeni* promoters mapped previously (Jobin *et al.*, 1997; Labarre *et al.*, 1996b).

DISCUSSION

This report presents the DNA sequence, characterization and mRNA analysis of the *trxA* gene of *O. oeni*. The primary sequence of the deduced protein revealed that TrxA has significant identity to a number of proteins belonging to the large family of thioredoxins, and in particular to TrxA from *B. subtilis* (48% identity) (Chen *et al.*, 1989), and that the Cys residues involved in catalysis are conserved. The C-X-X-C motif is critical in determining the exceptional reducing power of disulfide reductase (Konz *et al.*, 1998). Site-directed mutagenesis experiments showed that change of these two central residues can modulate the reducing power of DsbA, a member of the thioredoxin family (Grauschopf *et al.*, 1995). It has recently been reported that thioredoxin from anaerobic, amino-acid-utilizing bacteria possesses a modified consensus sequence at the active centre (Harms *et al.*, 1998). The thioredoxin of such bacteria did not react with thioredoxin reductase of *E. coli* and the authors suggested that these thioredoxins might form an evolutionarily separate group. One of the two amino acids between the cysteine residues is not conserved in *O. oeni* TrxA, in which a Thr residue is substituted for the highly conserved Gly residue. This substitution may alter the reducing power of the enzyme. Nevertheless, the protein encoded by the *O. oeni* *trxA* gene was able to complement *E. coli* mutant strains defective in thioredoxin activity. Taken together, these results led us to conclude that the *O. oeni* *trxA* gene encodes a functional thioredoxin.

Few transcriptional analyses of *trxA* bacterial genes have been reported so far. The only well-documented example concerns the *trxA* gene from *B. subtilis* (Scharf *et al.*, 1998). A variety of stresses, including heat and hydrogen peroxide, strongly enhanced the synthesis of thioredoxin in this bacterium. Transcription initiation of the monocistronic *trxA* gene occurs at two promoters with different profiles of induction in response to stress. One of these promoters is recognized by the RNA polymerase associated with an alternative σ^B factor (Hecker & Völker, 1998). In *Streptomyces coelicolor*,

the alternative sigma factor σ^R modulates expression of the *trxB* operon, encoding thioredoxin reductase and thioredoxin, in response to oxidative stress (Paget *et al.*, 1998).

The detection of an approximately equal level of *trxA* mRNA in growing cells and during stationary phase suggests that *trxA* expression is not growth-phase regulated. The level of *trxA* mRNA is strongly increased by hydrogen peroxide, indicating that thioredoxin is involved in response to oxidative stress, as in many other organisms (Fernando *et al.*, 1992; Kuge & Jones, 1994; Scharf *et al.*, 1998; Wiele *et al.*, 1997) whereas the *E. coli* and *Salmonella typhimurium* *trxA* genes are not known to be regulated (Farr & Kogoma, 1991). In addition to oxidative stress, *O. oeni* *trxA* is also induced by heat shock as is also the case in *B. subtilis* (Scharf *et al.*, 1998) and in human cells (Jacquier-Sarlin & Polla, 1996).

The 5' end of the *trxA* monocistronic transcript was identified in normal growth conditions and allowed us to identify a promoter with a consensus sequence typical for Gram-positive bacteria as well as for *E. coli* (Graves & Rabinowitz, 1986). An identical transcription initiation site was obtained with total RNA extracted from cells submitted to oxidative or heat stress. This result suggests that the same potential -35 and -10 regions are recognized by RNA polymerase in all growth conditions tested. During recent work in *B. subtilis*, at least four different classes of heat-inducible genes were distinguished. The genes of class I are controlled by a repressor which recognizes the CIRCE (controlling inverted repeat of chaperone expression) element (Hecker *et al.*, 1996). Class II genes are positively controlled by the alternative σ^B factor (Hecker & Völker, 1998). Recently, CtsR, a novel regulator of stress and heat shock response, has been shown to act as a repressor of stress genes belonging to class III (Derré *et al.*, 1999) and those of class IV are regulated by a still unknown mechanism. In lactic acid bacteria, heat-shock genes sharing characteristics of class I (Eaton *et al.*, 1993; Van Asseldonk *et al.*, 1993) and class III (Derré *et al.*, 1999) from *B. subtilis* have been reported. No CIRCE element was present in the vicinity of the *O. oeni* *trxA* start site of transcription. To our knowledge, no stress gene expressed under an unusual promoter sequence that may involve an alternative sigma factor has been reported so far in lactic acid bacteria. Moreover, the target consensus sequence corresponding to CtsR was not found in the promoter region of *O. oeni* *trxA*. Therefore, the induction of *O. oeni* *trxA* expression observed after heat or oxidative stresses may require another unknown regulator element, as has been suggested for the heat-shock genes belonging to class IV from *B. subtilis*.

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Structural and putative regulatory genes involved in cellulose synthesis in *Rhizobium leguminosarum* bv. *trifolii*

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Six genes involved in cellulose synthesis in *Rhizobium leguminosarum* bv. *trifolii* were identified using Tn5 mutagenesis. Four of them displayed homology to the previously cloned and sequenced *Agrobacterium tumefaciens* cellulose genes *celA*, *celB*, *celC* and *celE*. These genes are organized similarly in *R. leguminosarum* bv. *trifolii*. In addition, there were strong indications that two tandemly located genes, *celR1* and *celR2*, probably organized as one operon, are involved in the regulation of cellulose synthesis. The deduced amino acid sequences of these genes displayed a high degree of similarity to the *Caulobacter crescentus* DivK and PleD proteins that belong to the family of two-component response regulators. This is to our knowledge the first report of genes involved in the regulation of cellulose synthesis. Results from attachment assays and electron microscopic studies indicated that cellulose synthesis in *R. leguminosarum* bv. *trifolii* is induced upon close contact with plant roots during the attachment process.

Keywords: *Rhizobium leguminosarum* bv. *trifolii*, cellulose synthesis, two-component regulation

INTRODUCTION

Cellulose production has been documented in several bacterial genera such as *Acetobacter*, *Agrobacterium*, *Rhizobium* and *Sarcina* (Ross *et al.*, 1991). *Acetobacter xylinus* (previously *Acetobacter xylinum*) has served as a model organism in studies of bacterial cellulose synthesis at the molecular and biochemical level (Wong *et al.*, 1990; Saxena *et al.*, 1990, 1991; Saxena & Brown, 1995). Two operons from *Agrobacterium tumefaciens* with genes encoding proteins involved in cellulose synthesis (*celABC* and *celDE*) have also been characterized (Mathysse *et al.*, 1995a, b). The results suggest that cellulose synthesis in these two organisms involves at least two homologous genes, a cellulose synthase and an endoglucanase. The other three genes in the *Ag. tumefaciens* *cel* operons show no homology to known genes in *Ac. xylinus*, thus reflecting differences in the

mechanisms of cellulose synthesis in these two bacteria. Mathysse *et al.* (1995b) have suggested a model pathway for cellulose production in *Ag. tumefaciens*. In this model, cellulose synthesis proceeds via several lipid-linked intermediates, involving the CelE and CelB proteins. A cellulose synthase (the product of the *celA* gene) adds additional glucose moieties to these lipid-glucose intermediate products. The incorporation of the last intermediate into cellulose requires the CelC protein. The exact function of all the *cel* genes is not yet clear.

Here we report the cloning of genes involved in cellulose synthesis (the *cel* genes) from *Rhizobium leguminosarum* bv. *trifolii*. The primary sequence and the organization of the genes are similar to that of *Ag. tumefaciens* (Mathysse *et al.*, 1995a). In addition, we have identified two putative regulator genes, *celR1* and *celR2*, probably organized as one operon and most likely controlling cellulose synthesis. According to the sequence composition, these genes belong to the family of two-component response regulators (Parkinson & Kofoid, 1992). Surprisingly, they exhibit highest sequence homology to the *Caulobacter crescentus* pleD

Abbreviations: Amp, ampicillin; Km, kanamycin; Sm, streptomycin; Tet, tetracycline.

The GenBank accession numbers for the *celABC* and *celR1–celR2* sequences determined in this work are AF121340 and AF121341, respectively.

and *divK* genes, which are the key components in interconnecting polar morphogenesis and cell division events in this bacterium (Sommer & Newton, 1989; Hecht *et al.*, 1995; Hecht & Newton, 1995).

The identification of genes involved in the synthesis of cellulose fibres opens the possibility to more precisely investigate the biological significance of these structures in *Rhizobium*.

METHODS

Bacterial strains, plasmids and growth media. The wild-type strain R200 is a spontaneous streptomycin- and rifampin-resistant (Sm^r Rif^r) mutant of *R. leguminosarum* bv. *trifolii* strain 2046 from the culture collection at the Department of Microbiology, Swedish University of Agricultural Sciences. Strain R201 is a cellulose-overproducing mutant obtained after NTG treatment of strain R200. Strains R204, R205, R206, R207, R211 and R214 are cellulose-negative Tn5 mutants generated from strain R201. *Escherichia coli* strains XL-1 Blue and DH5 α were used for cloning. The pUC18 and Bluescript plasmids were used for cloning and sequencing. Vector pRK404A was used as a broad-host-range plasmid in complementation experiments. It is a derivative of plasmid pRK404 (Ditta *et al.*, 1985) with a unique *EcoRI* site in the multilinker, constructed by W. Buikema at the University of Chicago, USA. Plasmid pRK2013 (Figurski & Helinski, 1979) was used as a helper plasmid in bacterial conjugations. Plasmid pSUP2021 (Simon *et al.*, 1983) was used for Tn5 mutagenesis. *Rhizobium* strains were grown in TY medium (l^{-1} : 5 g tryptone, 3 g yeast extract and 1 g CaCl_2) or YMB medium (l^{-1} : 0.5 g K_2HPO_4 , 0.1 g NaCl, 10 g mannitol, 0.4 g yeast extract and 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), supplemented with Congo red (25 mg l^{-1}) when needed. *E. coli* strains were grown in Luria-Bertani liquid (LB) or agar (LA) media. When appropriate, antibiotics were added at the following concentrations: ampicillin (Amp), 50 $\mu\text{g ml}^{-1}$; kanamycin (Km), 20 $\mu\text{g ml}^{-1}$; tetracycline (Tet), 10 $\mu\text{g ml}^{-1}$; and streptomycin (Sm), 20 $\mu\text{g ml}^{-1}$.

Mutagenesis and isolation of mutants. Washed cells of *R. leguminosarum* bv. *trifolii* strain R200 (exponential-growth phase) were suspended in 10 ml 0.1 M citrate buffer pH 5.8 to a cell density of 5×10^8 c.f.u. ml^{-1} . Freshly dissolved NTG was added to a final concentration of 100 $\mu\text{g ml}^{-1}$ (Adelbey *et al.*, 1965). After incubation at 28 °C for 60 min, the cells were washed in saline, suspended in 50 ml YMB and incubated at 28 °C overnight to allow phenotypic expression. Cellulose-overproducing mutants were isolated as deep-red colonies after plating on YMB agar plates containing Congo red.

Random Tn5 mutagenesis of strain R201, forming deep-red colonies on YMB Congo red plates, was carried out using plasmid pSUP2021 as described by Simon *et al.* (1983). Cellulose-negative mutants were isolated as white colonies on YMB Congo red plates containing Km.

Cloning and sequencing. DNA manipulations were performed according to standard protocols (Sambrook *et al.*, 1989). Ligations were performed using the Ready-to-go kit from Pharmacia Biotech, and all plasmids were prepared using the QIAprep Spin Miniprep kit (Qiagen). DNA was sequenced using the Thermo sequenase dye terminator cycle sequencing kit (Amersham) and an ABI PRISM 337 DNA Sequencer (Perkin Elmer) according to protocols provided by the manufacturers. Sequences were analysed with the PC/GENE

software package. Database searches were carried out using BLAST (Altschul *et al.*, 1990). Protein alignments were carried out with the PC/GENE program PALIGN with parameters set at open gap cost 10 and unit gap cost 6.

Complementation assay. DNA fragments used in the complementation assays were cloned into the broad-host-range vector pRK404A. The recombinant plasmids were electroporated into *E. coli* DH5 α and introduced into strains R200 or R205 by triparental conjugation using the helper plasmid pRK2013 (Simon *et al.*, 1983). Matings were performed by mixing 3×10^8 cells of both donor strains and 1.5×10^9 recipient cells from exponential-phase cultures and filtering the suspension onto 0.45 μm Millipore filters. The filters were incubated for 3 h at 25 °C on TY nonselective plates. Subsequently, the cells were resuspended and plated on YMB plates containing Sm, Tet and Congo red.

Attachment assay. *R. leguminosarum* bv. *trifolii* strains were grown in TY medium until early stationary phase, washed twice with equal volumes of Fahraeus nitrogen-free medium (Fahraeus, 1957) and diluted five times with the same medium before being added to clover seedlings. The seedlings were produced from seeds of red clover cv. Vivi incubated on water agar for 48–52 h in the dark. The binding assays were performed in two different ways, essentially as described by Smit *et al.* (1986) or by Dazzo *et al.* (1984), respectively. In the first case five seedlings were incubated in 10 ml bacterial suspension in a 100 ml flask at room temperature under slow shaking. In the second case the binding assay was performed on microscope slides as follows: five seedlings were placed on a microscope slide and 100 μl bacterial suspension was dropped on the roots, which were then covered with a cover slide and stored at room temperature in a Petri dish sealed with Parafilm. Incubation time was varied from 2 h to overnight. After the incubation the seedlings were washed three times in 10 ml Fahraeus medium under vigorous shaking. The attachment of the bacteria to the root hairs was evaluated under a phase-contrast microscope. All binding experiments were repeated at least three times.

Electron microscopy. Bacterial suspensions were analysed by different techniques: positive and negative staining and shadow casting to select optimal electron microscopic contrast. Since the diameter of the cellulose microfibrils produced by the bacteria should correspond approximately to that of double-stranded nucleic acid, the contrasting technique, which is used for DNA or RNA (Höglund & Öhagen, 1997), was employed. Bacterial suspensions (taken directly from the growth medium or washed gently in 0.1 M Tris buffer pH 7.5) were adhered to carbon-coated grids, stained with 10^{-4} M uranyl acetate and rinsed with 95% ethanol. Rotary shadowing with platinum was then employed at low angle (8°) to allow visualization of thin fibres. Specimens were analysed with a Zeiss CEM 902 electron microscope, equipped with a N2(1) trap, at an accelerating voltage of 80 kV.

Nodulation test. Seeds of red clover cv. Brita were immersed for 5 min in a chlorhexidine/ethanol (60%) solution (5 mg ml^{-1}), washed in three changes of sterile water and distributed onto the surface of water agar plates, which were then incubated upside down for 2 d in the dark at room temperature. Seedlings with 5–7 mm roots were planted in the upper part of 10 ml agar slopes of Jensen agar (Vincent, 1970) in 150 $\times$ 20 mm tubes closed with transparent plastic caps. Tubes were illuminated for 16 h from a 400 W HPI-T lamp at 70 cm distance. After 2 d the seedling in each tube was inoculated with 1 ml bacterial suspension prepared from 10 ml

sterile tap water with 2 loopfuls from 5-d-old colonies grown on YMB agar. After inoculation early nodulation was followed for 3 weeks.

RESULTS AND DISCUSSION

Characterization of cellulose production in the overproducing mutant R201

Most strains of *Rhizobium* lack the ability to absorb the dye Congo red. However, there are exceptions to this generalized behaviour, as reported by Zevenhuizen *et al.* (1986). These authors showed that a few *Rhizobium* strains produced red to deep-red colonies on medium with Congo red and had cellulose contents of 2–10% of their dry weights. Binding of Congo red was positively correlated to the presence of microfibrils as observed under the electron microscope and, furthermore, these fibrils disappeared upon treatment with cellulase. These data suggested to us that binding of Congo red could be used in screening for cellulose-overproducing mutants. Such strains with an increased cellulose production would be a basis for generating cellulose-negative mutants since most wild-type strains do not produce enough cellulose to acquire significant colour in the Congo-red-based plate assay.

The wild-type strain R200 (as most *Rhizobium* strains) forms white colonies on YEM plates with Congo red.

After mutagenesis of this strain with NTG, deep-red-coloured colonies appeared at a frequency of approximately 5×10^{-4} . One such mutant, here called R201, was selected for further studies. In comparison to the wild-type strain, the mutant strain R201 flocculated heavily when grown in YEM broth and produced a pellicle in static cultures. It also produced an alkali- and acid-insoluble substance that was degraded by cellulase but not by protease treatment (data not shown).

Electron microscopy of cells from strain R201 showed an abundance of fibres or bundles of fibres surrounding the bacteria. Negative staining visualized the fibres as relatively thick bundles or a meshwork of these bundles. It was difficult to determine any specific localization of these structures or the site of emergence from the cell membrane (Fig. 1a). To avoid these problems and to see if the appearance of the fibres was dependent on the method of preparation, rotation shadowing was used, a technique suitable for visualizing thin structures (Höglund & Öhagen, 1997). Furthermore, the bacteria were not washed before application onto the grid to avoid aggregation of the cellulose. By this technique, much thinner microfibrils were seen emerging radially from all parts of the cell surface (Fig. 1b). A clear difference between these fibrils and the flagella was obvious (Fig. 1c). With reference to the thickness of flagella of 13–14 nm (Madigan *et al.*, 1997), the diameter

Fig. 1. Cellulose fibrils produced by *R. leguminosarum* bv. *trifolii* mutant strain R201. (a) Thick bundles of cellulose surrounding the cells (negative staining technique, washed cells). (b) Numerous thin cellulose microfibrils (indicated by C) are seen emerging radially from the cells (rotary shadowing technique, unwashed cells). (c) Part of a bacterium showing a flagellum (F) and cellulose fibres (C). Bar, 100 nm.

of the microfibrils ranged from approximately 1 to 2 nm. The fibrils are produced in the late exponential or early stationary phase since cells in early exponential phase were almost devoid of these fibrils, whereas most bacteria from an early-stationary-phase culture were heavily fibrillated. Cellulase treatment of the bacteria resulted in total disappearance of the fibrils.

According to Brown *et al.* (1976) and Wong *et al.* (1990), the newly polymerized subelementary cellulose strands in *Ac. xylinus* are extruded through pores in the outer membrane and crystallized into cellulose microfibrils by hydrogen bonding. The diameter of these microfibrils was reported to range between 1.6 and 5.8 nm. The very thin fibres of *R. leguminosarum* bv. *trifolii* protruding from the cell membrane (Fig. 1b, c) have a diameter of 1–2 nm and resemble the microfibrils described by Brown *et al.* (1976). The considerably thicker cellulosic structures visualized by negative staining (Fig. 1a) are probably aggregates of entwined fibrils.

Screening of 50 individual cells of the wild-type strain under the electron microscope did not detect any fibrillated cells. Likewise, no fibrils were detected on the cells of the cellulose-negative mutants R204 (*celA*::Tn5) and R205 (*celR2*::Tn5) (see below). These strains formed white colonies on agar plates with Congo red, confirming that binding of the stain by the mutant R201 resulting in red colonies is correlated to high production of cellulose fibrils. Although it has been reported that Congo red also can bind to outer-membrane proteins of various Gram-negative, animal pathogenic bacteria (Theisen *et al.*, 1993), our data support the observation by Zevenhuizen *et al.* (1986) that in *Rhizobium* the Congo red binding is related to the cellulose content of the bacteria. Thus in all subsequent experiments (e.g. the complementation of the cellulose-negative phenotype), the binding of Congo red was taken as a marker for cellulose production, without additional confirmative tests.

Identification of genes involved in cellulose synthesis by Tn5 mutagenesis

The cellulose-overproducing strain R201 was subjected to Tn5 mutagenesis. The screening of cellulose non-producing mutants was carried out on agar plates containing Km and Congo red. Eleven white colonies were isolated and total DNA from them prepared. The DNA was cleaved with *EcoRI* and subjected to Southern blot hybridization. In order to detect all possible insertion events, the whole pSUP2012 plasmid was cleaved with *HindIII* and used as template in the random priming labelling procedure to create a radioactive probe. All mutant strains showed a single radioactive band on the autoradiography plates. After prolonged cultivation some of the mutants exhibited weakly red or segmented colony types and were not investigated further. Six isolates, R204, R205, R206, R207, R211 and R214, gave stable white colonies on Congo red plates and were chosen for further analysis. The *EcoRI*

fragments carrying the Tn5 insertions from all six strains were cloned into pUC18. Since Tn5 does not contain any *EcoRI* cleavage sites, the cloned fragments contained flanking rhizobial DNA. Nucleotide sequences obtained from these clones were compared to sequences in the nucleic acid and protein databases. The results showed that three mutants, R204, R206 and R214, had Tn5 inserted in a region showing high sequence homology to the *Ag. tumefaciens celA* gene, encoding a cellulose synthase (Matthysse *et al.*, 1995a). Certain regions of the deduced protein sequence also showed homology to the BscA, AxcS and AcsA proteins, all three characterized as cellulose synthases in *Ac. xylinus* (Wong *et al.*, 1990; Saxena *et al.*, 1990; Saxena & Brown, 1995). Tn5 flanking sequences from strains R207 and R211 showed strong homology to the *Ag. tumefaciens* cellulose genes *celE* and *celB*, respectively, suggesting that the cellulose synthesis pathway in *Rhizobium* might be similar to that of *Ag. tumefaciens* (Matthysse *et al.*, 1995a, b). The CelE protein in *Ag. tumefaciens* is proposed to attach UDP-glucose to a lipid carrier, forming a lipid intermediate product. The *celB* gene product was also shown to be involved in the synthesis of lipid-linked intermediates, although the exact function of the protein is not known (Matthysse *et al.*, 1995b).

Sequences obtained from strain R205 did not show homology to the *Ag. tumefaciens cel* operon. Instead, they indicated that the Tn5 insertion was located in a regulatory gene. The involvement of this gene in cellulose synthesis is described below.

Organization of the *cel* genes is conserved in *Rhizobium* and *Agrobacterium*

To establish the organization of the *R. leguminosarum* bv. *trifolii cel* genes, we took advantage of the sequence homology to the *Ag. tumefaciens cel* genes as revealed by the database search. Partial *EcoRI* cleavage products of total DNA from strains R207 and R211 were ligated into pUC18. The ligation mixtures were electroporated into *E. coli* DH5 α and transformants were selected on LA plates with Amp and Km. From these clones, inserts from isolated plasmids, containing the putative flanking sequences of the previous *EcoRI* fragments, were analysed by *EcoRI* cleavage and sequencing (Fig. 2a–c). Comparison of the resulting DNA sequence with the homologous region in *Ag. tumefaciens* showed a similar organization of the corresponding genes in *R. leguminosarum* bv. *trifolii* (Fig. 2b). The sequence homology is limited to the region where the cellulose genes are located. Upstream of the *celA* and *celE* genes the sequences of *R. leguminosarum* bv. *trifolii* and *Ag. tumefaciens* diverge. The *R. leguminosarum* bv. *trifolii celABC* operon was completely sequenced. Alignment of the deduced *R. leguminosarum* bv. *trifolii* and *Ag. tumefaciens* proteins indicated that the CelA proteins of the two species showed 69.5% identity and 80% similarity, the CelB proteins showed 49% identity and 62% similarity, and the CelC proteins showed 45% identity and 55% similarity in their amino acid

Fig. 2. Strategy for defining the structure of the *R. leguminosarum* bv. *trifolii* *cel* genes. (a) *EcoRI* map of the *cel* region. (b) The location and the orientation of the *cel* genes is analogous to that of *Ag. tumefaciens* (Matthysse *et al.*, 1995a). The *celD* gene, present in *Ag. tumefaciens* but not detected in *R. leguminosarum* bv. *trifolii*, is depicted by a dotted line. (c) Some of the partial *EcoRI* clones of strains R207 (*celE*::Tn5) and R211 (*celB*::Tn5) are depicted below the original clones. Triangles indicate the Tn5 insertions.

sequences. The sequence of the *celA* gene offers an ORF with three alternative start sites. No obvious Shine–Dalgarno (SD) sequence is present upstream of the first and second ATG in the ORF, while the third ATG is preceded by a classical SD motif. This yields a deduced protein of 730 amino acids. Similarly, the *celA* gene from *Ag. tumefaciens* also has two alternative initiation codons (GenBank accession no. L38609). Alignment of the deduced CelA proteins from *Ag. tumefaciens* and *R. leguminosarum* bv. *trifolii* showed sequence homology starting from the second and the third methionine, respectively. The CelA sequence was also similar to several cellulose synthases from other organisms, for example *Ac. xylinus*, *Aquifex* and *Arabidopsis*, and also to a hypothetical protein of unknown function from *E. coli* and to NodC proteins (*N*-acetylglucosaminyltransferase, involved in the synthesis of nod factors) from several *Rhizobium* species, as revealed by a BLAST database search. The *celB* gene begins at a start codon overlapping the *celA* stop codon. The deduced *R. leguminosarum* bv. *trifolii* CelB protein (822 amino acids) was somewhat longer than *Ag. tumefaciens* CelB (698 amino acids). Alignment of the two CelB proteins showed a highly conserved part near the C terminus, displaying 78% amino acid sequence similarity between amino acids 410 and 640. In the N-terminus of both proteins there was a domain of 110 amino acids rich in prolines, although there was no high score in pairwise alignment. The stop codon for *R. leguminosarum* bv. *trifolii* *celB* overlapped the start codon for *celC* and was situated approximately 320 bp downstream of the proposed site for the *Ag. tumefaciens* *celC* start codon. Thus the same pattern of overlapping stop and start codons was observed for these three genes in *R. leguminosarum* bv. *trifolii*, indicating that the synthesis of the proteins is coupled. CelC protein showed significant sequence similarity to several bacterial endoglucanases, among them the *Ac. xylinus* CMC α protein, encoded by a gene linked to the cellulose synthase operon (Standal *et al.*, 1994). The involvement of a cellulase thus seems to be a common feature in the cellulose production pathway in *Acetobacter*, *Agrobacterium* and *Rhizobium*. *R. leguminosarum* bv. *trifolii* is also reported to possess extracellular, cell-

associated cellulase activity (Mateos *et al.*, 1992). However, at present there are no data available to correlate this activity to CelC.

The *celE* region was partially sequenced. The sequence data indicated the presence of a gene with considerable sequence similarity to *Ag. tumefaciens* *celE*, located downstream of the *celC* gene. Sequences obtained from the DNA region located immediately upstream of the *celE* gene did not display similarity to *Ag. tumefaciens* *celD*.

Interestingly, a gene with strong homology to sensor histidine kinases is situated just upstream of the *celA* gene (Fig. 2b). In a BLAST database search, the FixL protein from *R. leguminosarum* showed the highest score. FixL is an oxygen sensor and transduces the low oxygen signal by controlling the phosphorylation of FixJ protein (a transcriptional activator). Sequence similarities covered only the C-terminal kinase part of the FixL protein (Lois *et al.*, 1993). No similarities were detected between the membrane-spanning region and the haem-binding domains of FixL and the N-terminal sequence of the putative kinase. The possibility that this gene encodes the cognate kinase for the putative response regulators CelR1 and CelR2, involved in the regulation of the cellulose synthesis (see below), will be tested in the future.

Identification of putative components of a signalling pathway involved in cellulose synthesis

Sequence comparisons showed that the gene inactivated by Tn5 in strain R205 is homologous to several response regulators belonging to the so-called two-component regulatory systems. We named the new regulator gene *celR2*. Besides the similarity to the conserved N-terminal parts of several response regulators, the deduced amino acid sequence of *celR2* shows high sequence homology to *C. crescentus* PleD (Hecht & Newton, 1995) throughout its entire length (Fig. 3). In *C. crescentus*, the regulatory genes *pleC* (a histidine kinase) and *pleD* and *divK* (the cognate response regulators), are the key elements in coupling shifts in polar morphogenesis to cell cycle events (Sommer & Newton, 1989; Hecht *et*

CelR1	-	MPKQVMIVEDNELNMKLF RDLE ASGYTTIQ TRNGMEALDLARKHR PDLI	-50
DivK	-	MTKKVLIV EDNELNMKLF FDLLEA QGYETL Q TREGLSALS SIARENKPDLI	-50
CelR1	-	LMDIQ LE VS GL EV TK N KL ED DELHV IP VI AV TAFAM KGDEER IR QGGCE	-100
DivK	-	IMDIQ LE IS GL EV TK N KL ED DLAH IP V AV TAFAM KGDEER I-EGGCE	-99
CelR1	-	AYVSKPIS V PKF ETIKTYLG--DA	-123
DivK	-	AYISKPIS V VH FLETIKRLLERQPA	-124
CelR2	-	MTARILV DDIPAN V KL LE AR LL AEYFDVMTAADGY TALA ICERNQVD LI	-50
PleD	-	MSARILV DDIEAN V RL LE AK LT AEY EV STAMDGPT TAL AMAARICPT II	-50
CelR2	-	L LDIM MP GID G FEVCERLK AS Q KT AH IP V VM V TAL DQ PT DRV GL KAGAD	-100
PleD	-	L LDV MM PGMD G FT V CR KL KDD PT TRH IP V V LI TAL DGR G DRI Q GL ES GAS	-100
CelR2	-	D FL TK PVND QL IS RV KS LL RL KT LS DE LRIRADTA HT MGIDDL TR AGEG	-150
PleD	-	D FL TK PID D V ML FAR VS LT RF KL VI DE LQ REASGR RM GV--IAGAAAR	-148
CelR2	-	RADE T AQ V LL VD GRANSQ ER IIK AL KPVAD V L AL S DP QA AL FEAA S AFD	-200
PleD	-	LDGLGGR V L IV DDNERQ AQ RVAA EL GV EH RPV IE S DP E K AKI-SAGGPVD	-197
CelR2	-	L VIV NA NFD Y DP LR LC SQ LR SL ER TR FL P ILI ITE QGADNM V VR AL DL G	-250
PleD	-	L VIV NA AAKN FD GL R FT AA L R SE ER TR QL PVL AM VD P DRGR M V K ALE IG	-247
CelR2	-	V NDY II RPV DP NEL VAR SL TQ IR R KRY ND RLRAS V K QT IEL AV TD PL T GL	-300
PleD	-	V ND IL SR P ID P Q EL SAR V KT QT Q R KCTD YL RNNLD HS LE LA VT D Q L T GL	-297
CelR2	-	Y NR RY LD N HL N VL FN RS MA RG R PL SV LIT D ID RF K H VND TY GHD G DE VL	-350
PleD	-	H NR RY MT G QL DS L V KRAT L G DP V S ALL ID ID FF K I ND TF GHD I G DE VL	-347
CelR2	-	R EFSS RV SR T IR G AD L AC RY GG EE F V V MP DT S PE IA AA V AER L RA AI ES	-400
PleD	-	R EFAL RL AS N VR AI D LP CR Y GG EE F V V MP DT AL AD AL RIA ER IRM H V S G	-397
CelR2	-	A PF ML K HS GE AL N VT AS FG IAS RI AS VL TP Q LM K Q AD L AL YE AK NT GR N	-450
PleD	-	S PT V A H GRE ML N VT IS IG VSATAGE GD T PE ALL K RA DE GV Y Q AK AS GR N	-447
CelR2	-	RVV AA AV	-457
PleD	-	AVV G K AA	-454

Fig. 3. Alignment of the *C. crescentus* DivK and PleD proteins (Hecht *et al.*, 1995; Hecht & Newton, 1995) with the corresponding proteins, CelR1 and CelR2, in *R. leguminosarum* bv. *trifolii*. The alignment was carried out using the PC/GENE program PALIGN. Bold letters indicate identical amino acids, dots indicate similar amino acids and asterisks represent conserved amino acids involved in phosphorylation in response regulators. The DivK and CelR1 proteins exhibit 74% identity and 83.7% similarity, and the PleD and CelR2 proteins exhibit 50.2% identity and 64.3% similarity, in their amino acid composition.

al., 1995; Hecht & Newton, 1995; Wang *et al.*, 1993). The *divK* and *pleD* genes in *C. crescentus* are situated immediately next to each other (Hecht & Newton, 1995). Interestingly, sequencing the upstream region of *celR2* revealed a gene with very high sequence homology to *divK* (Fig. 3). In *R. leguminosarum* bv. *trifolii* these two genes are also located close to each other, being separated by only 18 bp. The *R. leguminosarum* bv. *trifolii* *divK* homologue, named *celR1*, encodes a protein of 123 amino acids, and *celR2* encodes a protein of 457 amino acids. In *C. crescentus*, both DivK and PleD regulators are phosphorylated by the same histidine kinase, PleC. DivK is an essential cell division regulator (Hecht *et al.*, 1995) and PleD is required in the regulation of polar morphogenesis during the swarmer-to-stalked-cell transition (Hecht & Newton, 1995). Interestingly, *Rhizobium* is also a polar bacterium in the sense that the bacteria adhere to the root hairs with one cell pole (Marshall *et al.*, 1975; Maddock *et al.*, 1993; Höflich *et al.*, 1995). *Bradyrhizobium japonicum* produces a polar lectin (Ho *et al.*, 1994) and also displays intracellular asymmetry concerning the localization of reserve polymers (Tsien & Schmidt, 1977). Dazzo *et al.* (1982) showed that plant extracts caused polar changes in the structure of the capsule in *R. leguminosarum* bv. *trifolii*.

However, this polar behaviour is so far not explained at the molecular level. It is an intriguing possibility that the *celR1*–*celR2* regulatory system might in some way be involved in maintaining the polarity in dividing *Rhizobium* cells. There may also be other traits that are co-regulated with cellulose synthesis.

Cellulose synthesis in strain R201 is not polar. The electron microscopy study clearly showed that the cell surface of the overproducing strain was completely covered by cellulose. However, the attachment of these cells to the root hairs was clearly polar as seen under the phase-contrast microscope (data not shown). The same polar attachment was also observed both for the wild-type and the cellulose-negative strain R204 (*celA*::Tn5).

The cloned *celR1*–*celR2* genes complement the R205 *Cel*[−] phenotype and trigger cellulose overproduction in wild-type R200

To confirm the involvement of *celR* regulators in cellulose synthesis, complementation of the *celR2* mutation in strain R205 (*celR2*::Tn5) was carried out with the functional regulator region cloned from the overproducing strain R201. A *Hind*III fragment of approxi-

Fig. 4. Complementation of the Cel^- phenotype of R205 ($celR2::Tn5$) with different DNA fragments from the *reg* region. The *reg* region is a ~6.5 kb *Hind*III fragment containing both regulator genes *celR1* and *celR2* and flanking DNA. The corresponding fragments were cloned into pRK404A, generating the clones pReg1–pReg4 and then conjugated into R205. The restriction map of the *reg* region and the location of the fragments is shown. E, *Eco*RI; H, *Hind*III; B, *Bam*HI. + indicates full complementation (bright red colonies on Congo red plates after conjugation), -/(+) indicates incomplete or delayed complementation (originally white colonies turning pale red after a prolonged incubation), and – indicates no complementation (white colonies).

mately 6 kb, containing both regulator genes (the *reg* region; Fig. 4), was cloned into the broad-host-range vector pRK404A and transferred into R205 in a triparental conjugation. This construct, pReg1, completely restored cellulose overproduction in R205 as shown by the intense red colour of the transconjugant colonies on Congo red plates. Deletion clones from the original *Hind*III insert were created and their complementation ability was tested. The results are summarized in Fig. 4. A shorter *Hind*III/*Bam*HI fragment of the *reg* region (pReg2) partially complemented R205. This was manifested as the emergence of white transconjugant colonies 3–5 d after plating on Congo red plates. After prolonged incubation for more than 7 d, these colonies turned pale red, whilst the colonies of the cellulose-negative strain (containing the vector plasmid) remained white.

Plasmid pReg3, containing a 2 kb *Eco*RI fragment, failed to complement the mutation in R205. This plasmid contains the intact *celR1* and the upstream region but lacks 249 bp from the 3' end of the *celR2* gene. As expected, the pReg4 construct, containing the 3' end of the *celR2* gene and the downstream region, was also incapable of complementation.

The complementation analysis supported the requirement for the *celR1*–*celR2* tandem gene structure in the activation of cellulose synthesis in *R. leguminosarum* bv. *trifolii*. The fact that the pReg2 construct, lacking the proximal region of the *celR1* gene, only showed weak and delayed complementation, indicates that *celR1* and *celR2* are organized in an operon. Although the transconjugants received an intact *celR2*, this was not sufficient for effective complementation. The reason for the weak expression after prolonged incubation in our transconjugants, despite the lack of the *celR1* promoter, is unclear, but might be explained by spurious transcription from within the vector sequence. In *C. crescentus*, these two genes are coupled and transcription is initiated from a single promoter upstream of *divK*. A construct containing the promoterless *divK* and *pleD* region failed to complement the *pleD* defect (Hecht & Newton, 1995).

Interestingly, the construct pReg1 also caused cellulose overproduction in the wild-type R200 when this strain was used as a recipient in the conjugation experiments. As the pReg1 construct, containing the *reg* region from the overproducing strain R201, might carry a mutation, the corresponding *Hind*III fragment (wild-type *reg* region) was cloned from strain R200 and designated pRegW. The conjugative transfer of plasmid pRegW into strains R205 and R200 yielded bright red transconjugant colonies in both cases. No visual difference was observed between transconjugant colonies harbouring pReg1 or pRegW on Congo red plates. This shows clearly that the enzymic machinery for cellulose production is fully functional in the wild-type R200, and can be triggered by increasing the gene dosage of *celR1*–*celR2*. This result further strengthens our hypothesis that *celR1* and *celR2* are involved in the regulation of cellulose synthesis. An example of a similar gene dosage effect was reported concerning the regulation of virulence (*vir*) genes in *Ag. tumefaciens*, where increasing the gene dosage of a response regulator gene (*virG*) resulted in increased *vir* gene expression and elevation of virulence (Jin *et al.*, 1987; Rogowsky *et al.*, 1987).

Nodulation assay

All *R. leguminosarum* bv. *trifolii* strains described in this paper were capable of nodulating red clover under the assay conditions used (data not shown). This result agrees with data published by Smit *et al.* (1987) who also compared cellulose-producing strains and cellulose-negative mutants for their ability to nodulate, without seeing any differences in this respect.

Cellulose synthesis is probably an induced event in *R. leguminosarum* bv. *trifolii* as indicated by attachment assays

Since cellulose fibres were undetectable by electron microscopy of wild-type *R. leguminosarum* bv. *trifolii* cells, we tested the different strains for their ability to

attach to the root hairs of clover seedlings. Earlier reports showed that the firm attachment and especially the formation of large aggregates of bacteria on root hair tips (so-called 'cap formation') is an attribute of the cellulose fibres (Napoli *et al.*, 1975; Matthysse *et al.*, 1981; Smit *et al.*, 1986, 1987, 1992). According to Smit *et al.* (1987), the caps are formed during the first 2 h of the attachment process.

We carried out binding assays essentially as described by Smit *et al.* (1986). The strains tested were wild-type *R. leguminosarum* bv. *trifolii* R200, the cellulose over-producing mutant R201 and the cellulose-negative Tn5 mutants R204 (*celA*::Tn5) and R205 (*celR2*::Tn5). After 2 h incubation with R201, virtually all root hairs had large clumps of adhered bacteria (caps) around the root hair tips and along the root hairs. Besides aggregates, we also observed single cells attached polarly to the different parts of the root. Strains R200, R204 and R205 behaved differently from strain R201. After 2 h incubation, 90% of the root hairs were covered with single cells attached to the tip and stem region. No bacterial aggregates were seen on the root hairs of the seedlings incubated with these bacteria. Even after prolonged incubation times of up to 16 h, no visible caps were formed. All three strains displayed this single cell type of attachment. Assuming a direct correlation between cap formation and the presence of cellulose fibres, this result indicated that the wild-type R200 did not produce cellulose under these conditions as its attachment behaviour was indistinguishable from that of the cellulose-negative mutants. This conclusion is strengthened by the fact that we failed to see cellulose fibres on the R200 cells from a liquid culture under the electron microscope (see above).

An alternative strategy for studying attachment was described by Dazzo *et al.* (1984). These authors observed the synthesis of extracellular fibrils only after 12 h incubation in an undisturbed hydroponic slide culture. We repeated this experiment with the wild-type and the cellulose-negative mutants. After 2 h incubation, only single cell attachment was seen for all strains. However, after overnight incubation, some aggregation of the wild-type bacteria around the root hair tips was observed, whilst the binding pattern of the cellulose-negative strains remained unchanged.

Smit *et al.* (1992) presented a two-step attachment model in which the protein rhicadhesin was responsible for specific binding of the bacteria to plant roots and the cellulose fibres were required for the second stage of firm anchoring and floc or cap formation. Cellulose production in *R. leguminosarum* was considered to be constitutive by these authors (Smit *et al.*, 1987). On the other hand, cellulose synthesis in *Ag. tumefaciens* has been shown to be induced by plant extracts (Matthysse *et al.*, 1981). Earlier data presented by Dazzo *et al.* (1984) also indicated that cellulose synthesis in *R. leguminosarum* bv. *trifolii* was induced by some compounds in root exudates. Our data are in agreement with Dazzo *et al.* (1984) and indicate that cellulose production in *R. leguminosarum* bv. *trifolii* is induced by contact between

roots and bacteria. The identification of genes probably involved in the regulation of synthesis of cellulose (see above) strengthens that observation. The deduced structure of these regulators suggests that they are involved in a signalling pathway. The data obtained so far allow us to hypothesize that some plant-derived compound(s) may act as signal(s) for turning on cellulose synthesis in *R. leguminosarum* bv. *trifolii*, and that CelR1 and CelR2 are likely components of such a signal transduction pathway.

The attachment mechanisms are considered to be similar in *Rhizobium* and *Agrobacterium*, proceeding according to the two-step attachment model (Smit *et al.*, 1992). The protein rhicadhesin, mediating the first stage in the attachment process, is compatible between members of the family *Rhizobiaceae* (Smit *et al.*, 1989; Swart *et al.*, 1993). Cellulose fibres are involved in the second attachment stage to ensure the firm binding and aggregation of bacteria around a possible infection site. A similar model was also proposed for the attachment behaviour of *Azospirillum* bacteria (Michiels *et al.*, 1991; Vande Broek & Vanderleyden, 1995), where a flagellar protein mediates the first binding step and a Calcofluor-binding polysaccharide is responsible for the second firm attachment step. In *Ag. tumefaciens*, the cellulose fibrils serve to bind the bacteria tightly to the plant host cells and mutants which cannot synthesize cellulose are reduced in virulence, but still capable of infection, at least under laboratory conditions (Matthysse *et al.*, 1981; Minnemeyer *et al.*, 1991). Similarly, Smit *et al.* (1987) showed that fibril-negative as well as fibril-overproducing mutants of *R. leguminosarum* showed a normal nodulation behaviour, which was confirmed for *R. leguminosarum* bv. *trifolii* in the present study. Thus although the second binding step seems to be conserved among different bacteria it is not a prerequisite for a successful interaction.

The structural and putative regulatory genes described in this report will be the basis for making suitable reporter gene constructs to further investigate the environmental signalling process leading to cellulose production in these bacteria.

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