Diversity of Methylotrophy Pathways in the Genus *Paracoccus* (*Alphaproteobacteria*)

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Abstract

Paracoccus denitrificans Pd 1222 is a model methylotrophic bacterium. Its methylotrophy is based on autotrophic growth (enabled by the Calvin cycle) supported by energy from the oxidation of methanol or methylamine. The growing availability of genome sequence data has made it possible to investigate methylotrophy in other *Paracoccus* species. The examination of a large number of *Paracoccus* spp. genomes reveals great variability in C_1 metabolism, which have been shaped by different evolutionary mechanisms. Surprisingly, the methylotrophy schemes of many *Paracoccus* strains appear to have quite different genetic and biochemical bases. Besides the expected 'autotrophic methylotrophs', many strains of this genus possess another C_1 assimilatory pathway, the serine cycle, which seems to have at least three independent origins. Analysis of the co-occurrence of different methylotrophic pathways indicates, on the one hand, evolutionary linkage between the Calvin cycle and the serine cycle, and, on the other hand, that genes encoding some C_1 substrate-oxidizing enzymes occur more frequently in association with one or the other. This suggests that some genetic module combinations form more harmonious enzymatic sets, which act with greater efficiency in the methylotrophic process and thus undergo positive selection.

Introduction

The genus *Paracoccus* (class *Alphaproteobacteria*, order *Rhodobacterales*, family *Rhodobacteraceae*) currently includes around 70 defined species and hundreds of strains whose taxonomic position has yet to be precisely assigned (NCBI Taxonomy, 10 January 2018). Representatives of this genus have been identified in diverse environments. The original isolate and the type species, *P. denitrificans*, was isolated from soil (Beijerinck, 1910), like many other *Paracoccus* spp. (Urakami *et al.*, 1990; Siller *et al.*, 1996; Tsubokura *et al.*, 1999). Numerous strains have been isolated from fresh water (Sheu *et al.*, 2018), seawater (Kim and Lee, 2015), sediments (G. Zhang *et al.*, 2016), activated sludge (Liu *et al.*, 2006), biofilters (Lipski *et al.*, 1998), or from environments linked to higher organisms, such as plant roots (rhizosphere) (Doronina *et al.*, 2002), marine bryzoans (Pukall *et al.*, 2003), insects (S. Zhang *et al.*, 2016), and human tissues (opportunistic pathogens *P. yeei* and *P. sanguinis*) (Funke *et al.*, 2004; McGinnis *et al.*, 2015).

The ubiquity of *Paracoccus* spp. is due to their great metabolic diversity and flexibility. All paracocci have an aerobic respiratory metabolism and utilize multi-carbon compounds. However, many of them can switch between different growth modes, using different carbon and energy sources, and employing various final electron acceptors. In

the absence of oxygen, some *Paracoccus* spp. conduct nitrate respiration (Baker *et al.*, 1998; Kelly *et al.*, 2006). This process leads to denitrification and has been applied for the removal of nitrates from wastewater (Liu *et al.*, 2012). In addition to 'standard' carbon sources, like sugars, amino acids and succinate, *Paracoccus* strains isolated from polluted environments can utilize xenobiotics, e.g. polycyclic aromatic hydrocarbons (PAHs), making them useful in bioremediation (Sun *et al.*, 2013). Numerous representatives of the genus can grow chemolithoautotrophically, coupling CO_2 assimilation with the oxidation of inorganic compounds or elements, such as thiosulfate, thiocyanate, elemental sulfur, molecular hydrogen, or ferrous ions (Kelly *et al.*, 2006). Finally, many *Paracoccus* spp. are methylotrophs. Most utilize methanol (MeOH) and methylamine (MA) as sole carbon and energy sources, e.g. *P. denitrificans* and closely related *P. versutus* and *P. kondratievae* (Kelly *et al.*, 2006). However, other strains isolated from environments polluted with C1 compounds are able to metabolize dimethylamine (DMA), trimethylamine (TMA), *N,N*-dimethylformamide (DMF) (Urakami *et al.*, 1990; Kim *et al.*, 2001; Sanjeevkumar *et al.*, 2013) or dichloromethane (Doronina *et al.*, 1998).

The purpose of this study is to describe the diversity of methylotrophy in the genus *Paracoccus* at the biochemical and genetic levels, including an examination of the origin and evolution of C1 metabolism in this group of bacteria.

Paracoccus denitrificans Pd 1222 as an example of an 'autotrophic' methylotroph

Since its isolation at the beginning of the 20th century (Beijerinck, 1910), *P. denitrificans* has been extensively studied and different aspects of its energy metabolism have been revealed. The composition of its core respiratory chain closely resembles that of the classic mitochondrial respiratory chain (unlike respiratory chains of many other bacteria, including *E. coli*), which made it a valuable model for studies on the energetic processes in eukaryotes (John and Whatley, 1975). However, the electron transport chain of *P. denitrificans* also has many branches at both the entrance and exit sides of the core. On the one hand, this allows the bacterium to utilize alternative final electron receptors, namely nitrate, nitrite, nitric oxide and nitrous oxide (which leads to denitrification – *P. denitrificans* is an important model in studies on this process (Baker *et al.*, 1998), thus permitting growth when oxygen is limited. On the other hand, different electron donors may be used. As a consequence, *P. denitrificans* has the ability to grow chemolithoautotrophically on inorganic energy sources such as hydrogen and thiosulphate (Friedrich and Mitrenga, 1981). Its autotrophic growth may also be supported by the oxidation of some organic C1 compounds, namely MeOH, MA and formate (Baker *et al.*, 1998). These compounds are oxidized to CO_2, the released electrons are used for oxidative phosphorylation, and the ATP and CO_2 produced are used in the Calvin cycle for biomass production. Thus, *P. denitrificans* is an example of an 'autotrophic methylotroph', which lacks a 'heterotrophic' pathway dedicated to the assimilation of reduced C_1 units (such as the serine cycle or ribulose monophosphate pathway), but it can assimilate carbon from C_1 compounds after their total oxidation (Baker *et al.*, 1998; Chistoserdova, 2011) (Fig. 6.1).

Since the 1970s, numerous studies have sought to understand the details of C_1 metabolism in *P. denitrificans* (Harms *et al.*, 1985; Baker *et al.*, 1998), especially in strain Pd 1222, which is readily transformed by conjugation to enable genetic manipulation (Devries *et al.*, 1989). The results of these studies have uncovered the properties of many *P. denitrificans* proteins involved in methylotrophy (mainly MeOH and MA dehydrogenases, as well as associated proteins, i.e. those involved in the transfer of electrons from the dehydrogenases to the respiratory chain), and have shed light on the regulation of their expression (Baker *et al.*, 1998).

The whole genome sequence of *P. denitrificans* Pd 1222 was obtained in 2006 (NCBI Genomes). It has an unusual structure consisting of two chromosomes (chromosome 1, 2.8 Mb, and chromosome 2, 1.7 Mb) and one large plasmid (plasmid 1 1650 kb). The availability of this sequence has permitted elucidation of the genetic basis of its methylotrophy. *P. denitrificans* Pd 1222 carries several gene clusters responsible for C_1 metabolism, dispersed across the three replicons. Genes for the enzymes involved in the oxidation of primary C_1 substrates to formaldehyde are located on chromosome 2 (gene cluster encoding MxaFI-type MeOH dehydrogenase and associated proteins) and

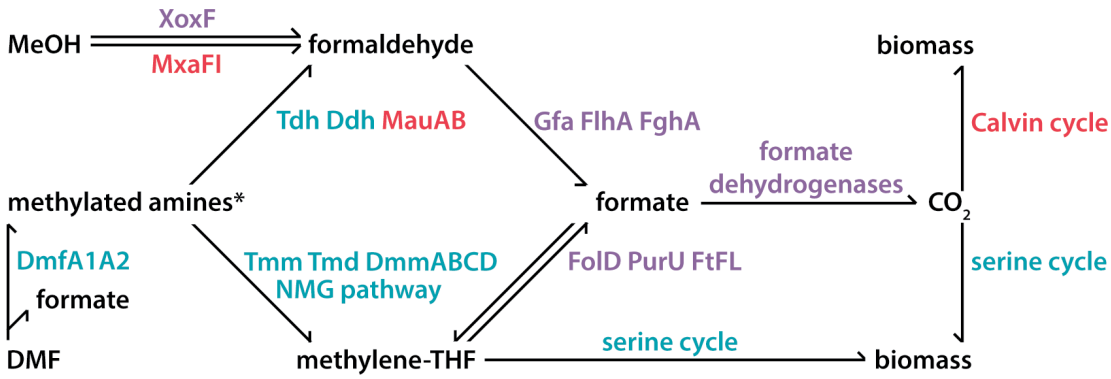

Figure 6.1 Summary of the methylotrophic pathways of *Paracoccus* spp. *P. denitrificans* Pd 1222 and *P. aminovorans* JCM 7685 are used as examples because these strains possess all of the methylotrophic pathways discussed in this study. The enzymes and pathways present in *P. denitrificans* Pd 1222 are shown in red, those present in *P. aminovorans* JCM 7685 are shown in blue, and those present in both strains are shown in violet. Ddh, DMA dehydrogenase; DmfA1A2, DMFase; DmmABCD, DMA monooxygenase; FghA, *S*-formylglutathione hydrolase; FlhA, *S*-(hydroxymethyl)glutathione dehydrogenase; FolD, methylenetetrahydrofolate dehydrogenase (NADP+)/methenyltetrahydrofolate cyclohydrolase; FtfL, formate-tetrahydrofolate ligase; Gfa, *S*-(hydroxymethyl) glutathione synthase; MauAB, MA dehydrogenase; MxaFI, MxaFI-type MeOH dehydrogenase; PurU, formyltetrahydrofolate deformylase; Tdh, TMA dehydrogenase; Tmd, TMA *N*-oxide demethylase; Tmm, TMA monooxygenase; XoxF, XoxF-type MeOH dehydrogenase. *Methylated amines – TMA, DMA and MA.

plasmid 1 (the *mau* genes encoding small and large subunits of MA dehydrogenase and associated proteins). The second step in the methylotrophy of *P. denitrificans* Pd 1222 is oxidation of formaldehyde to formate in the glutathione-dependent pathway, which is essential for growth of this strain on C_1 compounds (Harms *et al.*, 1996). Three enzymes of this pathway, *S*-(hydroxymethyl)glutathione synthase (Gfa), *S*-(hydroxymethyl)glutathione dehydrogenase (FlhA), and *S*-formylglutathione hydrolase (FghA), are encoded within chromosome 1. Interestingly, these glutathione-dependent formaldehyde oxidation genes occur in the immediate vicinity of genes encoding XoxF-type MeOH dehydrogenase and associated proteins. XoxF was recently confirmed as a MeOH-oxidizing enzyme (Keltjens *et al.*, 2014; Chistoserdova, 2016). However, its involvement in MeOH metabolism in *P. denitrificans* was suggested many years before (Harms *et al.*, 1996), although its redundancy with a MxaFI-type system remains unexplained. Formate is oxidized to CO_2 by two multi subunit (encoded in chromosomes 1 and 2) or one single subunit (encoded in chromosome 1) formate dehydrogenase. Finally, the Calvin cycle gene cluster, which is required for assimilation of CO_2, is located on chromosome 1 and consists of genes encoding two subunits of RuBisCO (*rbcL* and *rbcS*), as well as genes for fructose-1,6-bisphosphatase (*fbp*), phosphoribulokinase (*prk*), transketolase (*tkt*), fructose-1,6-bisphosphate aldolase (*fba*), RuBisCO activating protein (*cbbX*), and the Calvin cycle regulator (*cbbR*).

Parallel studies on the methylotrophy of the closely related *P. versutus* have shown that this species utilizes similar routes of C1 metabolism. The assimilatory pathway required for growth on C1 substrates includes highly similar MA dehydrogenase and Calvin cycle enzymes (Karagouni and Kelly, 1989; Baker *et al.*, 1998).

Paracoccus aminophilus JCM 7686 and *Paracoccus aminovorans* JCM 7685 as serine cycle methylotrophs specialized in DMF utilization

In 1990, the isolation of two DMF-degrading strains from a sample of DMF-polluted soil in Japan was reported (Urakami *et al.*, 1990). These strains, designated JCM 7686 and JCM 7685 were recognized as representatives of two new *Paracoccus* species: *P. aminophilus* and *P. aminovorans* (Urakami *et al.*, 1990). The methylotrophic pathways of these isolates were shown to be more complex than those of *P. denitrificans*, because they include enzymes

required for the utilization of a wider range of C1 compounds. Both strains are able to decompose DMF to formate and DMA, and oxidize TMA via trimethylamine *N*-oxide (TMAO) to DMA, and then degrade the resultant DMA to MA (Urakami *et al.*, 1990).

The entire genome sequences of *P. aminophilus* JCM 7686 and *P. aminovorans* JCM 7685 were obtained by our group (Dziewit *et al.*, 2014; Czarnecki *et al.*, 2017), facilitating the reconstruction of their methylotrophic pathways. As expected, genes required for the metabolism of additional C_1 substrates were identified in both strains. These encode small and large subunits of DMFase (DmfA1A2), as well as TMA monooxygenase (Tmm), TMAO demethylase (Tmd), and multi-subunit DMA monooxygenase (DmmABCD). Besides TMA and DMA monooxygenases, the *P. aminovorans* JCM 7685 genome also encodes TMA and DMA dehydrogenases (Tdh and Ddh, respectively), which may catalyse the oxidation of TMA and DMA (Fig. 6.1). The role of these genes in the metabolism of specific C1 substrates has been confirmed in both strains (Dziewit *et al.*, 2010, 2015; Czarnecki *et al.*, 2017).

Surprisingly, the enzymes involved in the metabolism of MeOH and MA by *P. aminophilus* and *P. aminovorans* differ from those employed by *P. denitrificans*. In the case of MeOH utilization, *P. aminophilus* and *P. aminovorans* do not possess a MxaFI-type MeOH dehydrogenase, and their growth on this compound relies fully on a XoxF-type dehydrogenase, as has been confirmed by mutational analysis (Dziewit *et al.*, 2015). In the case of MA, both strains have genes for an alternative MA oxidation pathway: the *N*-methylglutamate (NMG) pathway. In *P. aminovorans* the NMG pathway is the only pathway for MA oxidation, while in *P. aminophilus* it co-exists with the MA dehydrogenase pathway, which was previously characterized in *P. denitrificans* (Fig. 6.1). Furthermore, both strains lack RuBisCO genes, so cannot assimilate CO_2. Thus, their methylotrophy has to be supported by another pathway of C1 unit assimilation. A serine cycle gene cluster was found in both genomes; its involvement in methylotrophy has been confirmed in *P. aminovorans*, and the role of transcriptional regulator ScyR in its regulation was revealed (Czarnecki *et al.*, 2017). The serine cycle requires glyoxylate regeneration, which is accomplished by the action of the ethylmalonyl-CoA pathway in *P. aminovorans* JCM 7685 (Czarnecki *et al.*, 2017). All genes required for this pathway are also present in *P. aminophilus*, in the non-serine cycle methylotroph *P. denitrificans* and even in non-methylotrophic strains, as they are used for other purposes, such as growth on C2 compounds (Schneider *et al.*, 2012). The genes required for another glyoxylate-regenerating process, the glyoxylate shunt, are found in *P. aminophilus* and *P. aminovorans*, but, as they were unable to support methylotrophic growth in a strain with a blocked ethylmalonyl-CoA pathway (Czarnecki *et al.*, 2017), their role remains unclear.

Owing to the presence of different enzymes oxidizing primary C1 substrates and the serine cycle, the fate of C1 units released during methylotrophic metabolism is more complex in *P. aminophilus* and *P. aminovorans* than in *P. denitrificans*. In the serine cycle, carbon is assimilated in the form of methylene group bound to tetrahydrofolate (THF) and CO_2 (Fig. 6.1). Methylene-THF may be delivered directly by the C1-substrate-oxidizing enzymes TMAO demethylase and DMA monooxygenase or the NMG pathway, which do not release free formaldehyde, but transfer the C1 unit directly to THF (Fig. 6.1). On the other hand, the XoxF-type MeOH dehydrogenase, TMA dehydrogenase, DMA dehydrogenase and MA dehydrogenase release free formaldehyde, which has to be oxidized to formate by the glutathione-dependent pathway present in *P. aminophilus*, *P. aminovorans* and *P. denitrificans*. To feed the serine cycle, the formate has to be bound to THF, and then it has to be reduced to a methylene group in an energy-requiring process. The THF-dependent formate reduction pathway is found in all three *Paracoccus* spp., and requires the action of two enzymes: formate-THF ligase (FtfL) and 5,10-methylene-tetrahydrofolate dehydrogenase/methenyl-tetrahydrofolate cyclohydrolase (FolD). This pathway may also act in the opposite direction to oxidize the methylene group to formate. In this case, FolD promotes the reverse reaction itself, while the second reaction is catalysed by formyltetrahydrofolate deformylase (PurU), which is also present in all three species (Fig. 6.1). A special situation occurs during growth on DMF, where DMFase releases a C1 unit directly in the form of formate and the second product is DMA. The fate of C1 units has been analysed experimentally in *P. aminovorans*, where growth of a strain lacking the

glutathione-dependent pathway for formaldehyde oxidation on different C1 substrates was examined. As expected, this strain was unable to utilize MeOH (it has the XoxF-type MeOH dehydrogenase releasing free formaldehyde, which cannot be further oxidized without the glutathione-dependent pathway), but no effect on growth on MA was detected (it has the NMG pathway which produces methylene-THF, which directly enters the serine cycle or is oxidized in the THF-dependent pathway, without involvement of the glutathione-dependent pathway). Intermediate phenotypes were observed during growth on DMF, TMA or DMA, since monooxygenases can oxidize some portion of these compounds to produce methylene-THF, while another portion is oxidized by dehydrogenases to produce free formaldehyde (Czarnecki *et al.*, 2017) (Fig. 6.1). Like *P. denitrificans*, *P. aminophilus* and *P. aminovorans* have formate dehydrogenases to deal with an excess of formate (Dziewit *et al.*, 2015; Czarnecki *et al.*, 2017).

The genomic localization and clustering of methylotrophy genes of *P aminophilus* and *P. aminovorans* suggests that many of them could have been acquired horizontally to confer increased fitness for growth in DMF-polluted soil. Some DMFase genes of *P. aminophilus* are located on the small plasmid pAMI2 (18.6 kb), which also carries a genetic module putatively involved in its mobilization for conjugal transfer (Dziewit *et al.*, 2010). A closely related DMFase is encoded in the chromosomes of both *P. aminovorans* and *P. aminophilus* (84% aa identity of large subunits and 73% aa identity of small subunits). However, these chromosomal DMFase genes and their adjacent transcriptional regulator genes are surrounded by genes encoding transposases and other proteins typical of mobile genetic elements, which indicates their recent acquisition. Similarly, the TMA and DMA dehydrogenase genes of *P. aminovorans* JCM 7685, that are not found in any other *Paracoccus* strain (Czarnecki *et al.*, 2017), are located on the large extrachromosomal replicon pAMV3 (740 kb), which, like other replicons of this type, seems to be a reservoir of various horizontally transmitted genes.

A notable example of methylotrophy genes acquired by HGT are those clustered within a 40-kb methylotrophy island (MEI) located on the extrachromosomal replicon pAMV1 of *P. aminovorans* (Fig. 6.2). The genes present on this island are involved in all steps of methylotrophy: (i) oxidation of primary C1 substrates with methylene-THF generation (TMA monooxygenase, TMAO demethylase, DMA monooxygenase, the NMG pathway), (ii) oxidation of methylene-THF to formate (FolD and PurU), and assimilation of C1 units in the form of methylene-THF and CO_2 (the serine cycle). The closest homologue of this MEI was identified in *Paracoccus* sp. N5 (Beck *et al.*, 2015; Dziewit *et al.*, 2015; Czarnecki *et al.*, 2017), and similarly clustered genes are located in the chromosomes of many bacteria of the *Roseobacter* clade, including *Ruegeria pomeroyi* (Dziewit *et al.*, 2015). The MEI genes are also present in the *P. aminophilus* genome. However, in this case the island is divided in two, with one part encoding the serine

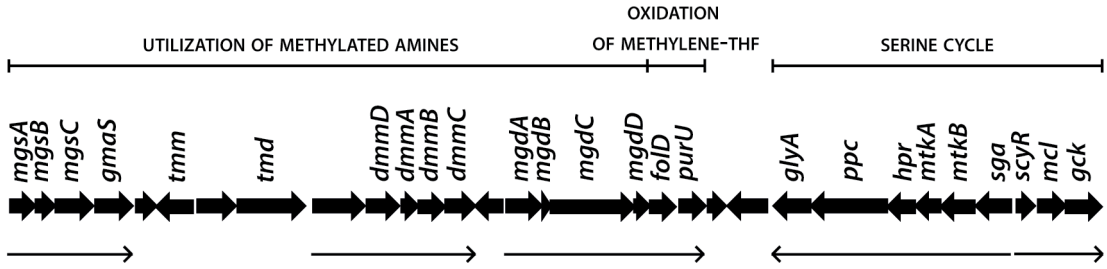

Figure 6.2 Genetic organization of the methylotrophy island (MEI) of *P. aminovorans* JCM 7685**.** The general functions of genes are indicated above their names. Potential operons are indicated by thin arrows. *dmmABCD*, DMA monooxygenase; *folD*, methylenetetrahydrofolate dehydrogenase (NADP+)/methenyltetrahydrofolate cyclohydrolase; *gck*, glycerate 2-kinase; *glyA*, serine hydroxymethyltransferase; *gmaS*, glutamate-methylamine ligase; *hpr*, hydroxypyruvate reductase; *mcl*, malyl-CoA lyase; *mgdABCD*, *N*-methylglut amate dehydrogenase; *mgsABCD*, *N*-methylglutamate synthase; *mtkAB*, malate-CoA ligase; *scyR*, serine cycle transcriptional regulator; *sga*, serine-glyoxylate aminotransferase; *tmd*, TMA N-oxide demethylase; *tmm*, TMA monooxygenase; *ppc*, phosphoenolpyruvate carboxylase; *purU*, formyltetrahydrofolate deformylase.

cycle enzymes located in the chromosome, and the second part, comprising the rest of the MEI, in extrachromosomal replicon pAMI6 (207 kb) (Dziewit *et al.*, 2015), whose genetic load is 40% homologous to that of *P. aminovorans* pAMV1. It appears that acquisition of the MEI (most probably from representatives of the *Roseobacter* clade (Dziewit *et al.*, 2015) could have enhanced the methylotrophic ability of *P. aminophilus* and *P. aminovorans*, which made them better adapted to living in DMF-polluted soil. To fully reconstruct the evolution of C1 pathways in the genus *Paracoccus*, deeper analyses are required, which consider not only the presence of given pathways (which has already been done (Dziewit *et al.*, 2015; Czarnecki *et al.*, 2017), but also their phylogenetic relationships.

Occurrence and phylogenetic relationships of methylotrophy genes in *Paracoccus* spp.

We previously examined 44 *Paracoccus* spp. genomes to determine the diversity of methylotrophy genes in this genus (Dziewit *et al.*, 2015; Czarnecki *et al.*, 2017). In this study we have broadened this analysis using additional recently deposited *Paracoccus* spp. sequences (in total, 62 *Paracoccus* genomes were available in NCBI GenBank on 11 May 2018) plus 10 *Paracoccus* spp. genomes obtained by our group, which will be fully described in a forthcoming publication (*P. bengalensis* DSM 17099, *P. ferrooxidans* NCCB 1300066, *P. haundaensis* LGM P-21903, *P. kondratievae* NCIBM 13773, *P. pantotrophus* DSM 11072, *P. solventivorans* DSM 11592, *P. sulfuroxidans* JCM 14013, *P. thiocyanatus* JCM 20756, *P. versutus* UW1 and *P. yeei* CCUG 32053). The majority of the studied *Paracoccus* strains (almost 65%), contain sets of genetic modules involved in all three steps of methylotrophy (Chistoserdova, 2011), which are potentially sufficient for methylotrophic growth.

Comparative analysis of this large number of *Paracoccus* genomes allowed us to distinguish two groups of methylotrophy-related genes based on their degree of conservation: (i) highly conserved, vertically transmitted genes, present in almost all of the genomes, and (ii) genes present only in some strains because of selective loss, horizontal gene transfer, or a combination of these two evolutionary mechanisms. The genes of the first group are usually located on chromosomes, while many of the genes of the second group are found on extrachromosomal replicons, or in the company of mobile genetic elements when present on chromosomes.

The first group includes genes comprising fundamental pathways, that are important for non-methylotrophic metabolism, to which further C1 pathways are appended. The first example of this group is a cluster consisting of genes for glutathione-dependent formaldehyde oxidation (*gfa, flhA, fghA*) and for MeOH oxidation by XoxF (Fig. 6.3). The compact and conserved nature of the gene cluster comprising both pathways suggests their cooperation. As mentioned above, both the XoxF and the glutathione-dependent pathways may be essential for some methylotrophic processes in *Paracoccus* spp. (Ras *et al.*, 1995; Harms *et al.*, 1996). However, the ubiquity of this gene cluster in non-methylotrophs indicates that its significance is more general. Its probable function in non-methylotrophic organisms is in the utilization of MeOH as an additional source of energy, without its assimilation into biomass. Despite its overall conservation, the cluster is truncated in some *Paracoccus* spp. and lacks the *xox* genes. For example, *P. halophilus* JCM 14014 does not have any homologues of *xoxF*. In *P. alcaliphilus* JCM 7364 the *xox* genes and the genes for the glutathione-dependent pathway are separated, being located on a large (430 kb) extrachromosomal replicon and on the chromosome, respectively.

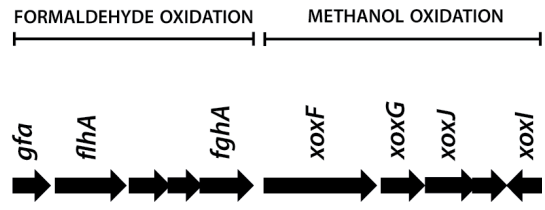

Figure 6.3 Genetic organization of the gene cluster for the glutathione-dependent formaldehyde oxidation pathway and the XoxF-type methanol dehydrogenase of *P. denitrificans* Pd 1222. The general functions of genes are indicated above their names. *fghA*, *S*-formylglutathione hydrolase; *flhA*, *S*-(hydroxymethyl)glutathione dehydrogenase; *gfa*, *S*-(hydroxymethyl)glutathione synthase; *xoxF*, XoxF-type MeOH dehydrogenase; *xoxG*, c-type cytochrome; *xoxI*, SRPBCC family protein; *xoxJ*, quinoprotein dehydrogenase-associated putative ABC transporter substrate-binding protein.

Another example of genes of the conserved group are those encoding the numerous enzymes of the ethylmalonyl-CoA pathway. These do not form a single gene cluster but are scattered throughout *Paracoccus* genomes. Enzymes of the ethylmalonyl-CoA pathway are involved in various processes, such as the utilization of C2 compounds as carbon and energy sources or synthesis of polyhydroxyalkanoates. As mentioned above, in some *Paracoccus* spp., the ethylmalonyl-CoA pathway is responsible for glyoxylate regeneration, which is crucial for functioning of the serine cycle during methylotrophic growth (Chistoserdova, 2011). A few *Paracoccus* spp. lack some enzymes of this pathway, e.g. *P. chinensis* CGMCC 1.7655 or strains of *P. sanguinis.* Nevertheless, a complete set of ethylmalonyl-CoA pathway genes was found in all strains that employ the serine cycle.

The next examples of conserved methylotrophy-linked genes in *Paracoccus* spp. are those involved in THF-dependent transformations of C1 units: *folD*, *purU* and *ftfL*. The encoded enzymes are important for the generation of C1-THF intermediates required by biosynthetic pathways, such as purine synthesis. As in the serine cycle C1 units are incorporated into biomass in the form of methylene-THF, the THF-dependent pathway constitutes a central metabolic process in *Paracoccus* serine cycle methylotrophs (Fig. 6.1). It should be noted that besides the conserved set of *folD*, *purU* and *ftfL* genes, there are also numerous homologues that are horizontally transferred, for example in the company of serine cycle genes or NMG pathway genes. It is possible that the serine cycle-associated and the NMG pathway-associated homologues are better adapted to co-operate with the methylotrophic pathways, while the conserved homologues are mainly responsible for anabolic housekeeping functions, but members of these groups are likely to be interchangeable to some extent.

The last representatives of the group of conserved genes are gene clusters encoding multi-subunit formate dehydrogenases, which are responsible for formate detoxification in both methylotrophs and non-methylotrophs, and form part of the core genome of *Paracoccus* spp.

The second group of genes, which only occurs in some *Paracoccus* strains, includes gene sets involved in C1 unit assimilation pathways, the Calvin cycle and the serine cycle, as well as genes involved in primary oxidation of C1 substrates. The Calvin cycle is widespread among *Paracoccus* species (Fig. 6.4) and a complete set of genes required for this process was identified in 56% of analysed strains. Phylogenetic analysis of the RuBisCO large subunit (RbcL) and phosphoribulokinase (Prk) indicates that there are at least two evolutionarily distinct lineages of Calvin cycle genes in this genus (Figs. 6.5–6.7). This is reflected in variations in the organization of Calvin cycle gene clusters in different *Paracoccus* spp. (Fig. 6.5). The Calvin cycle genes are generally found in chromosomes, with some exceptions, e.g. in *Paracoccus* sp. N5 and *P. kondratievae* they are located on large extrachromosomal replicons (1 Mb and 423 kb, respectively), most probably as a result of translocation from the chromosome.

Compared with the Calvin cycle, the serine cycle is less prevalent in *Paracoccus* spp. and its occurrence was predicted in 17% of the analysed strains. Serine cycle gene sets are present in a few different lineages of the genus (Fig. 6.4), and three types of gene organization were identified (Fig. 6.5). The occurrence of three gene organization schemes is probably an effect of the independent acquisition of the serine cycle from different sources. These separate events are reflected in the phylogenetic tree of phosphoenolpyruvate carboxylase (Ppc), the key enzyme of the cycle (Fig. 6.8). The first type of serine cycle gene organization occurs in the MEI of *P. aminovorans* JCM 7685 and, as mentioned above, is also found in many representatives of the *Roseobacter* clade, in the order *Rhodobacterales,* as are *Paracoccus* spp. The second type of serine cycle gene organization, found on the chromosome of *P. zhejiangensis* J6, for example, is also most similar to arrangements present in members of the *Roseobacter* clade. The third type of serine cycle gene organization, found in *P. denitrificans* ISTOD1, is typical for strains of the *Aminobacter* and *Labrys* genera (Beck *et al.*, 2015), within the order *Rhizobiales*. In some *Paracoccus* spp., two types of serine cycle module coexist in the same genome (Fig. 6.4). The serine cycle clusters may be located within extrachromosomal replicons, as in pAMV1 in *P. aminovorans* JCM 7685 and probably also ISTOD1 in *P. denitrificans* (the unfinished genome sequence of this strain does not allow confirmation of the genomic localization of this gene cluster, although it is present in a sequence scaffold containing a rep*ABC*

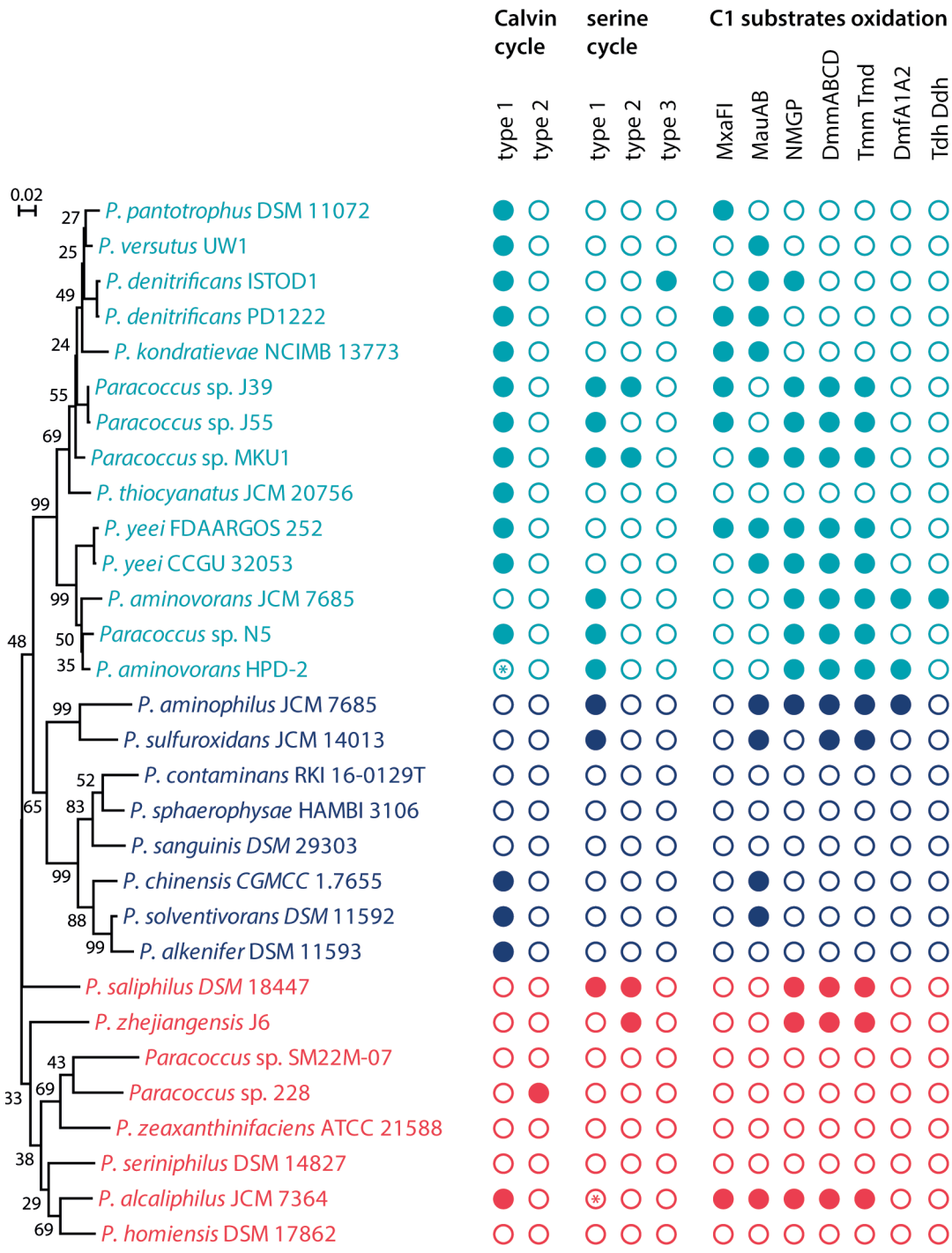

Figure 6.4 Occurrence of methylotrophy genes in *Paracoccus* spp. The maximum likelihood phylogenetic tree of *Paracoccus* spp. is based on the conserved gene for ethylmalonyl-CoA mutase (*ecm*). Where not indicated, the bootstrap support value is 100. Three sub-clades of *Paracoccus* spp., classified on the basis of 16S rDNA, *dnaA* (data not shown) and *ecm* comparisons, are indicated in light blue, dark blue and red. Ddh, DMA dehydrogenase; DmfA1A2, DMFase; DmmABCD, DMA monooxygenase; MauAB, MA dehydrogenase; MxaFI, MxaFI-type MeOH dehydrogenase; NMGP, *N*-methylglutamate pathway; Tdh, TMA dehydrogenase; Tmd, TMA *N*-oxide demethylase; Tmm, TMA monooxygenase. *Truncated gene clusters.

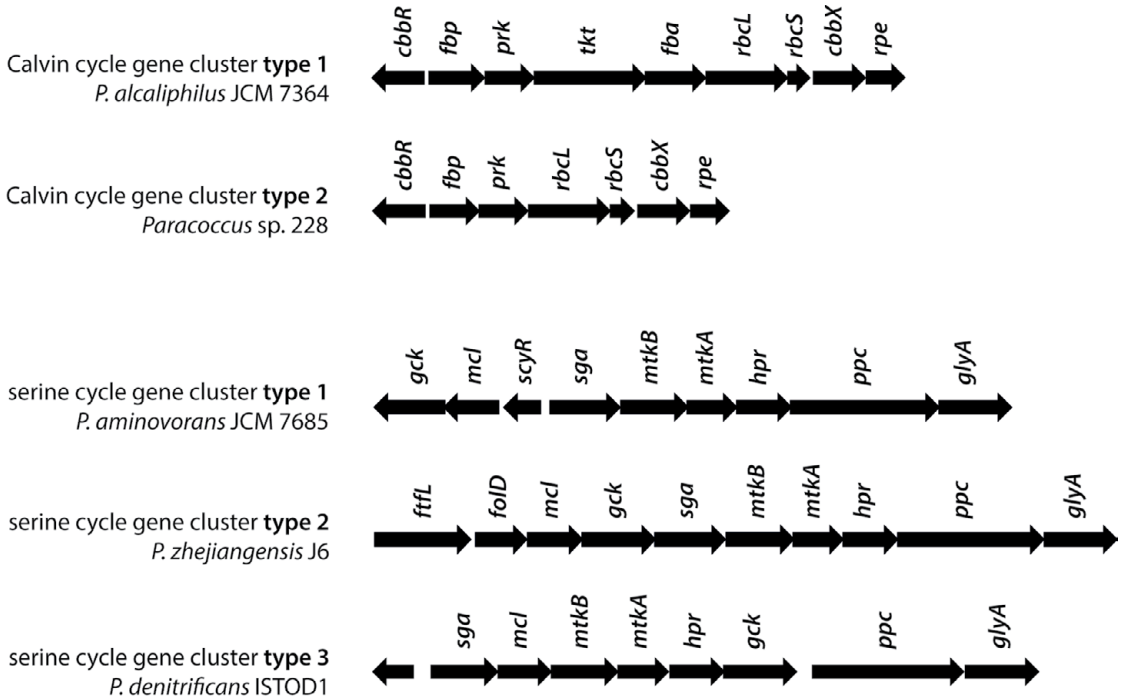

Figure 6.5 Organization of genetic modules for the Calvin cycle and the serine cycle in *Paracoccus* spp. The composition of the Calvin cycle gene cluster type 1 varies in some strains, e.g. it lacks the *rpe* gene in *P. denitrificans, P. pantotrophus*, *P. versutus*, *P. kondratievae* and *P. thiocyanatus*. It is also truncated in *Paracoccus* sp. N5 (lacks the *tkt*, *fba* and *rpe* genes) and in *P. alkenifer* DSM 11593 (lacks the *tkt* gene). In all these cases, homologues of the absent genes are present at other locations in the genome. An incomplete Calvin cycle gene cluster type 1 is found in *P. aminovorans* HPD-2 (lacks *rbcS*, pseudogenes of *rbcL* and *cbbX*). The serine cycle gene cluster type 1 occurs without the *gck* gene in *P. aminophilus* JCM 7686 and *P. sulfuroxidans* JCM 14013. In both cases, other homologues of *gck* are present in the genome. *cbbR*, Calvin cycle transcriptional regulator; *cbbX*, RuBisCO activating protein; *fba*, fructose-bisphosphate aldolase; *fbp*, fructose-bisphosphatase; *flfL*, formate-THF ligase; *folD*, 5,10-methylene-tetrahydrofolate dehydrogenase/methenyl-tetrahydrofolate cyclohydrolase (FolD); *gck*, glycerate 2-kinase; *glyA*, serine hydroxymethyltransferase; *hpr*, hydroxypyruvate reductase; *mcl*, malyl-CoA lyase; *mtkA*, malate-CoA ligase subunit alpha; *mtkB*, malate-CoA ligase subunit beta; *ppc*, phosphoenolpyruvate carboxylase; *prk*, phosphoribulokinase; *rbcL*, RuBisCO large subunit; *rbcS*, RuBisCO small subunit; *rpe*, ribulose-phosphate 3-epimerase; *scyR*, serine cycle transcriptional regulator; *sga*, serine-glyoxylate aminotransferase; *tkt*, transketolase.

replication-partitioning module, typical for large plasmids of *Alphaproteobacteria*). The serine cycle genes have a chromosomal location in *P. aminophilus* JCM 7685 or *P. sulfuroxidans* JCM 14013. The distribution of the serine cycle genes indicates that they were acquired horizontally several times in different lineages of the genus. Nevertheless, their prevalence could also have been shaped by gene loss. The best example is the incomplete serine cycle cluster of *P. alcaliphilus* JCM 7364, which lacks the *pcc* gene (there are no *pcc* homologues in the entire *P. alcaliphilus* genome).

One particularly interesting aspect is co-occurrence of the Calvin cycle and the serine cycle, and co-evolution of these two cycles in *Paracoccus* genomes. The phylogeny and distribution patterns of the key enzymes of these cycles (Figs 6.4, 6.6–6.8) indicate that the Calvin cycle is the more ancient in this group of bacteria, and that the serine cycle has been acquired independently in several lineages. In some strains, these two cycles coexist (Fig. 6.4), but in others, the Calvin cycle seems to have been lost after serine cycle acquisition. This phenomenon is perfectly illustrated by a group of three isolates: *Paracoccus* sp. N5, *P. aminovorans* HPD-2 and *P. aminovorans* JCM 7685. The first strain has the intact Calvin cycle gene cluster, the second one possesses an incomplete cluster lacking

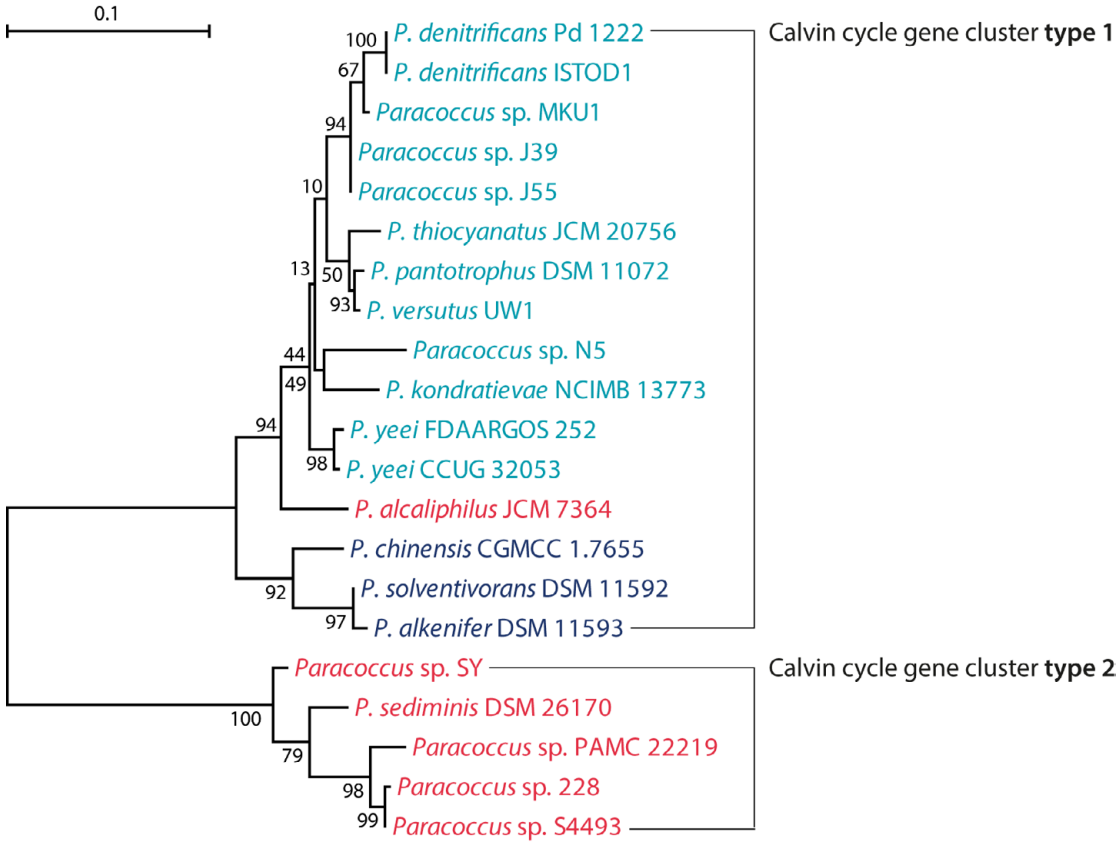

Figure 6.6 Maximum likelihood phylogenetic tree of RuBisCO large subunits (RbcL) of *Paracoccus* spp. Two phylogenetic groups matching different types of genetic organization of the Calvin cycle genes are indicated. Three sub-clades of *Paracoccus* spp. are coloured as in Fig. 6.4.

the *rbcS* gene but containing *rbcL* and *cbbX* pseudogenes, while the third strain does not possess the *rbcS, rbcL* nor *cbbX* genes (Fig. 6.4). However, not all of the Calvin cycle genes are lost in *Paracoccus* serine cycle methylotrophs lacking this cycle. These strains still have a truncated version of the Calvin cycle gene cluster, including its three first genes encoding the Calvin cycle regulator (CbbR), fructose-1,6-bisphosphatase (Fbp) and phosphoribulokinase (Prk). The phylogeny of the *Paracoccus* phosphoribulokinases (Fig. 6.7) indicates that the same reduction of the Calvin cycle gene cluster arose in different lineages where the serine cycle appeared. Moreover, retention of a reduced Calvin cycle gene cluster is typical only for the serine cycle methylotrophs, whereas the *cbbR, fbp* and *prk* genes are not present in *Paracoccus* non-methylotrophs. An identically organized gene cluster was detected in another serine cycle methylotroph, *Methylobacterium extorquens* PA1. This includes the gene encoding QscR, a transcriptional regulator with homology to CbbR, which is a global regulator of the serine cycle genes (Kalyuzhnaya and Lidstrom, 2003, 2005). Recently, QscR was shown to be regulated by phosphoribulokinase, and it was demonstrated that both the *qscR* and *prk* genes are essential for the methylotrophy of *M. extorquens* (Ochsner *et al.*, 2017). Parallel evolution of the same gene set in *Paracoccus* serine cycle methylotrophs (and *M. extorquens*) implies that there is a universal evolutionary linkage between the Calvin cycle and the serine cycle (Ochsner *et al.*, 2017). As the role of the *cbbR–fbp–prk* cluster is only regulatory, some strains may have evolved towards the loss of these genes. In the analysed strain set there is one example where partial loss of this regulatory cluster has occurred: *P. saliphilus* DSM 18447 has two different serine cycle gene sets, plus a remnant of the *cbbR–fbp–prk* cluster (it lacks *cbbR*, has a *fbp* pseudogene and an intact *prk* gene). However, the

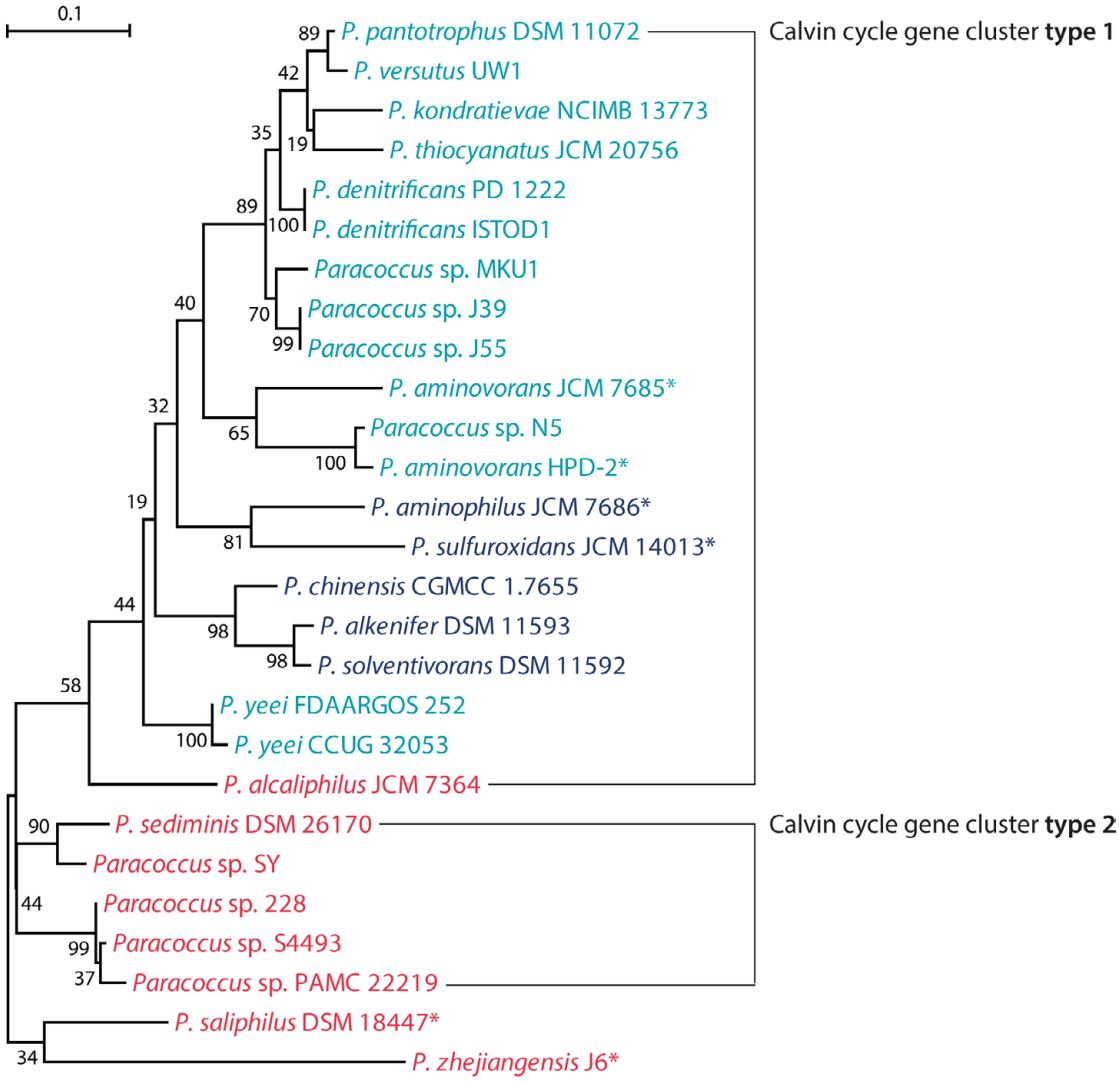

Figure 6.7 Maximum likelihood phylogenetic tree of phosphoribulokinases (Prk) of *Paracoccus* spp. Two phylogenetic groups matching different types of genetic organization of the Calvin cycle genes are indicated. Three sub-clades of *Paracoccus* spp. are coloured as in Fig. 6.4. *Strains with the serine cycle, which have truncated Calvin cycle gene clusters (and lack RuBisCO).

ability of this strain to grow methylotrophically has yet to be tested.

Among the genes involved in C1 metabolism, those encoding enzymes participating in the primary oxidation of C1 substrates, the first stage of methylotrophy, show the greatest variability in their occurrence. One prominent exception is the aforementioned conserved XoxF-type MeOH dehydrogenase. Conversely, a second type of MeOH dehydrogenase, the two subunit enzyme MxaFI, is found only in a few *Paracoccus* spp. (Fig. 6.4). A similarly limited distribution is observed in the case of MA dehydrogenase (Fig. 6.4). Both enzymes, which were thought to be reliable markers of methylotrophy in this genus based on studies on *P. denitrificans* Pd 1222, seem to be rather rare among *Paracoccus* spp. whose genomes have been sequenced. MxaFI-type MeOH dehydrogenase and MA dehydrogenase usually co-exist with the Calvin cycle (Fig. 6.4), which may result from adaptation of their mode of action to interact better with the Calvin cycle than with the serine cycle (release of the free formaldehyde, which is then oxidized to formate in the glutathione-dependent

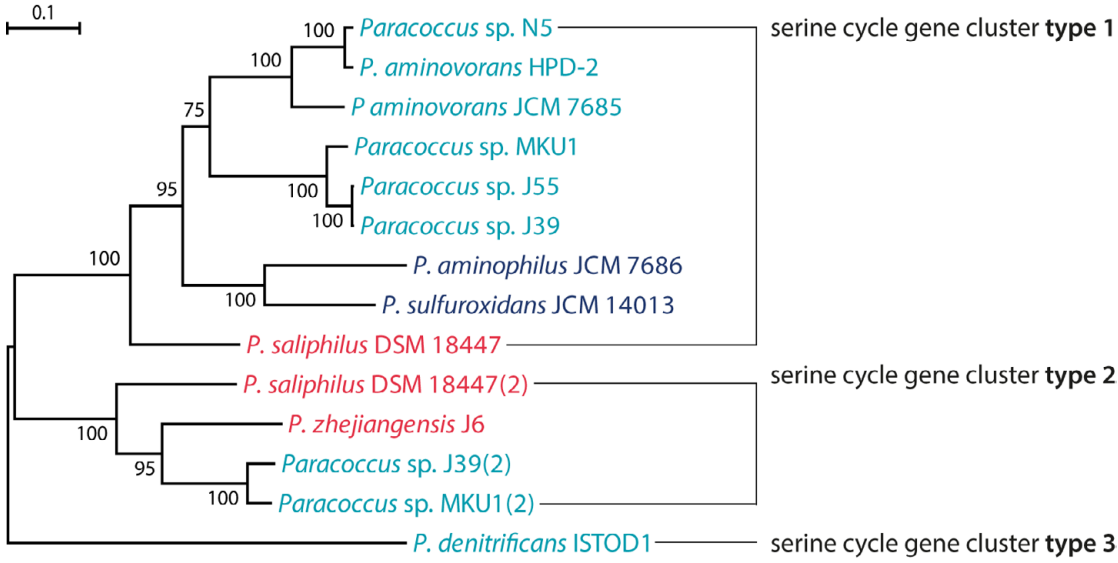

Figure 6.8 Maximum likelihood phylogenetic tree of phosphoenolpyruvate carboxylases (Ppc) of *Paracoccus* spp. Three phylogenetic groups matching different types of genetic organization of the serine cycle genes are indicated. Three sub-clades of *Paracoccus* spp. are coloured as in Fig. 6.4.

pathway, but not direct binding of the methylene group to THF). Genes of the NMG pathway for MA oxidation are mainly found in strains that possess the serine cycle (Fig. 6.4). The NMG pathway enzymes appear to be evolutionarily bound to the serine cycle enzymes, since their phylogenetic trees show a similar topology (Fig. 6.9). While the third type of serine cycle gene cluster (Fig. 6.4) is found in the company of only the NMG pathway genes, the first and the second types co-occur with other methylene-THF-producing enzymes, TMA monooxygenase, TMAO demethylase and DMA monooxygenase (Fig. 6.4), e.g. within the MEI of *P. aminovorans* JCM 7685 (Fig. 6.2) (Czarnecki *et al.*, 2017). The exception are strains of *P. yeei* which lack the serine cycle, but have the NMG pathway accompanied by DMA monooxygenase, TMA monooxygenase and TMA *N*-oxide demethylase genes (Fig. 6.4).

The DMFase genes *dmfA1A2* were only found in three strains, *P. aminophilus* JCM 7686, *P. aminovorans* JCM 7685 and *P. aminovorans* HPD-2, and always in association with serine cycle, NMG pathway, TMA monooxygenase, TMAO demethylase and DMA monooxygenase genes (Fig. 6.4). The methylotrophic potential of *Paracoccus* spp. does not seem to have been fully determined. Genes for TMA and DMA dehydrogenases were found only in *P. aminovorans* JCM 7685, but these enzymatic activities had already been identified in other *Paracoccus* isolates, and they may be important for the utilization of methylated amines when the oxygen concentration is changeable (Kim *et al.*, 2001, 2003). Moreover, one strain whose genome has yet to be sequenced, *P. methylutens* DM 12, expresses dichloromethane halogenase, which is required for dichloromethane utilization. Thus, further investigation of the content of *Paracoccus* spp. genomes may reveal interesting and unexpected features of their C_1 pathways.

Conclusions

From our detailed analysis of numerous *Paracoccus* spp. genome sequences, it is now clear that the archetypal autotrophic methylotroph, *P. denitrificans* Pd 1222, represents only a small part of the methylotrophic capacity present in the genus. Besides some generally conserved features, like the presence of the glutathione-dependent formaldehyde oxidation pathway and the ethylmalonyl-CoA pathway, methylotrophic *Paracoccus* spp. vary greatly in the genetic and biochemical basis of their C_1 metabolism. As anticipated there are differences in the sets of enzymes responsible for the primary oxidation of C_1 compounds, which are located at the periphery

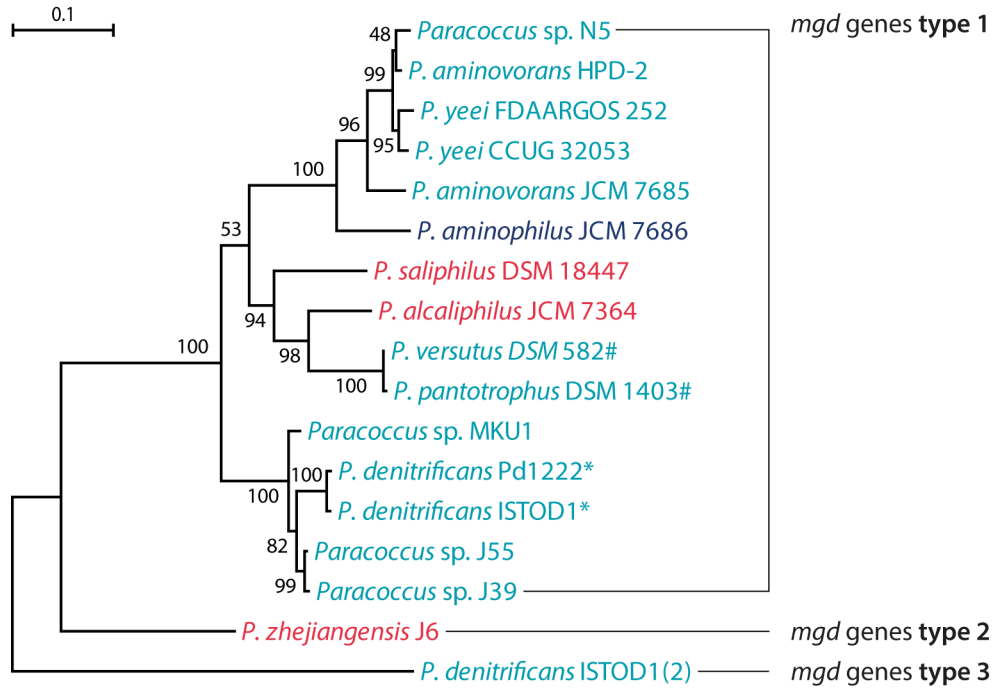

Figure 6.9 Maximum likelihood phylogenetic tree of *N*-methylglutamate dehydrogenase subunits C (MgdC) of *Paracoccus* spp. Three phylogenetic groups corresponding to the different types of serine cycle gene clusters in *Paracoccus* spp. (compare with Fig. 6.8) are indicated. Three sub-clades of *Paracoccus* spp. are coloured as in Fig. 6.4. #Strains with the NMG pathway, which do not have the serine cycle; *strains with orphan *mgd* operons, which occur without other genes of the NMG pathway.

of the C_1 metabolic net, and thus may be most 'exposed' to evolutionary changes. However, there is also considerable variation in the very nucleus of the C_1 metabolism: the C_1 assimilatory pathways. This variability is an effect of different evolutionary mechanisms, which are very hard to retrace. As was proposed previously, genetic modules representing different steps of methylotrophy may be reassembled in different genomes, generating new metabolic capabilities (Chistoserdova, 2011). This appears to be the case in *Paracoccus* spp. The more frequent coexistence of certain modules indicates that they may 'fit' together better, probably due to more efficient cooperation. This explains why the methylene THF-generating NMG pathway for MA oxidation is typically found with the methylene THF-consuming serine cycle, whereas the formaldehyde-producing MA dehydrogenase is found with the Calvin cycle. The opposite combinations of these pathways occur in some *Paracoccus* spp., but they are much less common.

The evolutionary tendencies in methylotrophy of the genus *Paracoccus* are the same as in the class *Alphaproteobacteria* as a whole, where the polyphyletic origins of C_1 metabolism have already been described (Beck *et al.*, 2015). Interestingly, these tendencies may be observed even on a microscale level, within strains of the same species. For example, comparison of two strains of *P. denitrificans*, Pd 1222 and ISTOD1, shows how evolutionary processes can easily rebuild a metabolic net. It is likely that the discovery of additional genetic modules associated with methylotrophy in this species will follow the sampling of new strains, especially those from environments where C_1 compounds are present.

Future trends

Although analysis of the increasing body of genomic data from *Paracoccus* spp. can give many interesting results, there is a need for experimental studies on the C_1 metabolism of these bacteria. The predicted functions of many genes have to be verified, the involvement of others confirmed (e.g. genes encoding putative transporters located in vicinity of

methylotrophy genes), and their regulatory mechanisms characterized. It would also be informative to determine whether serine cycle-based methylotrophy can be transmitted to other strains, e.g. via the transfer of the pAMV1 methylotrophy island. *Paracoccus* spp. seem to be ideal models for studying the evolution of methylotrophy. Studies on these bacteria may shed light on some unexplored molecular aspects of C_1 compound utilization, such as the relationship between metabolic routes representing different steps of methylotrophy (e.g. the serine cycle and the NMG pathway), and the regulatory dependencies between the Calvin cycle and the serine cycle. Such studies may also help clarify some ecological aspects, such as differences in fitness in particular niches between 'autotrophic' and 'heterotrophic' methylotrophs. A greater understanding of the *Paracoccus* C_1 metabolism will not only broaden general knowledge on methylotrophy, but may also assist the construction of novel methylotrophic strains that are adapted to perform industrially important processes.

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