Molecular Genetics of the Genus *Paracoccus*: Metabolically Versatile Bacteria with Bioenergetic Flexibility

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INTRODUCTION

The genus *Paracoccus* is one of the most distantly related of the *Proteobacteria* to *Escherichia coli* (178) as judged by 16S rRNA sequence. For many years, the sole representative of the genus was *Paracoccus denitrificans*, first isolated in 1908 by Beijerinck (13) as *Micrococcus denitrificans*. The original selection of this species was based on its ability to convert nitrate into molecular nitrogen. Improved molecular phylogenetics have led to the inclusion of *Thiobacillus versutus* (as *Paracoccus versutus* [145]) and *Thiosphaera pantotropha* (101, 178, 233) into the genus and to the addition of *P. kocurii* (203), *P. alcaliphilus* (301), *P. aminophilus* (300), *P. aminovorans* (300), *P. thiocyanatus* (145), and *P. solventivorans* (264). More recently, two other species have been characterized by using 16S rRNA (*P. marcusii* [112] and *P. alkenifer* [170]), but no other properties of these species have been published.

These newer species were isolated by using a range of organic and inorganic compounds, including acetone (*P. solventivorans*), dimethylformamide (*P. aminovorans* and *P. aminophilus*) and thiocyanate (*P. thiocyanatus*), as growth substrates. Recently, it has been shown that some strains of *P. denitrificans* can use carbon disulfide (139, 233). These properties raise the possibility of using *Paracoccus* species for bioremediation, particularly since most species in the genus can use nitrate and its reduction products as an alternative electron acceptor to oxygen during anaerobic respiratory growth (except *P. aminovorans*, *P. aminophilus*, and *P. alcaliphilus* [145]). Unifying characteristics of the species include an obligately respiratory mode of growth and the use of ribulose bisphosphate carboxylase/oxygenase to fix carbon during methylotrophic or chemolithotrophic growth. All these organisms are characterized by a high genomic guanine-plus-cytosine (G+C) content (63.8 to 70.2% [145]).

The electron transport chain used for aerobic growth by *P. denitrificans* has long been used as a model for the mitochondrial electron transport chain (137, 280), since it possesses a full complement of proteins with counterparts in mitochondria: electron transport flavoproteins, NADH-ubiquinone oxidoreductase, bc_1 complex, *c*-type cytochromes, and an aa_3-type terminal cytochrome oxidase (Fig. 1). This is in contrast to the usual bacterial model organism, *Escherichia coli*, which does not possess some of these complexes. Branches of the "conventional" electron transport chain (75a) allow the obligately respiratory members of *Paracoccus* to grow under different oxygen concentrations, to use N-oxides as alternative electron acceptors, and to use a variety of carbon sources, including amines and alcohols (Fig. 1). The interest in *P. denitrificans* electron transport has led to the striking achievement of the determination of the crystal structure of the terminal aa_3-type cytochrome *c* oxidase (136). Other redox proteins
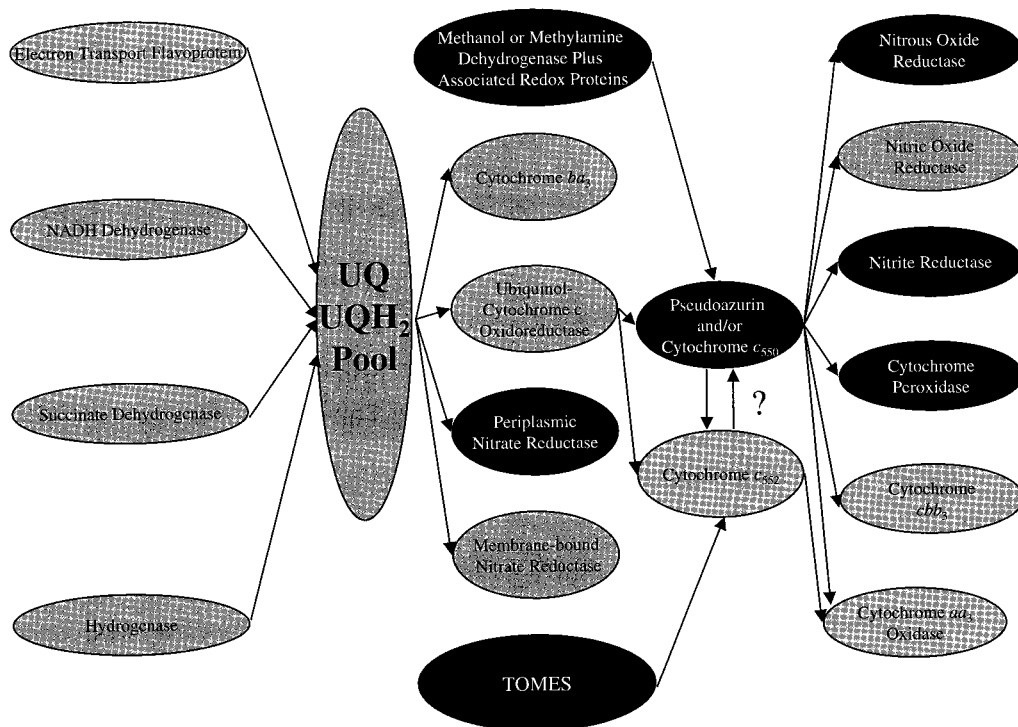
FIG. 1. Branched electron transport chains of *Paracoccus* species. Enzyme complexes colored black indicate a periplasmic location. Electron transfer between cytochromes c_{552} and c_{550} has not been demonstrated experimentally but is possible, given the redox potential of the proteins. The exact nature of the roles of cytochrome c_{550} and pseudoazurin is currently being studied. UQ = ubiquinone, UQH_2 = reduced ubiquinone. TOMES, thiosulfate oxidation multienzyme system.

isolated and structurally characterized from species within the genus include methylamine dehydrogenase, amicyanin, cytochrome c_{551} (45, 46, 73), cytochrome c_{550} (19), pseudoazurin (321), electron transfer flavoprotein (245), and cytochrome cd_1 nitrite reductase (6, 87).

Organisms such as *Paracoccus* species often have to face large fluctuations in the free-oxygen concentration. The adaptive responses of *P. denitrificans* to changing environmental conditions sometimes resemble that of *E. coli*, and parts of the signal transduction cascades appear to be common between these organisms. A typical example concerns the switch from aerobic to nitrate respiration. Optimal synthesis of nitrate reductase in both organisms requires a coordinated reaction to two different types of environmental trigger: the absence of oxygen and the presence of nitrate. The molecular basis for this type of regulation in *E. coli* is now well understood (165, 299). To date, two proteins similar to the fumarate/nitrate respiration (FNR) regulatory protein family (important in *E. coli* for the sensing of oxygen and the induction and repression of several operons) have been discovered in *P. denitrificans*. However, many other types of regulation must occur in order to account for the diversity of electron transport shown in Fig. 1.

The derivation of the strains of *P. denitrificans* from the first strain isolated by Beijerinck has been well reviewed by Goodhew et al. (101), who found that the type strain (ATCC 17741) was a direct subculture of the original strain isolated by Beijerinck. Relatively few new isolates of *P. denitrificans* have been found, and differences between these strains of *P. denitrificans* have been described (101, 199, 312). However, most of the molecular biology has been performed on a single strain, *P. denitrificans* Pd1222. The strains used and the various loci discussed in this review are listed in Table 1. The position of the strain initially named *Thiosphaera pantotropha* (247) has recently been the subject of some controversy. Although Ludwig et al. (178) reclassified *T. pantotropha* as *P. denitrificans*, there is doubt (233) about this reclassification (101, 139, 281). The strain of *P. denitrificans* used by Ludwig et al. itself appears to be not entirely a typical *P. denitrificans* strain as judged by analysis of either *c*-type cytochromes (101) or methyl fatty acids (5). More recently, an extensive survey of various *P. denitrificans* strains, based on a 16S rRNA analysis, has been undertaken (233). The outcome is a proposal to name *Thiosphaera pantotropha* as *Paracoccus pantotrophus*, a species to which several strains of *P. denitrificans* held in culture collections for many years may be transferred. These changes in nomenclature are likely to cause confusion. It is important to note that since 1993 some research groups have continued to use the name *Thiosphaera pantotropha* while others have adopted *P. denitrificans* GB-17. A careful reading of the literature is needed to identify which strain of *P. denitrificans* has been used in a particular study. At the time of writing, the proposal to revive *Thiosphaera pantotropha* under the name *Paracoccus pantotrophus* seems destined for acceptance (233); we have used the name *P. denitrificans* GB-17 in this review. It has been our experience that apart from the necessary considerations that must be taken into account as far as antibiotic resistance are concerned, molecular genetic techniques described for *P. denitrificans* may equally be applied to *P. denitrificans* GB-17 (*P. pantotrophus*). The commonly used *P. denitrificans* Pd1222 is intrinsically resistant to spectinomycin but sensitive to streptomycin, whereas the reverse is true for *P. denitrificans* GB-17 (*P. pantrophus*). The latter is resistant to lead and arsenic, but other *P. denitrificans* strains are not. This review focuses mainly on *P. denitrificans*, the species on which the majority of molecular biological work has been performed: where necessary, we distinguish between strains of *P. denitrifi-*

cans, but note that the species designated *P. denitrificans* GB-17 also has the strain numbers LMD 92.62 and LMD 82.5 in the literature. We would refer the reader to the forthcoming article by Rainey et al. (233) for more information.

The genus *Paracoccus* is member of a part of the alpha *Proteobacteria* known as the *Rhodobacter* group. *Paracoccus* is closely related to the physiologically well-studied photosynthetic species *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, but species of *Rhodovulum* (147), *Sagittula* (100), *Amaricoccus* (184), *Octadecobacter* (103), *Roseobacteria* (78), and *Tetracoccus* (27) are also members of the group.

The molecular biology of the genus has developed considerably since de Vries et al. (68) first obtained a mutant of *P. denitrificans* that was amenable to genetic techniques. The review by Steinrücke and Ludwig (277) considered a number of aspects of the molecular biology of *P. denitrificans*, including a proposed promoter structure (unique to the genus) and related aspects of gene regulation. A considerable amount of new information has become available, which in part does not confirm the earlier proposals regarding promoter structure and otherwise is of general interest in the context of bacterial respiration, for which *P. denitrificans* is a model organism. Thus, a new review is timely. We consider the molecular genetics of the commonly used strains of *P. denitrificans* and, to a lesser extent *P. versutus*, the organisms on which the majority of structural, biochemical, and genetic work has been done.

(The sequences referred to in this review and their annotations are from GenBank 106.0 (released March 1998) and EMBL 54.0 (released March 1998), plus their cumulative updates until 1 May 1998, held at the Oxford University Molecular Biology Data Centre, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, United Kingdom.)

GENETIC COMPOSITION OF *PARACOCCUS*

Relatively little work has been done on *Paracoccus* species to determine the overall genetic makeup of these bacteria, apart from their relatively high G+C content, which has been determined during classification studies (see, e.g., reference 199). However, since 82 loci have been sequenced (listed in Table 1), it is now possible to derive valuable information about the genomics of the genus.

The G+C content of the fragments of the *P. denitrificans* genome that have been sequenced to date is 65.63%. This compares well with the published genomic G+C content of 66.5% (145). The difference most probably arises because sequences submitted to the databases are biased toward coding regions of DNA. The total amount of *P. denitrificans* DNA sequenced (by 31 December 1997) is 166,864 bp, which can be estimated to be about 4% of the total genome (Table 2). This DNA contains 162 open reading frames (ORFs), of which 129 have had a function assigned to them, either by biochemical demonstration or by inference from closely related genes from other organisms. A compilation of this information for *P. versutus* is presented in Table 2.

Within the ORFs from *P. denitrificans* identified so far, there is a bias at the third codon to guanine or cytosine. These nucleotides occur in the third position in 84.03% of codons. The GC bias is also reflected in the frequency at the first (61.13%) but not the second (52.73%) position (Table 3). This codon usage is slightly different from that previously reported (277) because of the larger number of ORFs considered in this study. The additional ORFs included here also contain very rare codons (CTA and TTA for leucine for example), so that all combinations are represented. Hence, the tRNA composi-

TABLE 1. Isolated and characterized *Paracoccus* loci

Strain	Gene cluster	Accession no.	Known functions	Reference(s)
P. denitrificans 71.11	*adk*	U64203	ATP regeneration: adenylate kinase (*adk*)	66
P. denitrificans Pd1222[a]	*hemA*	U12508	Cytochrome *c* biosynthesis: 5-ALA synthase (*hemA*)	214
P. denitrificans Pd1222	*secF-hisH-ccmABCDG-ORF36-ORF117*	Z71971	Cytochrome *c* biosynthesis: secretory apparatus protein (*secF*), histidinol phosphate transaminase (*hisH*)	215
P. denitrificans Pd1222	*ccmF-ccmH*	AF023247	Cytochrome *c* biosynthesis	216
P. denitrificans LMD 92.63[b]	*narGHJI*	Z26255 Z37158	Denitrification: membrane-bound nitrate reductase α subunit (*narG*), β subunit (*narH*), γ subunit (*narI*)	23
P. denitrificans Pd1222	*nirISECFD*	U05002	Denitrification: nitrite reductase transcriptional regulator (*nirI*), structural gene (*nirS*), *S*-adenosyl-L-methionine uroporphyrinogen methyltransferase (*nirE*), small *c*-type cytochrome (*nirC*)	59
P. denitrificans LMD 92.63[b]	*nirSE*	U75413	Denitrification: nitrite reductase structural gene (*nirS*)	5, 6
P. denitrificans Pd1222	*nirIX*	AJ001308	Denitrification: regulation of *nir* gene expression	307
P. denitrificans Pd1222	*norCBQDEF*	U28078	Denitrification: nitric oxide reductase large subunit (*norB*), small subunit (*norC*)	60
P. denitrificans NCIMB 8944	*nosRZD*	X74792	Denitrification: nitrous oxide reductase transcriptional regulator (*nosR*), structural gene (*nosZ*)	125
P. denitrificans[c]	*nosZ*	AF016058	Denitrification: nitrous oxide reductase structural gene (*nosZ*)	255
P. denitrificans Pd1222	*nnr*	U17435	Denitrification: FNR-like transcriptional activator (*nnr*)	309
P. denitrificans LMD 92.63[b]	*pazS*	Z73141	Denitrification: pseudoazurin structural protein (*pazS*)	168
P. denitrificans Pd1222	*cycA-ctaDII*	Y07533	Electron transport: cytochrome c_{550} (*cycA*), iso-cytochrome *c* oxidase subunit II (*ctaDII*)	235
P. denitrificans Pd1222	*cycA-ctaDII*	M27304	Electron transport: cytochrome c_{550} (*cycA*), iso-cytochrome *c* oxidase subunit II (*ctaDII*)	315
P. versutus ATCC 25364	*ORF1-cycA-ctaDII*	X62808 S37058	Electron transport: cytochrome c_{550} (*cycA*), iso-cytochrome *c* oxidase subunit II (*ctaDII*)	296, 297
P. denitrificans Pd1235	*cycM*	X70367	Electron transport: cytochrome c_{552} (*cycM*)	294
P. denitrificans Pd1235	*qoxABCD*	X78196	Electron transport: cytochrome ba_3 quinol oxidase subunit II (*qoxA*), subunit I (*qoxB*), subunit III (*qoxC*), subunit IV (*qoxD*)	244
P. denitrificans ATCC 13543	*fbcFBC*	M17522	Electron transport: cytochrome bc_1 complex iron sulfur subunit (*fbcF*), cytochrome *b* subunit (*fbcB*), cytochrome *c* subunit (*fbcC*)	162
P. denitrificans Pd1222	*ctaDI*	X05829	Electron transport: cytochrome oxidase subunit I (*ctaDI*)	235
P. denitrificans PD 1235	*ctaC*	X05934	Electron transport: cytochrome oxidase subunits II (*ctaC*)	277
P. denitrificans Pd1222	*ctaC-ctaB-ORF1-ctaG-ctaE*	X05828	Electron transport: cytochrome oxidase subunits II (*ctaC*) and III (*ctaE*)	234
P. denitrificans S1657[c]	*ctaE*	X04406	Electron transport: cytochrome oxidase subunit III (*ctaE*)	251
P. denitrificans Pd1222	*ctaH*	Y08372	Electron transport: cytochrome *c* oxidase subunit IV (*ctaH*)	325
P. denitrificans[c]	*phaAB*	D49362	Energy storage: acetoacetyl-CoA reductase (*phaA*), β-ketothiolase (*phaB*)	336
P. denitrificans[c]	*phaC*	D43764	Energy storage: poly (3-hydroxyalkanoate) synthase (*phaC*)	298
P. denitrificans Pd1222	*pta-ORF3-ORF4-ORF1-ORF2-ORF5*	U08864	Insertion sequence IS1248: phosphate acetyltransferase (*pta*)	311
P. denitrificans Pd1222	*ORF3-ORF4-ORF1-ORF2-ORF5*	U08856	Insertion sequence IS*1248b*	312
P. denitrificans Pd1222	*ORF1-ORF2-flhA-clpP-ORF3-fghA-xoxF-cycB-xoxJI-ORF4*	U34346	C_1 metabolism: NAD-GSH-dependent formaldehyde dehydrogenase (*flhA*), *S*-formylglutathione hydrolase (*fghA*), PQQ-dependent dehydrogenase large subunit (*xoxF*), cytochrome c_{553i} (*cycB*)	116, 117, 237, 239, 240

TABLE 1—*Continued*

Strain	Gene cluster	Accession no.	Known functions	Reference(s)
P. denitrificans Pd1222	*ORF1-flhS-ORF2-flhR-abcABC-pqqE*	AJ223460	C_1 metabolism: transcriptional activator proteins (*flhS*, *flhR*), ABC transporter-type proteins (*abcA*, *abcB*, *abcC*), coenzyme PQQ synthesis protein E (*pqqE*)	241
P. denitrificans Pd1222	*mxaF*	M17339	Methanol oxidation: methanol dehydrogenase large subunit (*mxaF*)	114
P. denitrificans Pd1222	*mxaGIJ*	M57684	Methanol oxidation: cytochrome c_{551i} (*mxaG*), methanol dehydrogenase β subunit (*mxaI*)	314
P. denitrificans Pd1222	*ORF1-cycB-ORF2*	M75583	Methanol oxidation: cytochrome c_{553i} (*cycB*)	239
P. denitrificans Pd1222	*mxaZYX*	M92421	Methanol oxidation: two-component regulatory proteins (*mxaX*, *mxaY*)	118
P. denitrificans Pd1222	*ORF1-mxaACKLD-ORF2-ORF3*	AJ000884	Methanol oxidation	242
P. versutus ATCC 25364	*mauB-ORF1*	L08575	Methylamine oxidation: methylamine dehydrogenase α subunit (*mauB*)	130
P. denitrificans Pd1222	*mauB-ami*	X55665	Methylamine oxidation: methylamine dehydrogenase α subunit (*mauB*), amicyanin (*ami*)	317
P. versutus ATCC 25364	*ORF1-mauB-ami*	M58001	Methylamine oxidation: methylamine dehydrogenase α subunit (*mauB*), amicyanin (*ami*)	172, 297
P. denitrificans[c]	*mauDA*	M90098	Methylamine oxidation: methylamine dehydrogenase small subunits (*mauA*, *mauD*)	49
P. denitrificans[c]	*mauFBE*	M90099	Methylamine oxidation: methylamine dehydrogenase small subunits (*mauF*, *mauB*, *mauE*)	48
P. denitrificans Pd1222	*ORF1-mauRFB*	U12464	Methylamine oxidation: LysR-type transcriptional activator (*mauR*), methylamine dehydrogenase large subunit (*mauB*)	313
P. denitrificans Pd1222	*mauED*	X98581	Methylamine oxidation	303, 304
P. versutus ATCC 25364	*mauED*	L36951	Methylamine oxidation	130, 297
P. versutus ATCC 25364	*ORF5-mauF*	L36952	Methylamine oxidation	129
P. versutus ATCC 25364	*mauG*	L36953	Methylamine oxidation	130, 297
P. denitrificans Pd1222	*mauJGMN*	U15028	Methylamine oxidation: ferredoxin-like proteins (*mauM*, *mauN*), cytochrome *c* peroxidase-like protein (*mauG*)	304
P. denitrificans NDH-1[c]	*nqo1*	M64432, J05331	NADH dehydrogenase: 50-kDa subunit (*nqo1*)	332
P. denitrificans NDH-1[c]	*nqo2*	M74171, J05337	NADH dehydrogenase: 25-kDa subunit (*nqo2*)	331
P. denitrificans NDH-1[c]	*URF3-nqo3*	M84572	NADH dehydrogenase: 66-kDa subunit (*nqo3*)	334
P. denitrificans NDH-1[c]	*uvrA-nqo7-nqo6-nqo5-nqo4*	M93015	NADH dehydrogenase: nd3 subunit (*nqo7*), ndhK subunit (*nqo6*), 25-kDa subunit (*nqo5*), 48-kDa subunit (*nqo4*); DNA repair protein (*uvrA*)	333
P. denitrificans NDH-1[c]	*URF4-nqo8nqo9-URF5-URF6-nqo10-nqo11-nqo12-nqo14-birA*	L02354, L01096	NADH dehydrogenase: subunit VIII (*nqo8*), subunit IX (*nqo9*), subunit X (*nqo10*), subunit XI (*nqo11*), subunit XII (*nqo12*), subunit XIV (*nqo14*); biotin (acetyl-CoA carboxylase) ligase (*birA*)	335
P. denitrificans LMD 92.63[b]	*dctM-napEDABC*	Z36773	Nitrate reduction: periplasmic nitrate reductase molybdenum-iron-sulfur-heme subunit (*napA*), diheme subunit (*napB*); Membrane transport: integral membrane subunit of a dicarboxylate transporter (*dctM*)	24, 25, 35
P. versutus	*ORF1-ORF2*	U42228	Plasmid pTAV203	9
P. versutus	*repABC*	U60522	Plasmid pTAV203: replication proteins	8
Paracoccus sp. strain 164	*ori*	AF020624	Plasmid pTM164: origin of replication	266
P. denitrificans 71.11[c]	*secY*	U64202	Protein translocation: preprotein translocase (*secY*)	228
P. denitrificans Pd1222	*ORF1-fnrN-ORF278-ccoNOQPGH*	U34353	Regulation of anaerobic growth: transcriptional regulator (*fnrP*)	63
			Electron transport: alternative oxidase cytochrome cbb_3 monoheme subunit (*ccoN*), diheme subunit (*ccoQ*)	
P. denitrificans[c]	*rrfA*	X01501	Ribosome assembly: 5S rRNA (*rrfA*)	181

TABLE 1—*Continued*

Strain	Gene cluster	Accession no.	Known functions	Reference(s)
Paracoccus sp. strain Y4	*rrnA*	AB012914	Ribosome assembly: 16S rRNA (*rrnA*)	111
P. alcaliphilus JCM 7364	*rrnA*	D32238	Ribosome assembly: 16S rRNA (*rrnA*)	145
P. alkenifer	*rrnA*	Y13827	Ribosome assembly: 16S rRNA (*rrnA*)	170
P. aminophilus JCM 7686	*rrnA*	D32239	Ribosome assembly: 16S rRNA (*rrnA*)	145
P. aminovorans JCM 7685	*rrnA*	D32240	Ribosome assembly: 16S rRNA (*rrnA*)	145
P. denitrificans IAM 12479	*rrnA*	D13480	Ribosome assembly: 16S rRNA (*rrnA*)	146, 147
P. denitrificans LMG 4218[b]	*rrnA*	X69159	Ribosome assembly: 16S rRNA (*rrnA*)	178
P. kocurii JCM 7684	*rrnA*	D32241	Ribosome assembly: 16S rRNA (*rrnA*)	145
P. marcusii	*rrnA*	Y12703	Ribosome assembly: 16S rRNA (*rrnA*)	112
P. solventivorans DSM 6637	*rrnA*	Y07705	Ribosome assembly: 16S rRNA (*rrnA*)	264
P. solventivorans	*rrnA*	Y13826	Ribosome assembly: 16S rRNA (*rrnA*)	170
P. thiocyanatus THIO11	*rrnA*	D32242	Ribosome assembly: 16S rRNA (*rrnA*)	145
P. versutus IAM 12814	*rrnA*	D32243	Ribosome assembly: 16S rRNA (*rrnA*)	145
P. versutus IAM 12815	*rrnA*	D32244	Ribosome assembly: 16S rRNA (*rrnA*)	145
P. denitrificans KS1[b]	*rrnA*	U58015	Ribosome assembly: 16S rRNA (*rrnA*)	139
P. denitrificans KS2[b]	*rrnA*	U58016	Ribosome assembly: 16S rRNA (*rrnA*)	139
P. denitrificans KL1[b]	*rrnA*	U58017	Ribosome assembly: 16S rRNA (*rrnA*)	139
P. denitrificans LMG 4218[b]	*rrlA*	X87287	Ribosome assembly: 23S rRNA (*rrlA*)	179
P. denitrificans 71.11[c]	*rpsM*	U64204	Ribosome assembly: ribosomal protein S13 (*rpsM*)	228
P. denitrificans ATCC 13543[c]	*sdhCDAB*	U31902	Succinate oxidation: succinate dehydrogenase *b*-type cytochrome subunit (*sdhC*), hydrophobic membrane anchor (*sdhD*), flavoprotein subunit (*sdhA*), iron-sulfur protein subunit (*sdhB*)	69
P. denitrificans LMD 82.5[b]	*soxABCDEF*	X79242	Sulfur oxidation: protein B (*soxB*), sulfite oxidase (*soxC*), cytochrome (*soxD*), cytochrome (*soxE*)	326, 327
P. denitrificans Pd1222	*porG*	Y09451	Membrane proteins: porin structural protein (*porG*)	254
P. denitrificans Pd1222	*recA*	U59631	DNA recombination	75
P. denitrificans IFO 12442[c]	*tyrB*	Y08272	Amino acid biosynthesis: aromatic amino acid transferase (*tyrB*)	207

[a] *P. denitrificans* Pd1222 is a derivative of DSM 413.

[b] Now proposed to be *P. pantotrophus* (233).

[c] Classification as *P. denitrificans* or *P. pantotrophus* not yet determined.

tion of *P. denitrificans* cannot be deduced confidently from codon usage. A similar codon bias is also seen in *P. versutus* (data not shown), but guanine or cytosine occurs in the first position in 67.13% of codons. The codon usage tables used in this review (Genetics Computer Group format) are available on request from the authors for both *P. denitrificans* and *P. versutus*.

Megaplasmids and Genomic Structure

Bacterial genomes are generally believed to be a single circular DNA molecule, with the model being *E. coli*. The alpha subgroup of the *Proteobacteria*, a division which includes *Paracoccus*, contains notable exceptions to this: *Rhodobacter sphaeroides* has two circular chromosomes (282), and *Rhizobium meliloti* has three (267). *Agrobacterium tumefaciens* C58 also has two chromosomes, but one is linear and the other is circular (2). The reason why these bacteria have multiple replicons is obscure: although some genes are duplicated (for example, the two copies of the carbon dioxide fixation genes in *R. sphaeroides* on separate chromosomes [93]), genes forming enzymes for a complete pathway are scattered over all the replicons (see http://capsulapedia.uchicago.edu for emerging results on *R. capsulatus*). *P. denitrificans* has also proved to have an unusual genomic structure.

When chromosomal DNA from *P. denitrificans* Pd1222 was separated by pulsed-field gel electrophoresis, it became apparent that the genome consists of three distinct DNA molecules of 1.83, 1.16, and 0.67 Mbp (323), designated molecules I, II and III, respectively. The behavior of the molecules under various electrophoretic conditions suggested that at least the two smaller ones were linear. To determine if the molecules were large plasmids conferring specific properties to *P. denitrificans*, probes to respiratory genes were used to gain an insight into gene distribution. Genes coding for the aa_3-type oxidase were spread between molecules I and II, while ubiqui-

TABLE 2. Information for the ORFs sequenced from
P. denitrificans and *P. versutus*

Characteristic	*P. denitrificans*	*P. versutus*
% G+C from sequence	65.63	64.90
% G+C (experimental)[a]	66.5	66.8
Total DNA sequenced[b]	166,864	16,092
% of genome sequenced[c]	4.17	0.81
No. of ORFs identified	162	19
No. of ORFs (defined function)[d]	129	13
Total length of ORFs (bp)[e]	115,489	11,718

[a] Experimental data taken from reference 145.

[b] The total DNA sequenced includes all known strains of *P. denitrificans*, including strain GB-17.

[c] The total length of both genomes is taken to be 4,000 kbp, the size estimated for *P. denitrificans* by pulsed-field gel electrophoresis (323). The duplicate sequencing of the *cycA* (cytochrome c_{550}) locus has been taken into account, but all other sequences are taken to be unique and nonoverlapping.

[d] "Defined function" is taken to mean either that the gene product has had its biochemical effect identified experimentally or that the gene product has significant identity to a protein from another organism.

[e] The shorter total length of the ORFs compared to the total DNA sequenced does not reflect large intergenic regions in *Paracoccus*; rather, it reflects the fact that sequencing targeted to specific genes leaves regions to the 3′ and 5′ of the loci that are not of sufficient length to have the definition "ORF" applied to them.

TABLE 3. Codon usage in ORFs of *P. denitrificans*[a]

Amino acid	Codon	No.	No./1,000	Fraction	Amino acid	Codon	No.	No./1,000	Fraction
Gly	GGG	155	12.44	0.16	Trp	TGG	320	25.69	1.00
Gly	GGA	50	4.01	0.05	End	TGA	96	7.71	0.87
Gly	GGT	56	4.50	0.06	Cys	TGT	26	2.09	0.12
Gly	GGC	731	58.68	0.74	Cys	TGC	198	15.89	0.88
Glu	GAG	351	28.18	0.61	End	TAG	7	0.56	0.06
Glu	GAA	224	17.98	0.39	End	TAA	7	0.56	0.06
Asp	GAT	126	10.11	0.23	Tyr	TAT	178	14.29	0.57
Asp	GAC	425	34.12	0.77	Tyr	TAC	134	10.76	0.43
Val	GTG	344	27.61	0.46	Leu	TTG	78	6.26	0.07
Val	GTA	11	0.88	0.01	Leu	TTA	4	0.32	<0.01
Val	GTT	36	2.89	0.05	Phe	TTT	53	4.25	0.11
Val	GTC	361	28.98	0.48	Phe	TTC	419	33.64	0.89
Ala	GCG	608	48.81	0.39	Ser	TCG	349	28.02	0.45
Ala	GCA	123	9.87	0.08	Ser	TCA	65	5.22	0.08
Ala	GCT	80	6.42	0.05	Ser	TCT	44	3.53	0.06
Ala	GCC	745	59.81	0.48	Ser	TCC	130	10.44	0.17
Arg	AGG	142	11.40	0.14	Arg	CGG	242	19.43	0.24
Arg	AGA	57	4.58	0.06	Arg	CGA	55	4.42	0.05
Ser	AGT	21	1.69	0.03	Arg	CGT	30	2.41	0.03
Ser	AGC	172	13.81	0.22	Arg	CGC	497	39.90	0.49
Lys	AAG	279	22.40	0.81	Gln	CAG	253	20.31	0.81
Lys	AAA	64	5.14	0.19	Gln	CAA	60	4.82	0.19
Asn	AAT	53	4.25	0.19	His	CAT	118	9.47	0.55
Asn	AAC	229	18.38	0.81	His	CAC	95	7.63	0.45
Met	ATG	401	32.19	1.00	Leu	CTG	790	63.42	0.74
Ile	ATA	14	1.12	0.02	Leu	CTA	4	0.32	<0.01
Ile	ATT	48	3.85	0.08	Leu	CTT	87	6.98	0.08
Ile	ATC	533	42.79	0.90	Leu	CTC	103	8.27	0.10
Thr	ACG	230	18.46	0.32	Pro	CCG	458	36.77	0.54
Thr	ACA	54	4.33	0.07	Pro	CCA	64	5.14	0.07
Thr	ACT	21	1.69	0.03	Pro	CCT	61	4.90	0.07
Thr	ACC	416	33.39	0.58	Pro	CCC	272	21.84	0.32

[a] Including *P. denitrificans* GB-17.

nol oxidase genes were found on molecule III. The genes encoding specific pathways, if transcribed from separate loci, appeared to be randomly distributed: for example, a methanol oxidation gene (*mxa*) was found on molecule I but the cytochrome c_{550} structural gene (*cycA*) and the *S*-formylglutathione hydrolase gene (*fghA*) were found on molecule II. It thus seems likely that these three replicons comprise the *P. denitrificans* genome and will not be replicated independently of one another (323). However, the presence or absence of rRNA genes was not investigated, and so it was not possible to say which, if any, of these molecules were true chromosomes.

The composition of the genomes of other strains and species of *Paracoccus* varies. *P. denitrificans* GB-17 and DSM 65 both possess four DNA molecules of 2.2, 1.5, 0.71, and 0.5 Mbp (323) and are proposed *P. pantotrophus* strains (233). The electrophoretic characteristics of the 0.71-Mbp molecule indicate that this molecule is in closed-circular rather than linear form. Additionally, a much smaller molecule of less than 1 Mbp was seen in some preparations (323). A plasmid (pTAV1) of 107 kbp has been isolated from *P. versutus* and has been used to construct minireplicons (8, 9). *P. versutus* cured of the plasmid retained wild-type growth characteristics, except with respect to cesium and barium resistance (8). A second linear replicon (pTAV2) has also been found in *P. versutus* (201).

Restriction and Modification Systems and the SOS Response

The possession of an efficient means of ameliorating the effects of the introduction of foreign DNA into a cell is an important trait for a microorganism living in environments where mixed cultures occur. However, when these bacteria are transferred from their environment to the laboratory, DNA restriction and modification systems present a problem to the molecular geneticist. Studies of regulation in *P. denitrificans* NCIMB 8944 (traditionally used for biochemical studies) were hampered by the lack of a mutant suitable for the maintenance of plasmids without significant recombination into the genome. Stable inheritance of extrachromosomal material does, however, occur in *P. versutus*, as well as in *P. denitrificans* GB-17. Furthermore, the type culture of *P. denitrificans* (ATCC 17741) will maintain plasmids in the wild-type form of the strain (143).

An undefined *P. denitrificans* *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine chemical mutant (Pd1222) which had a recombination-minus phenotype and an enhanced frequency of conjugation was isolated from DSM 413 (68). The useful property of resistance to rifampin was subsequently introduced, and this antibiotic resistance can be used to select against *E. coli* strains present in bi- or triparental mating experiments. However, the

apparatus for recombination of plasmid DNA with the genome still remained in this mutant, and to manufacture a truly recombinant-deficient organism, Fernandez de Henestrosa et al. (75) isolated and mutated the *recA* gene of *P. denitrificans* Pd1222. This new derivative should prove valuable in future work.

Despite the high identity of *recA* proteins within the *Proteobacteria* (144) and the high identity within the coding regions (*P. denitrificans recA* is 88.6% identical to the *Rhodobacter sphaeroides* gene and 64.3% identical to that from *E. coli* [75]), regulation of *P. denitrificans recA* differed not only from that of *E. coli* but also from that of the phylogenetic near neighbor *R. sphaeroides*. No LexA binding site could be seen in the putative promoter region of *P. denitrificans recA*, but the use of a plasmid containing the promoter translationally fused to a reporter suggested that conditions for repression and activation of the gene in *P. denitrificans* were similar to those required by *E. coli*. Further evidence for differences in the details of control of the *recA* gene in *P. denitrificans* were obtained when the reporter gene was fused to *recA* promoters of *Rhizobium etli*, *R. sphaeroides*, and *R. capsulatus* (75). When these fusions were introduced into *P. denitrificans*, the reporter was induced (on the addition of mitomycin C, which induces the SOS response) only from the *Rhizobium etli* promoter. This was not the expected result in view of the closer phylogenetic relationship of *Paracoccus* and *Rhodobacter* than of *Paracoccus* and *Rhizobium*. Examination of the promoter sequences revealed little similarity between the *Rhodobacter* promoters and that of *Paracoccus*, but the *Rhizobium etli* promoter contained a similar region of dyad symmetry (5′-TTGN$_{10}$CAA-3′ in *P. denitrificans* and in *R. etli*, N = 11). Interruption of this inverted repeat in *Rhizobium etli* led to inactivation of the *recA* promoter (284). It would thus appear that *P. denitrificans* possesses a *recA* system more like that found in the rhizobia than in *Rhodobacter* species.

rRNA Genes

rRNA functions in the assembly of the ribosome but has assumed new significance with the realization that it can be regarded as a molecular clock (204, 329). The 5S, 16S, and 23S genes of *P. denitrificans* have been sequenced (Table 1), as have the 16S genes from all of the other species of *Paracoccus*. Unfortunately, due to the use of thermal polymerase amplification involving primers to conserved sequences within the genes, little information can be obtained about the promoters, which would be expected to be of the σ^{70} RNA polymerase (RNAP) type. The derivation of a consensus ribosome binding site from the 16S rRNA sequence has been discussed previously (277).

The transcript from the 23S gene is unusual in that it seems to be unstable in some preparations when isolated with total RNA from *P. denitrificans* GB-17 (*P. pantotrophus*), appearing on formamide-agarose gels as two smaller molecules (one the same size as 16S rRNA) cleaved at a distinct site (252). This phenomenon has also been noted in *R. capsulatus* (343). Since the integrity of the 16S rRNA transcript is often used as an indicator of the state of degradation of RNA, this may give a misleading result when considering the quality of a *P. denitrificans* GB-17 total RNA preparation. The instability of the 23S rRNA might indicate the presence of an intervening sequence (usually originating from an insertion sequence or other mobile genetic element, appearing as inverted repeats and/or an ORF[s] in the middle of some rRNA genes). Such intervening sequences have been found in several bacteria, including *Salmonella typhimurium*, and result in no apparent intact 23S rRNA in the cell (105).

Insertion Sequences

Bacterial insertion sequence (IS) elements are small, discrete elements of DNA that are integrated into the host genome or, more frequently, into naturally occurring plasmids in bacteria. The coding capacity of these elements is often limited to the synthesis of transposase, the protein which drives the transpositional event and allows the element to jump along the host DNA. The IS element IS*1248*, which was characterized in *P. denitrificans*, belongs to a larger family of elements that are found in strains belonging to different clusters of gram-positive as well as gram-negative bacteria. This family includes IS*869* and IS*427* of *Agrobacterium tumefaciens* (67, 224), IS*402* of *Pseudomonas cepacia* (77), IS*myco* of *Mycobacterium tuberculosis* (183), IS*1106* of *Neisseria meningitidis* (156), Tn*4811* of *Streptomyces lividans* (44), IS*Rm4* and a similar element from *Rhizobium meliloti* (84, 227, 268), and IS*1031* of *Acetobacter xylinum* (50). Trapping of IS*1248* occurred during plasmid transfer experiments with derivatives of suicide vector pRVS3, which appeared to be integrated into the genome via IS*1248*-mediated cointegrate formation (311). The finding that the vector was flanked by identical copies of the transposed IS element as well as of the target site, 5′-CTAG-3′, even suggested that integration had occurred via replicative transposition, an event which is preceded by a staggered cleavage of the IS target site, resulting in duplication of it. IS*1248* is 830 bp long and has 13-bp imperfect inverted repeats at the borders. Two of the five ORFs identified in IS*1248* correspond to counterparts from the other members of this IS family. Since these putative genes have the potential to encode proteins that are hydrophilic overall and have relatively high isoelectric points, they might be the candidates for the transposase function. Two sequences are found in the inverted repeats of IS*1248*, which have been suggested to be involved in the transpositional event. The first sequence, 5′-GANNNNTTGAT-3′, resembles the binding site for the integration host factor, which is involved in stimulation of transposition of a number of IS elements (90). The second sequence, 5′-GNNTCATAA-3′, is identical to that found in related elements and may be a recognition site for their transposases. IS*1248* is present in multiple (four to six) copies in the genome of many strains of *Paracoccus* (312), and the pattern of IS*1248*-hybridizing fragments appeared to be different in *P. denitrificans* Pd1222 and *P. denitrificans* GB-17 (*P. pantotrophus*). IS*1248* is not present in *P. versutus*, suggesting that it invaded *P. denitrificans* after these two species had branched from a common ancestor. This suggestion would support the idea of horizontal gene transfer (312).

Apart from the IS*1248*-mediated integration mechanism, *P. denitrificans* has a second mechanism involved in the integration of heterologous DNA into its genome (312). The result of the latter type of integration is different from that observed for IS*1248*, in that the integrated DNA is not flanked by two identical sequences. Furthermore, the DNA sequences of the donor backbone and the target DNA at the integration site were found to be similar and to resemble the *res* site found in transposons belonging to the Tn*3* family (90, 152). These *res* sites are an essential part of the transposon-mediated site-specific recombination system involved in cointegrate resolution. At least two copies of this integrative element are present in the genome of *P. denitrificans* (312).

Regulation of Transcription in *Paracoccus*

When considering how and when a particular gene from *Paracoccus* is transcribed, researchers find themselves in an unusual position. It is possible to define transcript start sites

TABLE 4. Palindromic sequences present in regions upstream of respiratory genes

Palindrome (consensus)	Sequence	Promoter	Distance relative to:	
			Transcriptional start[a]	Translational start
ACG...CGT	ACGG.CCGT	*fbc*	−22	52
	ACG...CGT	*etf*	NN[b]	90
T.GCA.....TGC.A	T.GCAGC.GCTGC.A	*nqo*	NN	125
	TCGCA.....TGCGA	*ccoN*	NN	45
	TCGCA.....TGCCC	*nap*	NN	11
	T.GCAA...TTGC.G	*ccoN*	NN	192
TCGC...GCGA	TCGCA.TGCGA	*ctaDII*	NN	82
	TCGCC.TGCGA	*cycA*	81	133
	TCGCA.GGCGA	*qox*	44	98
	TCGCT.ACCGA	*qox*	−34	20
TGCC.GGCA	TGCT.GGCA	*ccoG*	NN	79
	TGCC.GGCA	*ccoG*	NN	174
	TGCC.GGCA	*qox*	129	183
CCTGC.....GCAGG	CCTGCCG.CGGCATG	*ctaC*	NN	25
	CCTGC.....GCAGG	*ccoN*	NN	207
ATC....GAT	ATCCCGGTAT	*ctaDI*	NN	42
	ATCCCGAGAT	*ccoG*	NN	272
	ATTCCGGGAT	*ccoN*	NN	151
	ATCG..CGAT	*nap*	NN	294
	ATC.GC.GAT	*cycM*	NN	5
	ATC.GC.GAT	*sdh*	−5	128
TGC......GCA	TGCG....CGCA	*nap*	NN	124
	TGCG....CGCA	*ccoG*	NN	46
	TGC.TTAG.GCA	*fbc*	126	200
	TGC.A..T.GCA	*qox*	5	59

[a] Minus sign indicates that it is located downstream.
[b] NN, not known.

and some regulatory protein binding sites (such as FNR-type proteins [310]) but not to determine where RNAP might bind or even which type of RNAP is effective. Consensus sequences that have been proposed previously (277) for *Paracoccus* are, as discussed below, unsuitable. In the absence of any direct biochemical or genetic evidence for the presence of an RNAP of the σ^{70} type in *Paracoccus*, it is difficult to define clearly the elements of promoters from this genus that may be involved in transcription. The promoter regions that have been sequenced rarely contain the typical −10 or −35 motifs, and workers studying *Paracoccus* frequently note that its promoters rarely function in aerobically grown *E. coli*.

An obligately respiratory organism such as *P. denitrificans* achieves metabolic flexibility by having many alternative electron transport chains. The bacterium must have some overall control of these branched electron transport pathways it possesses: in many cases, the concentration of more than one respiratory enzyme is either elevated or diminished under a particular growth condition, suggesting that a single regulatory protein has pleiotropic control over the expression of their allocated genes. Comparison of the promoter regions in front of the known respiratory genes and gene clusters revealed a number of sequences with a minimum of 8 bases conserved in two or more of the putative promoter regions. Palindromic sequences, which may be probable candidates for binding transcriptional activators or DNA binding proteins, can be selected from these conserved sequences. A list of these sequences is presented in Table 4. Whether these sequences indeed act as regulatory elements is speculative at the moment, but these palindromes do not resemble those noted in *R. capsulatus* (5′-GTGTAART-N$_6$-TTACAC-3′ [1]), nor, in most cases, do they conform to the consensus sequence for *E. coli* transcriptional regulatory factors (5′-TGTGT-N$_{6–10}$-ACACA-3′ [95]).

Promoter structure in *Paracoccus* and the *Rhodobacter* group of the alpha *Proteobacteria*. There has been no further review of *Paracoccus* promoter sequences since Steinrücke and Ludwig (277) deduced a consensus sequence (5′-TCGGGGGN-N$_{(18 \pm 2)}$-GATNGS-3′) based on promoters from *Paracoccus*, *Rhodobacter*, and *Bradyrhizobium*. Surprisingly, little attention has been paid in general to promoters in the alpha-*Proteobacteria*, the division of the *Eubacteria* to which *Paracoccus* belongs. Although alternative polymerases (e.g., RpoN [37] of *R. capsulatus*) have been isolated, purified, and characterized, work on the binding of housekeeping holopolymerase to constitutively induced promoters is just beginning (54, 180), with *Rhodobacter* as the model organism. However, alignment of *Paracoccus* and *Rhodobacter* promoters (Table 5) indicates that most constitutive promoters have some sequences in common.

Few genes from *Paracoccus* have had their transcription start sites determined, an essential step in defining the exact position of possible promoter sequences. The start sites for *qoxA* (quinol oxidase [346]), *sdhC* (succinate dehydrogenase [69]), *ctaC* (cytochrome oxidase [277]), *fbc* (*bc$_1$* complex [277]), *porG* (porin [254]), *pazS* (pseudoazurin [168]), and *cycA* (cytochrome *c$_{550}$* [279]) have been published, and determination of the *nir* genes is in progress (5). Of these, one would expect the promoters for succinate dehydrogenase, cytochrome oxidase, *bc$_1$* complex, quinol oxidase, and porin to be under the control of a housekeeping polymerase, in that they are constitutively produced. They all have an unusual base usage biased towards A and T in the 40 bp upstream of the transcript start site (41 to 60% G+C, compared with 66.5% G+C for the whole genome), the expected promoter location. A · T base pairing in the −10 (TATAAT) and −35 (TTGACA) regions of the model *E. coli* σ^{70}-dependent promoter is thought to con-

TABLE 5. Promoters from the *Rhodobacter* group of the alpha *Proteobacteria*

Strain/promoter[a]	Promoter region[b]			Reference(s)
Ec/consensus[a]	TTGACA	N$_{15-19}$	TATAAT	120
Group A				
Rc/bchC	ATCAAA<u>TTGACA</u>GTCGGGCGTGTAAGTTC<u>AATGAT</u>ACACACAGG			54
Rs/groE	CCCCCG<u>TTGACA</u>GGTGCCGGACGCTCTCA<u>TATCTCT</u>CGCGTCG			166
Pd/qoxA*	<u>TTGACC</u>TAGATCAAGGTAATTCACCCCGCCATGTTGC			346
Rc/radC	GAGATC<u>TTGACA</u>GAGGCGCAGCCGGTTGCCG<u>TCTCTT</u>CCGACC			197
Rs/trxA	GACAAC<u>TTGACG</u>CGGCAGGGGGCCATCCT<u>TACTTT</u>CCAAGCA			222
Rc/recA	ATTTGA<u>TTGCA</u>ATGTTCCGCAATTGTACTT<u>CATACC</u>ATGAGAA			76
Rc/cpeA	ATGCAA<u>TTGGA</u>ATTTATCAAAATCGCCTTC<u>TAGTTT</u>CAATCGC			81
Rc/puc	TTACAC<u>TTGATC</u>GCCGACACTTGGGCTCC<u>CATAGT</u>GCGTCTCA			54
Rc/fdxA	CTGCTC<u>TTGATT</u>GATCGCCCCCGGAGGGC<u>TAGGACA</u>TCCC			72, 248
Rc/atpH	TGG<u>TTGCGA</u>GGGTCTTGATGCTCTGC<u>TAGACGC</u>AACCCCCGA			31
Rs/hupS	TTCTCA<u>TTGGCG</u>GAAACCGGCCCAACCATG<u>AGAATT</u>CCACTCTA			288
Rs/coxII	TTTTCC<u>TTGTCGT</u>CGCCAAAACAATATGG<u>TCTCAAT</u>CGGTTCA			79
Rc/lepB	TTGACT<u>TTGCCC</u>CCGCATCGGCCAAGGAA<u>GACAAGG</u>TTTCAAC			154
Rc/dnaK	CAGTTC<u>TTGCAG</u>GGCTATTTTCCCCTCCT<u>TATATAC</u>GCCGG			198
Rs/rrnB	ATCCGC<u>TTGCGC</u>CCGGGGCCGTCTGCTCC<u>TAGAAAC</u>CGCTTC			71
Rs/rrnA	TTCCTC<u>TTGCGGG</u>TTTTTTTGCGGTTCCC<u>TAGATAG</u>CGCCTC			71
Rs/rrnC	TTCCTC<u>TTGCGGG</u>TTTTTTTGCGGTTCCC<u>TAGATAG</u>CGCCTC			71
Rc/rpoD	TTTGA<u>TTCGCC</u>CCTGTGATTCGCCGCCGTGA<u>TTCGAT</u>AAAAC			223
Group B				
Pd/sdhC	ATGTGATCACAGCTGCCGTTTGCGTGATCAC<u>AAAAAT</u>GGAAA			69
Rc/porA	GGCCGCTTCGGTCCCGTTTTCGTTGCAA<u>TATGAGG</u>CGTGG			289
Rs/cycF	TTCCAGTATGTCTGTCGCGACCGGCGGGC<u>TAGATTT</u>CCGGGA			79, 80
Rs/norC	CCCGCAACCTCTCCGGCGCCGGCGCGGGC<u>TAGAGG</u>AGGTCCA			7
Pd/ctaC*	GATAGGTATGGCTTGCCGCCGGGGTAAGA<u>TATGGTT</u>CTGGTG			277
Pd/fbc*	CCGCTGGACTGACGGGGATTTGATCGC<u>TAGAAC</u>CGCGC			277
Pd/porG*	ATGCGGGGGGGAAAGCGCGACACCACTTG<u>CATACCC</u>CAAAC			254
Rc/pufG	CTCTGGACCGGATCGTGTCGCAACACCCGGT<u>TCTGAC</u>ACGGA			11
Rc/pufQ	CGCGCGACGGGCACCCCCTTCATGG<u>TTACAT</u>GGGTA			54
Rs/chrR1	GCCTGATCCAGACTGGCCCGGCCGCCG<u>TAAGAAGG</u>ACGTTA			257
Rs/chrR2	GCGGATGCAAGCCGGGGAGGGTTTCC<u>TATCTT</u>CACCTCCGG			257
Rc/cycA	ATATGCGACCTTTTGCCTTGTGG<u>TAAAGC</u>GTGGACCG			54, 70
Pd/pazS	TTCTCAAGCCGAATGCCCTGTACCGGACC<u>TAAACC</u>AGGCACG			168
Group C				
Pd/cycA1	CCTGTATTCTGCCCGCTTGGCACATGATAGCCCTGTCAATCG			279
Pd/cycA2	TCTGCCCGCTTGGCACATGATAGCCCTGTCAATCGGGGAAGCG			279
Rc/fruB	TTTTCGCGCGGAACTCTGCCCCATGGGCGATGGCCG			54
Rc/xdhA	TTCAACGCGCCGCGGGCCGAAATCCTTGCCAGCGCCAAAGCC			167
Rs/crtA	GGGCGGACATTAGTCGCGAAGTCGCACCGCCCTCAAGCCGCA			164
Rs/ctaD	GGAACAAGACCGGCGTCTACAGATATCCGGGAGATGCTGGCA			79
Rs/cycA	CCGGAACGCGCGGCCCGCAGTAGTGATTGTGTGCCGGCGGCA			70
Rs/fbcA	GCGCCGCAAGATCGAGCCCGACCCGCGCGAGCCGCGCTACCT			89
Rs/nirK	CGCAAACTCCGCCTCTCCAGAGGATCTACCGATCGGGTCAT			7
Rs/pucT	GAATCTGTCAGCGCAATGTGACACCCATAATGCGAGCCGGGG			94
Rs/pufG	ATCCGCCGCGCGACGGGCACCCCCTTCATGGGTTACATGGGT			151

[a] All promoters have had their transcript start sites (the last base in each sequence) determined by primer extension or S1 nuclease (*) assays. Group A consists of promoters possessing either or both of the −10 and −35 hexamers and are ranked according to the identity of the −35 region to that of the *E. coli* consensus. Group B consists of promoters with a −10 region 5 to 9 bp from the transcript start site but no −35 region. The promoters in group C have RNAP binding sites that cannot be identified easily, but this does not necessarily disqualify them from σ[70] RNA polymerase dependency. *R. capsulatus glnB, nifA1,* and *nifA2* promoters are not included because they have been shown to be RpoN-dependent (82). *Pd, Paracoccus denitrificans; Rc, Rhodobacter sphaeroides; Rs, Rhodobacter capsulatus; Ec, E. coli.*
[b] Underlined hexamers have 50% or more identity to the appropriate *E. coli* consensus.

tribute to local melting of the double helix, allowing holo RNAP to function (reference 36 and references therein).

Since comparatively few *Paracoccus* promoters have been thoroughly characterized, little can be deduced if they are considered in isolation. However, if the reasonable assumption is made that transcription in closely related bacteria will be very similar, results obtained within the *Rhodobacter* group (essentially *R. capsulatus* and *R. sphaeroides* as well as *P. denitrificans*) can also be considered. Although this allows the study of 45 promoters, the majority of these are from genes which are considered to be highly regulated. Data for more promot-

ers from genes with housekeeping functions would provide more information for derivation of a consensus for the equivalent of the σ[70] RNAP binding site. However, alignment of the promoters with respect to their transcript start sites does reveal sequences in some promoters that resemble those of the *E. coli* σ[70] consensus sequence.

Although studies of mutants with site-directed mutations of the *bch* operon of *R. capsulatus* (180) indicated that transcription was dependent on bases at −10 and −35, no promoter yet characterized from *Rhodobacter* or *Paracoccus* has sequences (Table 5) that conform exactly to the canonical *E. coli* σ[70]

motif (5′-TTGACA-N$_{(15–19)}$-TATAAT-N$_{(5–9)}$-3′, first derived by Hawley and McClure [120]). In vitro and in vivo studies of five *R. capsulatus* σ^{70}-dependent promoters (54) have led to a loose definition of the likely housekeeping holopolymerase binding sites: the −35 hexamer was found to be TTGACN, and the −10 motif was of such variable composition that "AT rich" was a sufficient description (54). Examination of the *R. sphaeroides rrn* operon promoters again revealed −35 regions resembling those of *E. coli* (Table 5) but found less similarity in the −10 region (70): it could have been expected that the *rrn* operons would have strong promoters conforming to the consensus σ^{70} for the genus.

Alignment of the promoters from the *Rhodobacter* group still gives no clear picture of a consensus sequence (Table 5). The promoters fall into three groups: those with a sequence at −35 with 50% or more identity to the *E. coli* consensus; those with a sequence at −10 with 50% or more identity; and those that have polymerase binding sites which are not easily identifiable from sequence data alone. Most of the *Paracoccus* promoters fall into one of the first two categories, but, surprisingly, neither of the *cycA* promoters (279) can be included in these groups. Although the influence of σ^{70} cannot be ruled out completely, since footprinting studies have not been performed, it has been demonstrated that *cycA* is transcribed under all the conditions tested (279, 315), including aerobic or anaerobic growth on succinate and aerobic growth on methanol or methylamine. Given the results obtained with *R. capsulatus* purified σ^{70} (54), it seems likely that *qoxA* is transcribed with the aid of this sigma factor, since the hexamer TTGACC appears 35 bp upstream of the transcription start site (Table 5). However, the definite assignment as "σ^{70} dependent" to the *Paracoccus* promoters that have a −10 hexamer alone is questionable. The constitutive expression of succinate dehydrogenase, cytochrome oxidase, the bc_1 complex, and porin has been experimentally determined, but the variation in composition and the position of candidate −10 hexamers show that the sequence results are only a very preliminary indication.

Considering all the promoters aligned in Table 5, the shortcomings of a sequence-based approach are illustrated. For example, the *R. sphaeroides ctaD* (cytochrome oxidase) gene has no clear −10 or −35 sequences, yet *P. denitrificans ctaC* has a −10 hexamer 67% identical to that of the *E. coli* consensus. However, the alignment does show that TTG in the −35 hexamer is a common characteristic of *Rhodobacter* group promoters. Because it is so difficult to identify candidate promoter regions, we suggest that definition of polymerase binding sites should be attempted only if the promoter/operator has been mapped by either S1 nuclease protection assay or primer extension experiments.

Even though it is not yet possible to derive a consensus sequence for a *Paracoccus* promoter, there are indications that a σ^{70}-type RNAP is present in the cell. The best evidence for this in *P. denitrificans* comes from studies with a broad-host-range vector containing the promoter of bacteriophage T4 gene 32 fused to *xylE* (85). After construction of the vector in *E. coli*, the plasmid was introduced by conjugation into *Agrobacterium*, *Erwinia*, *Xanthomonas*, *Pseudomonas*, and *Paracoccus* species and the transcription of the fusion was studied in comparison with that in *E. coli*. The transcription start site of the plasmid-borne fusion was identical in all the genera studied and was found to be downstream of hexamers resembling a σ^{70} promoter. In addition, the resulting mRNA molecules were subject to posttranscriptional modification and were processed in a similar manner. Although it could be argued, in the absence of σ^{70} footprinting studies in each strain, that the transcript arose from fortuitous promoters, the

exact coincidence of the transcript start sites is indicative of a common core polymerase subunit.

Although the promoters in the *Rhodobacter* group appear to be anomalous compared to the canonical σ^{70} promoter, recent work (106) has suggested that all the eubacteria have a very similar principal sigma factor. Examination of the variation in sigma factor protein sequences suggests that all housekeeping polymerase-dependent promoters have DNA sequences at −10 and −35 which bear some resemblance to the classical binding sites proposed by Hawley and McClure (120). The differences exhibited by the promoters in Table 5 could be explained by the interaction of polymerases with other unknown protein factors, rather than some intrinsic difference in the holopolymerase itself. The requirement for other factors may go some way to explaining the inability of *E. coli* to initiate transcription from the *Paracoccus* promoters tested so far.

Termination of transcription. To date, no direct experimental evidence exists for any termination event in *Paracoccus*. However, mRNA analysis and other indirect evidence suggests that *Paracoccus* possesses both factor-dependent and factor-independent pathways for termination of transcription. A truncated form of the Rho-dependent terminator gene (*rho′*) of *R. sphaeroides* 2.4.1 was lethal in the wild-type organism but partially interfered with the transcription termination machinery of *E. coli*. When *rho′* was introduced into *P. denitrificans* ATCC 17741, the construct was also found to be toxic (99). This suggests not only that a Rho-like system exists in the genus *Paracoccus* but also that the mechanism of termination is the same for *R. sphaeroides* and perhaps that the structure of Rho in these organisms is similar as well.

Some of the stem-loop structures found downstream of genes such as *cycH* (involved in cytochrome *c* biogenesis [215]) conform to the classical factor-independent structure (a stem-loop followed at the 3′ end by an AT-rich region). The run of A or T is particularly noticeable against the GC-rich *Paracoccus* DNA, but it includes some guanine and cytosine residues. A similar factor-independent termination site can be proposed when considering the region between *nirS* (cytochrome cd_1 nitrite reductase) and *nirE* (a putative methyl transferase) of *P. denitrificans* (59). Studies involving Northern blotting indicate that this terminator is functional during denitrifying growth in *P. denitrificans* (252).

Many transcriptional terminators have been deduced from putative stem-loop structures in DNA sequences. Stem-loops with highly negative Gibbs free energy can be found within one of the methanol oxidation operons (*mxaFJGIR*). However, these structures may equally confer stability to mRNA, forming the sort of stem-loop structures found in the *puf* operon of *R. capsulatus* (121).

In summary, the genus *Paracoccus* contains bacteria with multiple replicons. Genes and insertion sequences are distributed among these replicons, and to date no megaplasmid has been assigned a specific function. Despite a growing amount of sequence data and analysis, no obvious consensus promoter sequence is immediately apparent by simple inspection. This has led to the idea that *P. denitrificans*, along with the members of the alpha *Proteobacteria*, has very different promoter regions from those in *E. coli*. However, careful analysis of a variety of available data suggests that at least for some of the genes there is similarity to typical σ^{70} hexameric sequences. It also seems probable that there are novel regulation mechanisms (e.g., involving different regulators or sigma factors) yet to be discovered in *P. denitrificans* and its relatives.

TABLE 6. Well-characterized *Paracoccus* *c*-type cytochromes

Cytochrome *c*	Mol mass (kDa)	No. of *c* heme centers	Gene	Role or probable role
Cytochrome c_{550}	14	1	*cycA*	General periplasmic electron carrier
Cytochrome c_1	45^a	1	*fbcC*	Component of bc_1 complex
Cytochrome c_{552}	22	1	*cycM*	Electron donor to aa_3-type cytochrome oxidase
Cytochrome c_{551i}	18	1	*mxaG*	Electron acceptor for MDH
Cytochrome c_{553i}	23	1	*cycB*	Putative electron carrier during methylotrophic growth
Cytochrome c_{551} (putative)	40	2	*soxD*	Electron transport during growth on thiosulfate
Cytochrome c'	12	1		Unknown
Cytochrome *c* peroxidase	42	2		Removal of hydrogen peroxide from the periplasm
Cytochrome cd_1	65	1	*nirS*	Nitrite reductase
CcoO	30	1	*ccoO*	Component of cbb_3-type cytochrome oxidase
CcoP	45	2	*ccoP*	Component of cbb_3-type cytochrome oxidase
MauG	40	2	*mauG*	Synthesis of prosthetic group of MADH
NapB	15	2	*napB*	Component of periplasmic nitrate reductase
NapC	27	4	*napC*	NirT homologue, electron donor to periplasmic nitrate reductase
NirC	10	1	*nirC*	Unknown
NorC	14	1	*norC*	Component of nitric oxide reductase
SoxE	26	1	*soxE*	Electron transport during growth on thiosulfate

^{a} Cytochrome c_1 migrates with an apparent molecular mass of 60 to 68 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

REGULATION OF RESPIRATORY GENES

A number of respiratory systems and their underlying biosynthesis genes from *Paracoccus* have been characterized by molecular biological methods. The regulation of these genes has some features in common with the regulation of the genes in the more intensively studied organisms such as *E. coli*. The loci sequenced, their accession numbers, and the strain of origin are listed in Table 1. The respiratory pathways of *Paracoccus* are dependent on many metalloproteins, the best characterized of which are *c*-type cytochromes (Table 6). Although the biochemical mechanisms by which these proteins are synthesized are only just becoming understood, *P. denitrificans* has proved to be a good model organism for these studies, producing *c*-type cytochromes aerobically as well as under oxygen limitation.

Cytochrome *c* Biogenesis

Cytochromes *c* are distinguished from cytochromes of other classes by covalent attachment of the heme moiety to the cytochrome polypeptide via thioether links between the two protoporphyrin IX vinyl groups and the thiol groups of two cysteine residues in the conserved motif Cys-X-Y-Cys-His. The process of *c*-type cytochrome biosynthesis thus includes posttranslational modification of the apocytochrome polypeptide. A number of lines of evidence indicate that in gram-negative bacteria this process takes place in the periplasm, although this has yet to be rigorously demonstrated experimentally. Eight genes required for *c*-type cytochrome maturation have now been identified in *P. denitrificans*; all are clearly homologous to genes found in a number of other gram-negative bacteria including *Bradyrhizobium japonicum*, *R. capsulatus*, and *E. coli* (for comprehensive reviews, see references 218 and 285). The organization of the known *P. denitrificans* *c*-type cytochrome biosynthetic genes resembles that in *R. capsulatus*, in that they are distributed over at least three loci, but differs from that in *E. coli*, in which genes are clustered at a single locus (*ccmAB-CDEFGH*), and in the *Rhizobiaceae*, in which the genes are present at two loci (*cycHJKL* and *cycVWZXY*). Southern blotting of a cosmid library suggests that the three loci are separated by at least 20 kbp in the *P. denitrificans* genome (200). The *E. coli* nomenclature has been adopted for the *P. denitrificans* genes; an exception is *cycH*, which has no clear equivalent in *E. coli* (although it exhibits some similarity to the C-terminal region of *ccmH*). No gene corresponding to *ccmE*/*cycJ* has been identified in *P. denitrificans* but has for *R. capsulatus* (see http://capsulapedia.uchicago.edu).

ccmA, ccmB, ccmC, ccmD, and ccmG. *ccmA*, *ccmB*, and *ccmC* appear to encode the components of a membrane transporter of the ABC (ATP-binding cassette) superfamily. The corresponding hypothetical transporters in *B. japonicum* and *R. capsulatus* have been suggested to translocate heme or apocytochromes to the periplasm; however, sequence analysis indicates no similarity between CcmB and CcmC (or their homologues) and the membrane-integral components of transporters mediating the uptake of heme or other iron complexes. Supplementation of growth media with heme did not stimulate *c*-type cytochrome formation in mutants disrupted in *ccmA* or *ccmB*, although it elevated the levels of soluble hemoproteins and membrane-bound cytochromes *b*, suggesting that exogenous heme can traverse both outer and inner membranes in *P. denitrificans*. Expression of an apocytochrome c_{550}-alkaline phosphatase fusion protein and of apocytochrome cd_1 was unaffected in a *ccmB*::Tn5 mutant. These results suggest that the substrate for the putative CcmABC transporter may be neither heme nor *c*-type apocytochromes (217).

CcmD is predicted to comprise a single membrane-spanning α-helix and a small (about 30 amino acids) cytoplasmically oriented hydrophilic domain. Its function is obscure. The *ccmG* gene encodes a soluble periplasmic thioredoxin-like protein; disruption of *ccmG* (*P. denitrificans* DP307; *ccmG*::Ω) not only abolished *c*-type cytochrome biogenesis but also almost completely eliminated assembly of the aa_3-type cytochrome oxidase and rendered the mutant strain incapable of growth on rich media such as Luria-Bertani medium (215). Dithiothreitol promoted the growth of DP307 on rich media and substantially restored assembly of the aa_3-type cytochrome oxidase, although it did not restore *c*-type cytochrome biogenesis. Assembly of the disulfide-bridged proteins methanol dehydrogenase and *E. coli* alkaline phosphatase was unaffected in DP307. CcmG is proposed to act in vivo to reduce disulfide bonds in certain protein substrates including *c*-type cytochrome polypeptides and/or polypeptides involved in *c*-type cytochrome biogenesis (216).

cycH. Disruption of *cycH* (*ccmI* has also been suggested as a suitable name [218]) results in loss of soluble *c*-type cyto-

chromes, but low levels of membrane cytochromes *c* (estimated at 5 to 10% of wild-type levels) remain. Thus, CycH is not absolutely required for *c*-type cytochrome assembly in *P. denitrificans*, but it clearly increases the efficiency of the process manyfold (215). Analysis of a *cycH-lacZ* fusion indicates that it is expressed during aerobic growth but is induced fourfold under anaerobic growth conditions and that this induction is mediated by the transcriptional activator FnrP but not by the closely related protein Nnr (210). FnrP and Nnr are discussed further in the context of the regulation of denitrification (see below).

***ccmF* and *ccmH*.** The *P. denitrificans ccmF* and *ccmH* have recently been established (225). CcmF is predicted to be a membrane-integral protein with 11 or more membrane-spanning α-helices, and, as such, it is potentially a transporter; however, supplementation of growth media with heme did not stimulate *c*-type cytochrome formation in a mutant disrupted in *ccmF*. CcmH has a Cys-X-X-Cys motif and thus may be a protein-disulfide oxidoreductase, but a *ccmH* mutant has yet to be constructed and characterized.

***hemA*.** While not sensu stricto a *c*-type cytochrome biogenesis gene, the *P. denitrificans hemA* gene (coding for 5-aminolevulinic acid [5-ALA] synthase) was identified during screening for mutants defective in *c*-type cytochrome assembly. A transposon mutant in which Tn*5::phoA* had integrated in the *hemA* promoter region, reducing but not eliminating *hemA* expression, was obtained. This had the effect of reducing the levels of *a*- and *b*-type cytochromes and membrane-bound *c*-type cytochromes in the mutant strain to about 50% of those in Pd1222 and virtually eliminating the formation of soluble periplasmic cytochromes *c*. Disruption of the *hemA* structural gene led to 5-ALA auxotrophy, indicating that *P. denitrificans*, like *R. capsulatus* but unlike *R. sphaeroides* (127, 195), possesses only one 5-ALA synthase (confirmed by Southern blotting) and that no 5-ALA synthase-independent route of 5-ALA synthesis exists in *P. denitrificans* (214).

Genes of Oxygen Respiration

All species of *Paracoccus* are obligately respiratory and have no pathways that allow them a fermentative mode of growth. Although *P. denitrificans* is noted for the similarity of its aerobic electron transport chain to that of mitochondria, it differs from them in that it uses alternative terminal oxidases depending on the aerobic state of the immediate environment. The genes of oxygen respiration must be regulated in response to oxygen, up to the point of anaerobiosis. In this scenario, nitrate (if present in sufficient concentration) may act as an alternative electron acceptor to oxygen.

NADH-ubiquinone oxidoreductase. The NADH-ubiquinone oxidoreductase holoenzyme from *P. denitrificans* is thought to contain at least 14 subunits, whereas that from mitochondria is considerably more complex, with 28 additional subunits (126, 258). Despite the difference in subunit composition, the function of the two enzymes is the same and there is considerable protein sequence homology between equivalent subunits (331, 332). Therefore, the *Paracoccus* proteins are named after their mitochondrial counterparts (Nqo1, Nqo2 etc.). The genes coding for these subunits are found in an operon between an ORF possibly coding for the *Paracoccus* UvrA (a DNA repair enzyme), and another gene (ORF240) similar to *birA* (biotin [acetyl coenzyme A (CoA) carboxylase] ligase). *P. denitrificans* UvrA has 74 and 71% identity to the equivalent *E. coli* (131) and *Micrococcus luteus* (263) proteins, respectively, while *P. denitrificans* BirA is 31% identical to the equivalent *E. coli* protein. The proposal that a bacterium such as *Paracoccus* is

the forerunner of the eukaryotic mitochondrion (137) receives little support from the gene order of this *nqo* operon: the arrangement of the genes is more similar to that of chloroplasts (e.g., liverwort [202]) than to that of the bovine mitochondrion.

The region upstream of the initiation codon of *nqo7* (the first subunit to be transcribed), is rich in long inverted repeats. However, there has been no attempt to define the base pair composition of a promoter. Termination appears to be Rho independent, probably occurring at a potential stem-loop structure between *nqo14* and *birA*. A second inverted repeat is found within the coding region of *birA*, but it is more likely that this serves to stabilize *birA* mRNA than that it is a duplicated terminator.

Although 14 subunits of the NADH-ubiquinone oxidoreductase have had genes assigned to them (331–335), three ORFs within the operon are still of unknown function. N-terminal sequencing of the subunits of the holoenzyme has not revealed sequences similar to those of the putative products of the translated ORFs, and no homology to these unknown proteins can be found in the databases. Steinrücke and Ludwig (277) note that URF2 is similar to *ctaG* of the *P. denitrificans* cytochrome oxidase operon (275, 331, 332, 334).

Succinate dehydrogenase. Succinate dehydrogenase of *P. denitrificans* has been purified (226) and shown to have four subunits, and the genes for these subunits appear to be in an operon (69). The enzyme contains covalently bound flavin, iron-sulfur centers, and cytochrome *b*, thus showing considerable amino acid sequence similarity to its mitochondrial counterpart. The promoter of the *P. denitrificans sdhCDAB* operon has been characterized (69), but sequence analysis alone does not provide much information on this constitutively expressed cluster (160, 226). The presence of σ^{70} hexamers at -10 with respect to the transcription start site (Table 5) suggests that the genes are transcribed with a σ^{70}-like RNA polymerase.

The cytochrome bc_1 complex. After an early report on the purification and characterization of cytochrome c_1 from *P. denitrificans* as a polypeptide with an unusually high molecular mass (177), the bc_1 complex was isolated initially as a "super-complex" along with cytochrome *c* oxidase and a membrane-bound cytochrome c_{552}, yielding high quinol-oxidizing activity (26). Subsequently, its subunit composition was confirmed unequivocally (340), showing that only the three subunits carrying redox centers make up a complex that is also fully competent in free energy transduction (341). The cloning of the corresponding genes (162) revealed an operon structure, *fbcFBC*, coding for the Rieske FeS subunit, the cytochrome *b*, and the cytochrome c_1 subunit. The latter is unique in having an additional N-terminal domain of around 150 amino acids (compared to the eukaryotic mitochondrial proteins, explaining the higher molecular weight of the *P. denitrificans* protein), with a characteristic composition (40% alanine, 38% acidic residues, and no basic residues [162]); its function is still not understood. While cytochrome *b* shows an amazingly high degree of sequence identity to other bacterial and mitochondrial subunits (see references 155, 285, and 290 for reviews), the existence of an additional transmembrane helix in the N terminus of the protein has been suggested (153) on the basis of using monoclonal antibody fragments in conjunction with electron microscopy. Gene and operon deletion studies, as well as expression of the *fbc* operon from a multicopy plasmid (92), resulted in a considerable overexpression of the complex in the homologous host. Once again, an *E. coli* σ^{70}-like -10 region (TAGAAC; Table 5) can be found in the promoter region.

Cytochrome aa_3. *P. denitrificans* can use several terminal electron acceptors during aerobic growth, the best character-
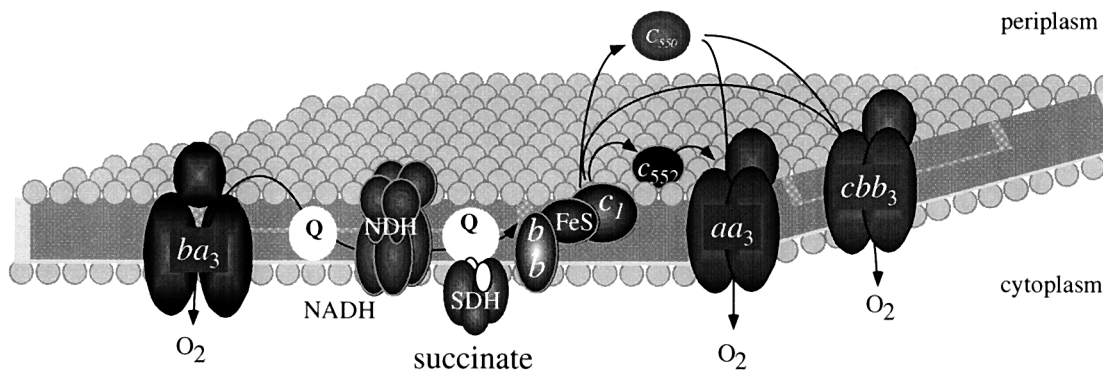
FIG. 2. Branched electron transport pathway of *P. denitrificans* under various conditions of oxygen limitation. FeS, iron-sulfur center; NDH, NADH-ubiquinone oxidoreductase; SDH, succinate dehydrogenase; Q, ubiquinone pool.

ized of which is the aa_3-type cytochrome c oxidase (cytochrome aa_3). The biochemistry of this multisubunit enzyme has been reviewed extensively (62, 88, 109, 206, 291). The structure of the holoenzyme has been determined to a resolution of 2.8 Å by X-ray crystallography (136).

The cytochrome oxidase of *P. denitrificans* is a four-subunit enzyme (136). Three of these subunits have eukaryotic equivalents encoded mitochondrially, but the mitochondrial holoenzyme has an additional 10 subunits which are coded for in the nucleus (38, 291). Initially, only two subunits could be isolated from *P. denitrificans* (176). By use of labeled oligonucleotides, designed to hybridize to the conserved regions of the mitochondrially encoded subunit 3 of aa_3, Raitio et al. (234) demonstrated that *P. denitrificans* possessed an equivalent polypeptide. Subunit I contains a heme a and a heme a_3 prosthetic group, which, together with Cu_B, forms the active site. Subunit I is transcribed from the *ctaDII* gene, which was cloned independently by Raitio et al. (235) and van Spanning et al. (315). The product of this gene is a constituent of the aa_3 complex under physiological conditions, although an isogene (*ctaDI*) exists. The isogenes have nearly 90% identity (235) and are found in different loci on the *P. denitrificans* genome. The *ctaDI* gene can be expressed in *P. denitrificans* only if it is maintained on a plasmid, possibly due to a gene dosage or repressor titration effect.

The genes coding for cytochrome oxidase subunits II and III (208, 234, 278) are found in an operon, *ctaCB*-ORF1-*ctaGE*. CtaC (subunit II) contains a copper A center, which is the entry point of electrons to the aa_3 holoenzyme (123), although alternative electron pathways have been proposed. The *ctaE* gene codes for subunit III, an integral membrane protein of unknown function (108). The two remaining *cta* genes in the operon code for enzymes involved in posttranslational processing of subunit I (276, 278). CtaB catalyzes the conversion of heme b to heme o (91). The insertion of heme has been postulated to be mediated by CtaG (276). The function of the ORF found in the operon is currently unknown. The promoter region of the *ctaCB*-ORF1-*ctaGE* operon has been examined in some detail. The transcription start site was mapped with S1 nuclease (278) and was found to be 34 bp upstream of the translation initiation codon. Although there is a clearly discernible −10 region (that might indicate binding of a σ^{70} RNA polymerase), when the operon was expressed in *E. coli*, it was thought that initiation of transcription originated in the plasmid vector used, rather than from the native promoter (278).

Cytochrome oxidase was considered for many years to be composed of three subunits, although Haltia had copurified a small polypeptide with the oxidase (110). Determination of the crystal structure of the holoenzyme revealed that this polypeptide was a fourth subunit (136, 205), consisting of a cytoplasmic and a transmembrane domain. The peptide sequence enabled recovery of the gene (*ctaH*) from a *P. denitrificans* genomic library (325). Deletion of the *ctaH* gene did not seem to have any effect on the assembly of the other three subunits or their prosthetic groups or on the in vitro activity (325). Despite a similar spatial location (although no homology) to subunit IV of quinol oxidases, no role could be assigned to this polypeptide, and it has been speculated that it is an evolutionary remnant (325).

The *ctaH* gene is transcribed from its own promoter at a locus removed from *ctaDI*, *ctaDII*, and *ctaCB*-ORF1-*ctaGE*. Both the *ctaCB*-ORF1-*ctaGE* and *ctaDII* loci have upstream regions rich in inverted repeats, but to date it has not been possible to relate this to regulation. Since *P. denitrificans* uses three different terminal oxidases, maximally expressed under different oxygen concentrations (Fig. 2), there must be fairly stringent control of expression of terminal electron transfer complexes. The region upstream of *ctaH* has some elements in common with the *ctaC* promoter (325), but no clear picture has yet emerged of the elements responsible for the coregulation of the four *cta* loci.

The cbb_3-type oxidase. Bacteria that rely entirely on respiration for the liberation of free energy are challenged under near-anoxic conditions since the terminal oxidases of the aa_3 and bo_3 or ba_3 type are unable to function at exceptionally low oxygen concentrations. One of the strategies for survival under these conditions is the recruitment of a cbb_3-type oxidase, which has a relatively high affinity for oxygen. This type of oxidase was first encountered in endosymbiotic rhizobia, which use it during nitrogen fixation in the root nodule (142, 182, 230, 231). The finding that its derived K_m value for oxygen is 7 nM may explain why the cbb_3-type oxidase supports the growth of the bacteroids in these nodules, where the free-oxygen concentration is only 3 to 22 nM (230).

The oxidase has three subunits, which are encoded by the *ccoNOQP* gene cluster: CcoN, CcoO, and CcoP (63). The last two subunits are membrane-bound mono- and diheme c-type cytochromes, respectively, which may function as electron entry sites. CcoN is the catalytic subunit I with two hemes b and a copper ion. The architecture and metal-heme arrangement are similar to those of subunits I of the other members of the heme copper oxidases and to that of subunit I of nitric oxide reductase (42, 302). The role of CcoQ is not yet clear.

Apart from *P. denitrificans*, other bacteria are able to syn-

thesize an oxidase of the cbb_3 type (63, 286). The importance of this oxidase for *P. denitrificans* has been stressed by the observations that a mutant lacking the *ccoN* gene was unable to grow under microaerobic conditions (61). Detailed studies on the properties of the cbb_3-type oxidase of *P. denitrificans* have been facilitated by the construction of a mutant strain that lacks the other types of oxidase. Proton translocation measurements of this mutant showed that the oxidase has the capacity to pump protons (63). Recent analyses of growth efficiencies of the wild-type and a set of double oxidase mutant strains, as determined from chemo- and auxostat experiments, have suggested that the cbb_3-type oxidase transduces free energy as efficiently as the aa_3-type oxidase does (306). However, amino acid residues implicated in the pathway of pumped protons and chemical protons, which are conserved in aa_3-type cytochrome *c* and bo_3-type quinol oxidases (128, 136), are not present at the corresponding positions of the cbb_3-type oxidases (63, 302). It may thus be that the proton channel in the cbb_3-type oxidases is composed of residues different from those of the other types or that the mechanism of proton translocation is not yet understood properly.

Expression of the *cco* gene cluster in *P. denitrificans* is under control of the FnrP protein as judged by the presence of an FnrP binding site, TTGAC-N_4-ATCAA, in the *ccoN* promoter region and by the finding that FnrP mutants showed a decreased expression of this type of oxidase (310). This conclusion is in agreement with the observation that synthesis of the cbb_3-type oxidase increases with decreasing oxygen concentrations (32).

Quinol oxidase. Initially it was speculated that quinol oxidase activity in *Paracoccus* could be ascribed to cytochrome *o* (51), based mainly on spectroscopic data. Less than a decade ago, more detailed studies indicated the presence of a proton-pumping quinol oxidase in whole cells (232). The complex thought to be responsible was purified shortly afterward from membranes of *P. denitrificans* (175). Several characteristics of this complex, such as its function and subunit pattern, suggested some similarities to the bo_3 quinol oxidase studied extensively in *E. coli* (reference 47 and references therein). This was substantiated by sequencing of the genes for the *Paracoccus* quinol oxidase (62).

The *Paracoccus* quinol oxidase is a member of the superfamily of heme copper oxidases (250). Six conserved histidine residues, which may act as ligands to the two heme components and one copper atom, were identified in the largest of the genes coding for subunit I (62). The four genes of the *qox* locus are arranged in an operon-like fashion (244), and they all show a remarkable degree of identity when translated not only to the four subunits of their *E. coli* counterparts but also to other oxidases.

Detailed biochemical and spectroscopic studies have shown that the quinol oxidase of *P. denitrificans* carries one heme *b*, one heme *a*, and one copper atom as redox-active groups in subunit I, QoxB. The last two constitute the binuclear center of oxygen reduction (345, 346). The observation that only one copper atom is present per enzyme complex reflects the fact that quinol oxidases do not possess a binuclear Cu_A center in their homologue of subunit II. The ba_3 heme composition of this oxidase is essential for maintaining its catalytic competence, as was seen when an inactive quinol oxidase variant was isolated from a *Paracoccus* strain unable to synthesize heme *a* (346). Nevertheless, there are conflicting reports on the heme composition of the quinol oxidase, based on heme analysis of membranes from an aa_3-type cytochrome oxidase mutant (62, 307).

Recently, two-dimensional crystals of the bo_3-type quinol oxidase of *E. coli* were analyzed by cryoelectron microscopy (98). Although the resolution of about 6 Å is too low to identify individual residues, it definitely shows a transmembrane helix and subunit arrangement similar to the structure of the aa_3-type cytochrome oxidase of *P. denitrificans* (136), apart from differences in the nonhomologous subunits IV. Given the close relationship of the *P. denitrificans* quinol oxidase to its *E. coli* homologue, a similar spatial structure can be inferred from these results. Another indication of their structural similarity is the retention of some enzymatic activity (about 20% both in membranes and in the isolated complex compared to the native *P. denitrificans* quinol oxidase) after replacement of the *qoxA* gene coding for subunit II of the *P. denitrificans* enzyme by the equivalent gene, *cyoA*, from the bo_3-type quinol oxidase of *E. coli* (324). It is not yet clear whether the chimeric quinol oxidase retains the ability to translocate one proton across the membrane per electron transferred to oxygen, as do the parent oxidases.

The *qox* promoter region contains both an FNR box (34 bp upstream of the transcript start site [Table 5]) and a sequence (TTGACC) very close to the *E. coli* σ^{70} −35 hexamer (TTGACA). The presence of a binding site for FnrP (310) in the promoter region of the *qox* operon encoding the quinol oxidase might indicate regulation of transcription in response to the level of oxygen or changes in the redox potential. However, no significant decrease in expression was observed with a synthetic promoter/reporter gene construct when reporter levels were measured under aerobic growth conditions compared to those measured under anaerobic growth conditions (346). Surprisingly, the same study revealed the positive influence of nitrate and nitrite (added to the growth medium) on the expression of quinol oxidase under aerobic conditions. Additional regulatory studies are needed before the role and importance of this oxidase for the metabolic flexibility and growth of *Paracoccus* can be assessed.

Cytochrome c_{550}. Cytochrome c_{550} is believed to function as an electron donor in several respiratory pathways including those for denitrification (30, 185), methanol oxidation and methylamine oxidation (57, 62, 74). The *P. denitrificans* locus coding for cytochrome c_{550} is found just upstream of *ctaDII* (235, 315), separated by a putative Rho-dependent terminator. A similar gene order is found in the *P. versutus* locus, and the two species have 89% DNA identity over comparable regions. Truncated versions of the *cycA-ctaDII* locus from *P. versutus* fused to a reporter and transferred to *E. coli* showed that the two genes are separated by a strong terminator. No activity from the *P. versutus ctaDII* promoter region could be demonstrated in *E. coli* (296).

The gene coding for cytochrome c_{550} (*cycA*) is subject to extensive regulation, since the expression is raised above a basal level in response to the induction of various respiratory pathways (209, 211, 275). Dual transcription start sites (separated by only 8 bp) for the gene have been determined by primer extension studies (279), but it still remains unclear which elements in the putative promoter regions are responsible for the regulation of the gene. Furthermore, examination of promoters by the authors of this review revealed no clear −10 or −35 sequences in the appropriate positions upstream of either transcription start site proposed (Table 5).

Cytochrome c_{552}. Among the many *c*-type cytochromes found in *P. denitrificans* (Table 6), a membrane-bound polypeptide, cytochrome c_{552} (CycM), is the most likely mediator between the cytochrome bc_1 complex and the heme aa_3 cytochrome *c* oxidase, as suggested by a number of arguments. (i) Under certain solubilization conditions, a complex of this cytochrome either with the oxidase or with oxidase and the bc_1
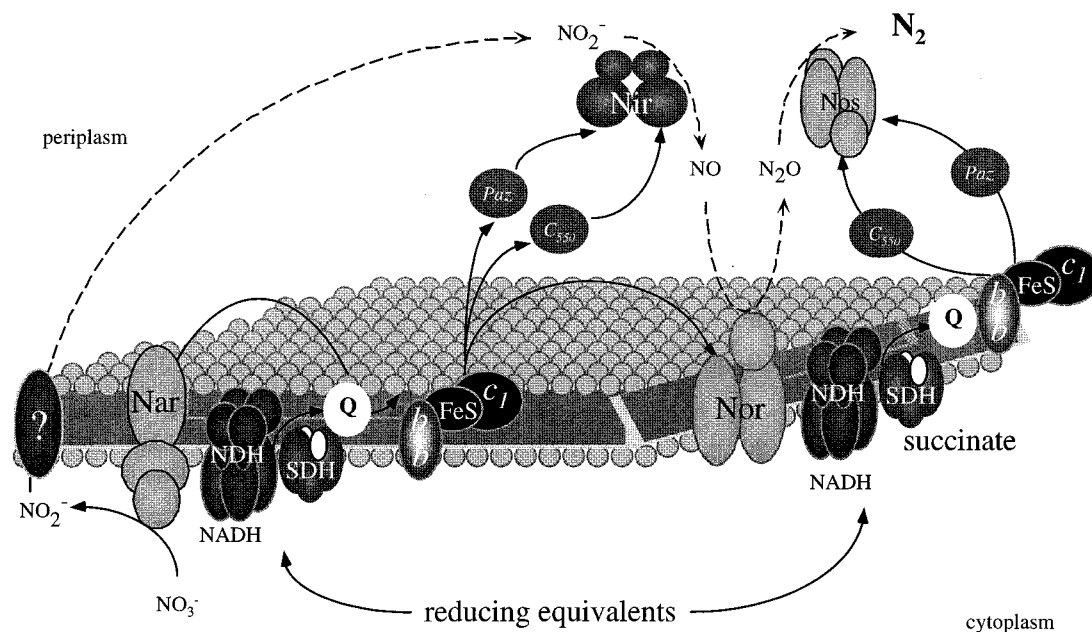
FIG. 3. Denitrification pathway of *P. denitrificans*, with succinate as the carbon and energy source. FeS, iron-sulfur center; NDH, NADH-ubiquinone oxidoreductase; SDH, succinate dehydrogenase; Nar, nitrate reductase; Nor, nitric oxide reductase; Nir, nitrite reductase; Nos, nitrous oxide reductase; Q, ubiquinone pool; ?, unknown transporter. The exact electron pathway(s) through the *c*-type cytochromes and pseudoazurin is not known.

complex has been isolated from membranes (26, 109); a high rate of electron transfer points at its efficient role as a redox link. (ii) Specific antibodies obtained against the purified protein (294) inhibited electron transport in membranes between NADH and oxygen but had no effect when partial reactions were assayed with mitochondrial cytochrome *c*. (iii) Cloning of the gene encoding cytochrome c_{552} (294) showed a tripartite structure of the polypeptide with an N-terminal membrane anchor; membranes isolated from gene deletion mutants (293) were blocked in their electron transport from NADH to oxygen via complexes I, III, and IV, and this inhibition could be partially overcome by the addition of mitochondrial cytochrome *c*.

Electron transport flavoprotein. Electron transport flavoproteins (ETF) of bacteria transfer electrons between flavoprotein dehydrogenases and the respiratory chain. The electron acceptor of ETF is presumably ubiquinone in *P. denitrificans*. Although the mitochondrial ETF accepts electrons from a wide variety of dehydrogenases, the *P. denitrificans* protein shows a more restricted substrate range. ETF has been isolated in abundance from trimethylamine-grown cells but has also be shown to transfer electrons from glutyryl coenzyme A (CoA) dehydrogenase (135). However, it does not accept electrons from periplasmic dehydrogenases such as methanol dehydrogenase and methylamine dehydrogenase (56, 273).

The genes encoding the two subunits of the heterodimeric protein were not given a gene designation and will be called *etfS* and *etfL* (for the small- and large-subunit genes, respectively, of this heterodimer) for the purposes of this review. These two genes are found in a short operon (12), much like the orthologous gene cluster *fixAB* of *Azorhizobium caulonidulans* (4). No gene coding for ETF-ubiquinone oxidoreductase has yet been discovered in *Paracoccus*. The *etfSL* locus is characterized by an abundance of inverted repeats upstream and downstream of the structural genes, and in *P. denitrificans*,

these repeats probably prevent expression of the genes in heterologous systems (12).

Interest in *P. denitrificans* ETF centers on the similarity of the protein to the mammalian mitochondrial equivalent. Some human diseases are associated with mutations in ETF, and although *P. denitrificans* ETF differs considerably in substrate range, the potential for overexpression has made it an attractive model. The differences between the human and *Paracoccus* ETFs have been overcome to some extent by the production of chimeric proteins: these and the wild-type proteins have been the subject of X-ray crystallography studies, yielding structures to a resolution of 2.5 Å (245).

Respiratory Denitrification Genes

Denitrification proceeds via nitrogen oxide intermediates (Fig. 3). A membrane-bound nitrate reductase with its active site facing the cytoplasm yields nitrite, which is converted to nitric oxide (NO) by a periplasmic reductase. The NO is further reduced by a periplasmic membrane-bound nitric oxide reductase, and molecular nitrogen is generated by a soluble periplasmic nitrous oxide reductase. This reaction pathway is also seen in other bacteria, including *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, and *R. sphaeroides* (forma denitrificans), and parts of the pathway are found in fungi (157). The bacterial enzymes of denitrification are essentially similar in all organisms, except that there are two types of nitrite reductase: a copper-containing protein and a cytochrome containing *c* and d_1 hemes. The two types of nitrite reductase have yet to be found together in a single bacterial species (342). *P. denitrificans* has the latter. The enzymology of denitrification has been reviewed thoroughly (22, 347, 348, 350).

Organization of denitrification genes. In *P. denitrificans*, *nir* genes (associated with nitrite reduction) and *nor* genes (associated with nitric oxide reduction) are clustered on a 17.7-kbp fragment, essentially transcribed divergently from one another
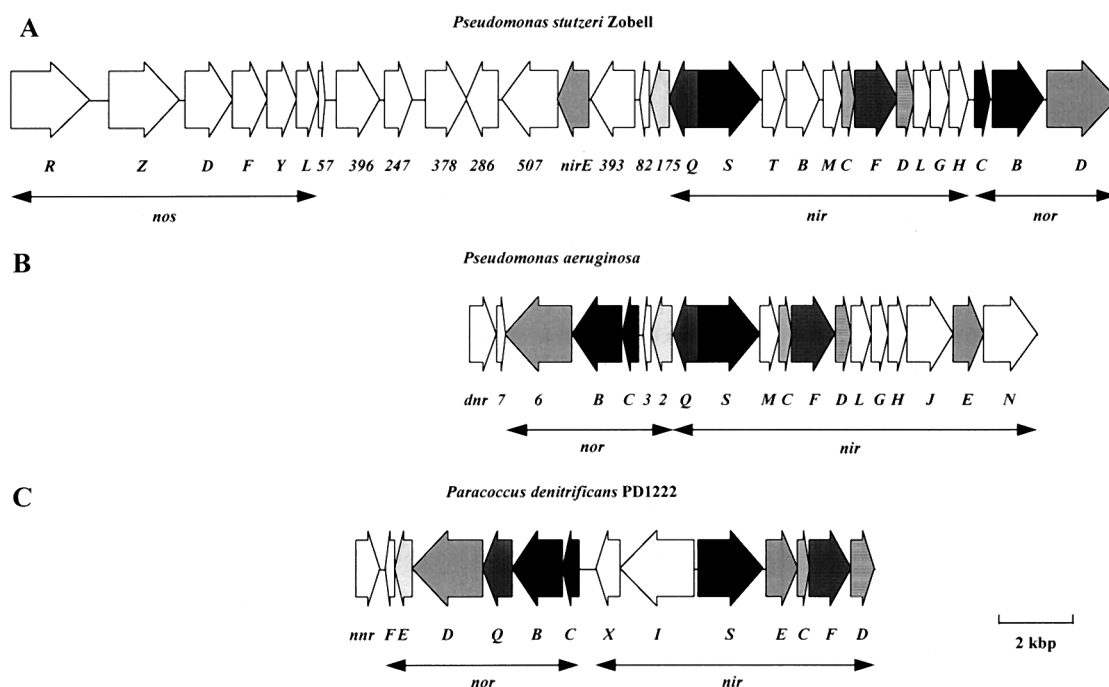
FIG. 4. Denitrification gene clusters of *Pseudomonas stutzeri* (A), *Pseudomonas aeruginosa* (B), and *P. denitrificans* Pd1222 (C). Reproduced with permission from reference 251a.

(Fig. 4). There seems to be little variation in genetic organization between the strains of *P. denitrificans* studied (252). In *Pseudomonas stutzeri*, the other organism for which the genetics of denitrification have been extensively studied, the *nos* genes (nitrous oxide reductase associated) are also found in the same 30-kbp region of the genome as the *nir* and *nor* genes (34, 97, 347, 348, 350). The order of the genes within the *nir*, *nor*, and *nos* clusters varies among organisms, but the structural genes (*nirS* and *norCB*) are transcribed from the 5′ end of an operon (34, 59, 252). It is probable that other *nir*, *nos*, and perhaps *nor* genes remain undiscovered in *P. denitrificans*, given that the *Pseudomonas stutzeri* cluster contains at least five genes so far unidentified in *Paracoccus* (348). It is not yet known whether the nitrate reductase (*nar*) genes map to the same region of the chromosome as the *nir*, *nor*, and *nos* genes in any of the denitrifying bacteria.

Nitrate reductases. Two nitrate reductases, a membrane-bound enzyme induced during oxygen limitation and nitrate availability and a constitutively expressed periplasmic reductase, can be active during denitrification (16). Insertion of Tn5 into a structural gene of the membrane-bound nitrate reductase of *P. denitrificans* resulted in anaerobic overexpression of periplasmic nitrate reductase activity (15).

(i) Membrane-bound nitrate reductase. The nitrate reductase induced during denitrification is of a similar type to that found in *E. coli*. It is a three-subunit protein, with the transmembrane γ subunit anchoring the cytoplasmically exposed α and β subunits to the membrane. Deletion of the γ subunit stops nitrate reductase activity in whole cells, but the activity of the remaining αβ dimer can still be detected in a nonphysiological assay with methyl viologen as an electron donor (16). To date, only the 5′ region of *narG* (the α subunit), plus *narH* (the β subunit), *narJ* (unknown function), and *narI* (the γ subunit) have been sequenced (23), and so it can be assumed only that the genes form an operon much like that found in *E. coli*.

(ii) Periplasmic nitrate reductase. The periplasmic nitrate reductase is a molybdopterin protein containing both heme and nonheme iron (18, 25, 35, 259). The structural and biosynthetic peptides required for construction of the holoenzyme are coded for by the *napEDABC* operon (24). The large and small subunits are coded for by *napA* and *napB*, respectively. Sequence analysis shows that the large subunit contains the molybdenum center and an iron-sulfur center whereas the smaller subunit has two heme binding sites. The exact functions of the cytoplasmic NapD and membrane-bound NapE are currently under investigation, but it is thought that NapC is a tetraheme *c*-type cytochrome (247a) and the electron donor to the holoreductase (24); evidence for this role has been presented for *R. sphaeroides* NapC (243). It has similarity to the tetraheme cytochrome NirT (implicated in denitrification in *Pseudomonas stutzeri* [97]).

The induction of the *nap* gene cluster does not follow that of denitrification. Although the proteins are expressed during anaerobic growth, maximal expression is found during aerobic growth on substrates such as butyrate (260). Examination of the region upstream of *napE* reveals no easily distinguishable binding sites for regulatory elements.

Nitrite reductase. The reduction of nitrite to nitric oxide is the first committed step of the denitrification process. The NO produced can be used as a substrate only by nitric oxide reductase and must be quickly removed from the cell because it is toxic at quite low concentrations. The physiological electron donors to nitrite reductase in *P. denitrificans* are cytochrome c_{550} (CycA) and the copper-containing pseudoazurin, PazS. No confusion of azurin with pseudoazurin should occur: they are connected merely by both being copper proteins (168, 189, 192, 321). The existence of two donors with two differing metal centers probably allows the organism to maintain the electron transport chain under different metal ion-limited conditions.

Nitrite reductase has been the subject of study for a number of years, and the protein was first purified by Newton (196).

The enzyme has an alternative function as a cytochrome oxidase, but this is probably nonphysiological. The enzyme has two hemes as prosthetic groups, a normal c-type heme and a unique d_1 heme. Both of these hemes are added to the reductase in the periplasm or during the preprotein's translocation to the periplasm (211, 212). To date, the d_1 heme has been found only in other bacterial cytochrome nitrite reductases. A crystal structure (87) reveals that the two hemes lie in distinct domains: one domain somewhat resembles the class I c-type cytochromes such as c_{550}, with the c-type heme covalently attached to the N terminus of the domain; the second domain contains the d_1 heme noncovalently held in a complex β-sheet structure. The structure of the d_1 domain can be regarded as an eight-bladed propeller (87), similar to the structure of methanol dehydrogenase (6) but with a d_1 heme active site held in the center.

(i) Biosynthesis of nitrite reductase. The structural gene for cytochrome cd_1 nitrite reductase (*nirS*) was sequenced by de Boer et al. (59). The structural gene maps close to those of nitric oxide reductase but is transcribed in the opposite direction (Fig. 4). Immediately upstream of *nirS* lie two ORFs (*nirI* and *nirX*), which, by interruption mutagenesis, are found to be essential for nitrite reduction. Neither NirI nor NirX is thought to have any function in the biosynthesis of nitrite reductase, but they both influence the transcription of the gene (307). Downstream of *nirS* lie four other ORFs, also essential for nitrite reduction, *nirE*, *nirC*, *nirF*, and *nirD* (59), which may be transcribed from a promoter in the *nirS-nirE* intergenic region (252). Interruption of these genes by introduction of a kanamycin cassette does not have any effect on the transcription of the structural gene or on the insertion of c-type heme into the polypeptide (59), but no d_1 heme appears to be made in these mutants. NirE resembles, by homology, CysG of *E. coli*, a uroporphyrinogen methyltransferase implicated in the synthesis of the sulfite reductase prosthetic group, siroheme (53). It thus seems probable that NirE is involved in the addition of methyl groups to a precursor to form d_1 heme, but since the biosynthetic pathway for this prosthetic group remains uncharacterized, it is not possible to determine exactly how NirE acts. NirC codes for a low-molecular-weight class I c-type cytochrome, which plays an undefined part in the formation of d_1 heme. This cytochrome has not been seen in heme-stained extracts of *P. denitrificans* (252).

nirF codes for a protein with some degree of identity to the d_1 domain of the nitrite reductase structural gene. Sequence identity of NirF to the NirS d_1 domain may support the postulate that NirF is similar to this domain (97). Although NirF has aspartate residues in a similar spacing to NirS and other propeller structures (a motif that may be common to many propeller folds [6]), no clear structural similarity can be predicted (6). It has been suggested that NirF may be acting as a scaffold on which the d_1 heme is assembled prior to insertion into nitrite reductase (97), but the periplasmic location of the protein and the difference in deduced structure argue against this theorem (6). The last gene in the cluster so far identified is *nirD*, an ORF of unknown function.

Nitric oxide reductase. For some time it was a matter of debate whether nitric oxide was really an intermediate in denitrification, but the matter has been resolved by the finding of a separate nitric oxide reductase complex (39, 40), the trapping of NO during denitrification (39, 102, 161), and, ultimately, the interruption of the genes encoding the nitric oxide reductase proteins (34, 60), yielding a mutant that is unable to grow anaerobically.

The nitric oxide reductase of *P. denitrificans* has been purified (40, 96) as a two-subunit protein. The larger subunit binds heme b, while the smaller one binds heme c. The complex has also been shown to contain nonheme iron (96, 122). The smaller subunit is anchored to the membrane via a single α-helix, with the c-heme domain acting as a soluble periplasmic polypeptide. The large subunit has 12 membrane-spanning helices, anchoring all of the protein within the membrane except for the N and C termini. Despite the similarity to heme copper oxidases (249, 302), which can act as primary proton pumps (62, 63, 219, 236, 250), nitric oxide reductase has no proton translocation activity (17, 39, 261).

The genes associated with nitric oxide reductase are found in a cluster upstream of the *nir* locus (60). A small ORF, *norC*, codes for the c-heme subunit, and *norB* codes for the larger b-heme subunit. The *norCB* genes appear to be cotranscribed with four other genes that are essential for the maturation of nitric oxide reductase (*norQDEF*), although their exact function remains to be determined. NorQ contains two putative nucleotide binding motifs and has been found in other denitrifiers including *Pseudomonas stutzeri* (351) and *R. sphaeroides* (7). NorQ has been implicated in the formation of nitrite reductase in *Pseudomonas stutzeri* (141). The gene product of *norD* appears to be cytoplasmically located like NorQ, suggesting that both influence transcription rather than protein maturation. A knockout of *Pseudomonas stutzeri norQ* affects *nirS* and *norCB* activities; a knockout of *Pseudomonas stutzeri nirQ* also affects *nirS* and *norCB*. The *norQ* knockout has the same effect in *P. denitrificans*, but as yet *nirQ* has not been isolated. The genes *norE* and *norF* have no known function. NorF of *P. denitrificans* has no known orthologues, but NorE is similar to cytochrome oxidase subunit III (60). Knockouts of *norE* and *norF* affect only nitric oxide reductase activity (60). Several experiments suggest that the nitrite reductase and nitric oxide reductase are in some way linked, perhaps as a multienzyme complex. Both de Boer et al. (59) and Jungst et al. (140) describe a lowering of nitric oxide reductase activity on insertional mutagenesis of *nirS*, despite there being no obvious transcriptional link between *nirS* and *norCB* in either *Paracoccus* or *Pseudomonas* (Fig. 4).

Nitrous oxide reductase. The formation of molecular nitrogen from nitrous oxide is carried out in *P. denitrificans* by a periplasmic copper nitrous oxide reductase (21). The redox centers of this protein are of a copper A type and an unusual copper Z type (reference 350 and references therein). The *nosZ* gene coding for the structural protein of nitrous oxide reductase has been sequenced, along with part of an upstream ORF cotranscribed with it, named *nosR* (125).

NosR of *P. denitrificans* is the counterpart of the *Pseudomonas stutzeri* NosR protein (55). Protein sequence analysis indicates that NosR is a membrane protein with relatively large soluble domains facing the periplasm and cytoplasm. The cytoplasmic domain harbors two cysteine signatures indicative of the presence of [4Fe-4S] clusters. NosR has a high degree of homology to NirI (307). Other structurally related proteins are NapH, MauN, and CcoG (21, 22). Disruption of the *nosR* gene from *Pseudomonas stutzeri* yielded a mutant unable to transcribe the *nos* gene cluster (55). It was suggested that NosR itself was the transcriptional activator based on the identification of a helix-turn-helix-like DNA binding motif. Other examples of transcriptional regulators that may bind both the membrane and DNA have been found in other organisms, including ToxR (which regulates cholera toxin expression in *Vibrio cholerae* [187]).

Pseudoazurin. Despite the roles in electron transport assigned to CycA (cytochrome c_{550}), a knockout of its gene (315, 316) did not lead to cessation of anaerobic or methylotrophic growth. This does not necessarily contradict the postulate that

cytochrome c_{550} is involved in these processes (Fig. 1–3, 5), but it suggests that the role of this cytochrome can be duplicated by another protein; the blue copper protein pseudoazurin (PazS) has been proposed as a candidate (132, 189, 192, 193) for a substitute in denitrification. A *cycA pazS* double deletion is currently under construction.

The *pazS* gene may be under the control of an Fnr-like protein, as indicated by the presence of an FNR box 42.5 bp upstream of the transcript start site (168), but there is no experimental evidence whether this putative transcriptional activator is FnrP or Nnr (see below). There is also a −10 hexamer (TAAACC) with low similarity to that of *E. coli* (TATAAT [Table 5]). In addition, the promoter/operator region of *pasZ* contains some apparently anomalous motifs. An apparent NtrA box can be found in the promoter region, but it is centered at −82 bp with respect to the transcript start site determined during growth on nitrate. Since σ^{54}-controlled genes usually have the NtrA box from −24 to −11, this may be either the binding site for a second promoter or coincidental (253). Another puzzling nucleotide structure is found just upstream of the transcript start site. In this case, a palindrome is formed in the 5′ end of the mRNA. This might be expected to increase mRNA stability, but it appears to inhibit isopropyl-β-D-thiogalactopyranoside (IPTG)-dependent expression in *E. coli* (168). Its removal leads to a twofold elevation in the yield of the protein when expressed heterologously, a finding previously observed with the *Ralstonia (Alcaligenes) eutrophus* S-6 pseudoazurin (337).

Regulation of denitrification and integration with oxygen respiration. Oxygen concentration can be sensed by a protein designated FNR (Fumarate Nitrate Reduction), which in *E. coli* activates the transcription of the genes involved in anaerobic metabolism (107, 269, 271, 299). FNR has four important functional domains. First, the N-terminal domain contains three cysteine residues. Together with a fourth cysteine residue located in the central domain, they may bind a redox-sensitive [4Fe-4S] cluster (10, 107, 150). Second, a sequence of residues in the central domain of FNR forms a loop-like structure that contacts RNA polymerase (322). Third, FNR contains an α-helical domain surrounding Asp154, which is probably involved in dimerization (10, 262). Finally, at the C-terminal end it contains a helix-turn-helix DNA binding motif, which recognizes the DNA target site with the consensus sequence TT-GAT-N_4-ATCAA (the FNR box). Two invariant amino acid residues (Glu209 and Ser212) in this region are essential for binding to the FNR box (269, 270). Anaerobic transcription activation by FNR depends on the presence of the redox-sensitive [4Fe-4S] cluster. Since this cluster is rapidly destroyed upon exposure to oxygen (104, 150, 265), increasing activation of FNR may be expected at decreasing oxygen concentrations. In its active form, FNR may dimerize with a partner molecule and bind strongly to the FNR box. Usually, this box is located directly upstream of the RNA polymerase binding site at the −35, −10 promoter consensus sequences. Such an organization of binding sites allows physical contact between the FNR dimer and RNA polymerase. Once this contact occurs, the transcription of a number of target genes essential for anaerobic metabolism gets underway.

Although expression of the *E. coli nar* operon requires active FNR, optimal expression of this locus is achieved only when the nitrate-sensing regulatory device is activated too. This device is composed of two proteins, a protein histidine kinase (NarX) and a DNA binding response regulator (NarL), a set of proteins that belongs to the family of two-component regulators. Since the behavior of *P. denitrificans* with respect to the on-off set of nitrate reductase synthesis is similar to that of *E.*

coli, one may expect that both organisms will make use of similar protein-based signal transduction circuits. Partial evidence for this suggestion has recently been obtained from the identification of the *P. denitrificans* counterpart of the *E. coli fnr* gene, designated *fnrP*. Its product, FnrP, resembles FNR to a large extent, and all four functional domains that are found in FNR are also present in FnrP. The *fnrP* gene was identified close to the *ccoNOQP* locus encoding the *cbb₃*-type cytochrome *c* oxidase. A mutation in *fnrP* had pleiotropic effects during growth at oxygen depletion. The mutant was unable to express membrane-bound nitrate reductase (the product of the *narGHJI* operon) and cytochrome *c* peroxidase (encoded by the *ccp* gene [307]), whereas it had attenuated levels of the cbb_3-type cytochrome oxidase and increased levels of the quinol oxidase (encoded by the *qoxABCD* locus). Target sites of FnrP, 5′-TTGAC-N_4-ATCAA-3′, which resemble the *E. coli* FNR box were identified in the promoter regions of the *cco*, *qox*, and *fnrP* genes and operons (those of the *ccp* gene and the *nar* operon have yet to be sequenced). In the promoter region of the *qox* locus, this box appears to be located within the RNA polymerase binding site rather than upstream of it, which may explain the observed repression of transcription of the *qox* locus by FnrP. The presence of such a box in the *fnrP* promoter suggests that its expression is autoregulated, as is the case for *fnr* from *E. coli*. The expression of two other gene clusters that are important for anaerobic growth with nitrate, the *nir* and *nor* gene clusters encoding nitrite reductase and nitric oxide reductase, respectively, turned out to be under the control of NNR, which is a second FNR homologue found in *P. denitrificans* (309). NNR shares all but one of the features of FNR homologues: it does not contain the redox sensitive [4Fe-4S] cluster. The *nnr* gene is located close to the locus that encompasses the *nir* and *nor* gene clusters. A mutation in the *nnr* gene resulted in an inability to synthesize nitrite reductase, while the expression of nitric oxide reduction was decreased to about one-quarter of the wild-type level. The expression of other gene clusters (*ccoNOQP*, *qoxABCD*, *narGHJI*, and *ccp*) was not affected by the *nnr* mutation. DNA binding sites for NNR, which are virtually identical to the FnrP binding site, were found in the promoter regions of both gene clusters. Despite this degree of identity, NNR and FnrP were unable to take over each other's tasks in gene regulation during denitrification. Apparently, expression of each set of target genes requires additional transcription factors that are dedicated to either NNR- or FnrP-mediated control. Alignment studies of a number of FNR homologues of different bacterial origins have led to the suggestion that this family of regulators may be divided into three more or less discrete groups, each with proteins harboring specific signatures of invariant amino acid residues within their functional domains. Such a specificity in a resulting RNAP contact site may thus reflect specificity of proteins from each group for a particular type of sigma factor. Sigma factors are proteins that bind reversibly to the catalytically active core RNAP and are required for transcription initiation. Since NNR and FnrP fall into two different groups, this could mean that the dedicated transcription factors suggested for NNR and FnrP are specific sigma factors. In many bacterial species, there are sigma factors other than the primary ones; the latter are σ^{70} homologues that are involved in transcription of the majority of housekeeping genes. Alternative sigma factors often control the transcription of coordinately regulated sets of genes from promoter sequences that are different from those recognized by the primary sigma factor. Examples of these alternative sigma factors are RpoN (control of genes involved in nitrogen metabolism), RpoH (control of heat

shock genes), and RpoS (control of gene expression at the stationary phase of growth). Examination of the upstream regions of the genes that are regulated by NNR (*nirS*, *nirI*, and *norCB*) reveal no obvious σ^{70} RNAP binding sites; instead, other common motifs that might have some function in transcription have emerged (5).

The nature of the signals sensed by FnrP and NNR is still not fully established. FnrP closely resembles *E. coli* FNR in that it contains the cysteine signature for ligating a redox-sensitive [4Fe-4S] cluster. In *E. coli*, the concentration of molecular oxygen is the most important factor involved in modulation of FNR activity. In vitro experiments showed that the [4Fe-4S] cluster is rapidly destroyed by molecular oxygen, resulting in inactive FNR (165). FnrP of *P. denitrificans*, however, is still capable of activating the transcription of its target genes at aerobic growth conditions. Under these conditions, FnrP activation appears to increase at increasing reduction levels of the components of the respiratory network. These observations suggest that the [4Fe-4S] cluster of FnrP requires reduction for its full activation and that it is less sensitive to molecular oxygen than is the *E. coli* FNR protein. It has been shown that the latter protein may acquire an enhanced stability toward oxygen when the Asp residue at position 154 is changed into an Ala residue. Interestingly, there is an Ala residue in *P. denitrificans* FnrP at the position corresponding to Asp154 of the *E. coli* FNR protein.

Despite the diversity of promoters, comparison of the anaeroboxes allows the deduction of some *Paracoccus* promoter features. To date only two NNR/FNR-regulated promoters have had their transcription start sites determined. The genes for pseudoazurin (*pazS* [168]) and NNR-controlled nitrite reductase (*nirS* [252]) have anaeroboxes. In both cases, the FNR-like box is centered at a position 41.5 bases upstream of the transcript start site.

The closely related bacterium *R. sphaeroides* also has an NNR-like (NnrR) system, *nirK*, controlling a copper-containing nitrite reductase (287). Again, this system works in parallel with a conventional FNR-type protein. By using *lacZ* fusions to *nirK* and organic compounds from which NO was generated in the medium of *R. sphaeroides*, it was shown that NO was the signal to which the *R. sphaeroides* Nnr was responding (163). It is postulated that *nirK* is switched on for most of the time, albeit at very low levels. In the presence of nitrite (from nitrate reductases), a small quantity of NO can be produced. NO is argued to be a biologically very active signal molecule by analogy to eukoryotic NO-responsive systems and because of the low levels of exogenous NO required to stimulate *R. sphaeroides nirK* fusions (163). NO switches on NnrR, which in turns stimulates transcription of *nirK*, so that more NO is produced. The transcription of *norCB* is partially dependent on the same NnrP protein; therefore, NO does not accumulate to toxic levels (7, 154).

NO is also a regulator of nitrite reductase in *P. denitrificans* (307); the oxygen tension of the cell is not the sole modulator of transcription and hence of the activity of the denitrification enzymes. This may give some credence to the suggestion that *P. denitrificans* LMD 82.5 can denitrify aerobically (14, 29, 39, 41, 58, 171, 246, 247). The presence of nitrate could indirectly act as a stimulus, since nitrate would be converted to nitrite by nitrate reductase(s) and NO would be generated by nitrite reductase, which is present at low levels in *P. denitrificans* even under aerobic conditions (Northern blotting experiments revealed a low level of transcription of the *nirS* gene) (252). Experiments are under way in several laboratories to find strategies to characterize completely the signal transduction pathway of the denitrification cluster.

Genes for Autotrophy

When *P. denitrificans* experiences a shortage of heterotrophic substrates, it can switch to chemoautotrophic growth, during which hydrogen or thiosulfate is oxidized (86, 138, 327). The carbon supply is provided by the fixation of atmospheric carbon dioxide via the Calvin cycle under these growth conditions. The bacterium is also able to grow on C_1 substrates such as methanol or methylamine (reviewed in reference 119), and these processes are also considered autotrophic, since these carbon sources are completely oxidized before assimilation (318).

Methanol dehydrogenase. Not all *Paracoccus* species can utilize methanol. *P. denitrificans* grows well on this substrate, but the closely related *P. denitrificans* GB-17 is unable to use methanol as a sole source of carbon and energy. The latter strain does not carry the full complement of methanol oxidation genes, but mutagenic events (191) can restore the methanol dehydrogenase (MDH) activity specifically by the extension of the substrate range of an alcohol dehydrogenase (238).

In the presence of methanol as the sole source of energy, *P. denitrificans* induces the expression of the *mxa* gene cluster, encoding MDH and its dedicated electron acceptor cytochrome c_{551i} (119, 316). Both proteins are located in the periplasm, and in subsequent redox steps they connect the oxidation of methanol to the reduction of cytochromes c_{550} and c_{552}. Methanol-oxidizing systems are found in a wide variety of methylotrophic bacteria (3). Apart from *P. denitrificans*, much of the current knowledge about the physiology and molecular genetics of this system has been obtained from studies on the facultative methylotrophic bacteria *Methylobacterium extorquens* AM1, *Methylobacterium organophilum* XX, and *Methylobacterium organophilum* DSM 760 (reviewed in reference 169). These studies revealed that the synthesis of a fully active methanol-oxidizing pathway requires a total of at least 32 genes, among which are the *pqq* genes involved in cofactor biosynthesis and a comprehensive set of so-called *mxa*, *mxb*, *mxc*, and *mxd* genes, some of which encode the structural enzymes, others of which encode enzymes involved in calcium insertion, and yet others of which encode proteins involved in regulation of gene expression and protein activity (169).

Methylamine dehydrogenase. Growth on methylamine is accompanied by the appearance in the periplasm of methylamine dehydrogenase (MADH) and its electron acceptor, the blue copper protein amicyanin, both of which are encoded by the *mau* gene cluster (48, 49, 304, 313). Electrons derived from the oxidation of methylamine are transferred via amicyanin to the electron transport network at the level of cytochrome *c* (64, 133, 295, 314). The *mau* gene cluster of *P. denitrificans* consists of 11 genes, 10 of which encode the structural proteins or proteins involved in cofactor biosynthesis and are transcribed in one direction whereas the 11th regulatory gene (*mauR*) is located upstream and is divergently transcribed (48, 304, 313).

In addition to the proteins expressed specifically during and involved in the oxidation of methanol and methylamine, a cytochrome c_{553i} is expressed during growth on these C_1 substrates (33, 134). The gene encoding this cytochrome (*cycB*) has been cloned and shown to be part of the gene cluster designated *xox* (239). Surprisingly, the products of the genes located upstream and downstream of *cycB* (*xoxF* and *xoxJ*, respectively) had a large degree of identity to the α subunit of MDH and MxaJ (a protein of unknown function), respectively. The role of this putative oxidizing branch still remains obscure, since mutational analysis of this gene cluster did not reveal a clear function for the proteins (240).
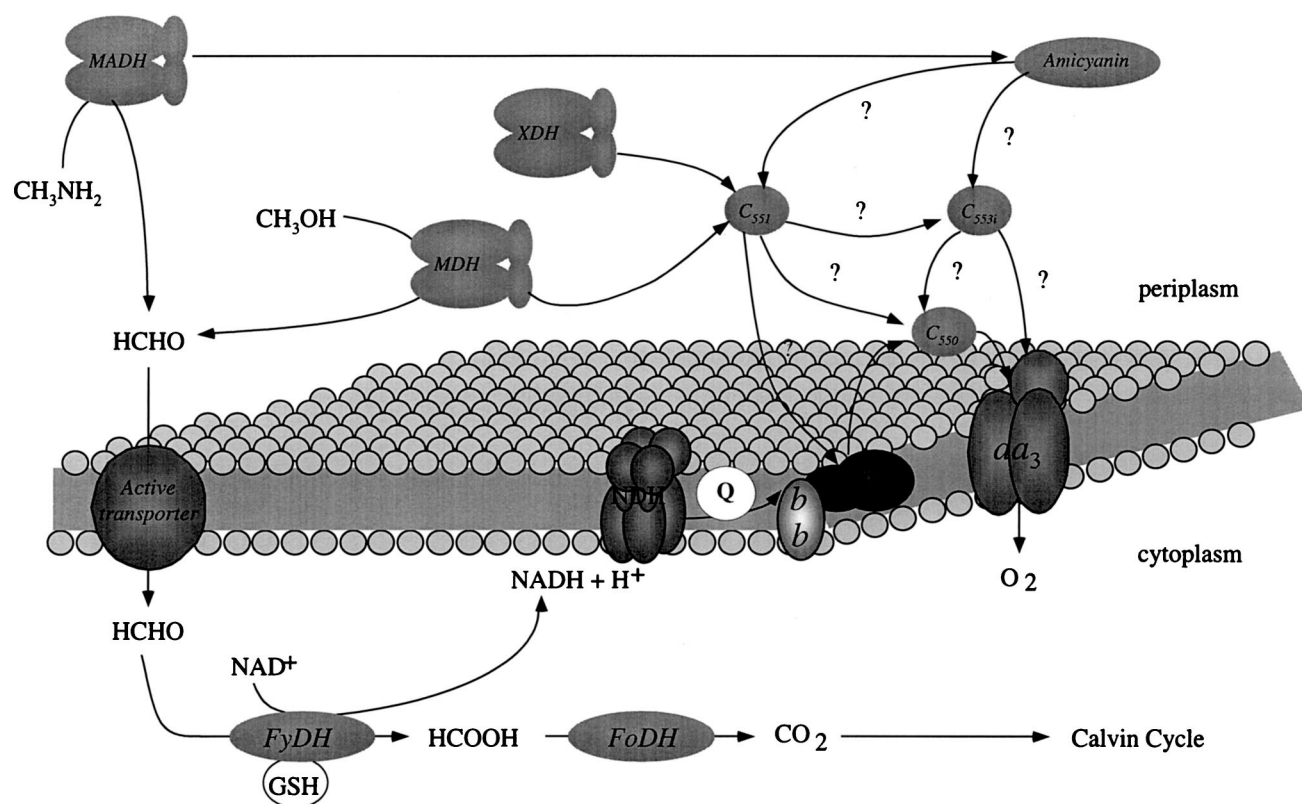
FIG. 5. Electron transport pathway of *P. denitrificans* during growth on methylamine and methanol. FyDH, formaldehyde dehydrogenase; FoDH, formate dehydrogenase; FeS, iron-sulfur center; NDH, NADH-ubiquinone oxidoreductase; GSH, glutathione. The exact electron pathway(s) through the *c*-type cytochromes is not known.

Formaldehyde dehydrogenase. The oxidation product of both methanol and methylamine is formaldehyde, which is translocated across the membrane to the cytoplasm. This transport process has been suggested to involve a specific formaldehyde carrier (159). In the cytoplasm, formaldehyde is further oxidized to formate via two consecutive reactions. In the first reaction, a NAD-linked glutathione-dependent formaldehyde dehydrogenase (FlhA) catalyzes the oxidation of formaldehyde into *S*-formylglutathione (305). In the second reaction, the latter molecule is converted into formate by *S*-formylglutathione hydrolase, which is a human esterase D homologue (117). The *flhA* gene cluster of *P. denitrificans* encoding the two enzymes has recently been identified upstream of the *xox* gene cluster (240). Finally, formate is oxidized to carbon dioxide predominantly by a NAD-dependent formate dehydrogenase (3). It has been reported, however, that *P. denitrificans* can synthesize isoenzymes of the formaldehyde and formate dehydrogenases that are NAD independent (113). A schematic view of the respiratory network that operates during C_1 metabolism is presented in Fig. 5.

Regulation of the metabolism of C_1 compounds. Expression of the components of the methanol- and methylamine-oxidizing branches is subject to complex regulatory cascades that have to orchestrate the optimal composition of the respiratory network under these growth conditions to maintain respiratory electron flows at competitive rates on the one hand but prevent the accumulation of the toxic intermediate formaldehyde on the other hand. Moreover, part of these signal transduction systems coordinate gene expression according to an energetic hierarchy, ensuring that the C_1-oxidizing branches are ex-

pressed only when the cell is unable to metabolize more energetically favorable substrates (119). This view is corroborated by extensive physiological and biochemical studies on this type of metabolism. MADH and amicyanin are synthesized only when the cell uses methylamine (and to a lesser extent other alkylamines) as the sole source of energy (118, 213, 317). MDH and XDH and their dedicated cytochromes are expressed not only during growth on methanol but also during growth on methylamine or choline. Since the oxidation of all these carbon sources yields formaldehyde, it has been postulated that the latter molecule is an important trigger in the regulation of expression of these gene clusters (113). The synthesis of all the enzymes involved in the consecutive steps of methanol or methylamine oxidation is blocked if the cells can metabolize multicarbon substrates in addition to these C_1 growth substrates (118, 213, 313). Growth during C_1 metabolism also affects the regulation of expression of the other components of the branches, cytochrome c_{550} and the cytochrome c oxidases. The amount of these components increases two- to fivefold under these conditions compared to that obtained during heterotrophic growth (279, 313). Apparently, this increased supply meets the demands of increased velocities of electron transfer within this part of the respiratory network. To date, a number of different regulatory proteins that control the expression of the *flh*, *xox*, *mxa*, and *mau* gene clusters (115), as well that of the *cco* and *qox* gene clusters, have been identified (308–310).

Expression of the *mxa* genes is controlled by the products of the *mxaXYZ* genes, which are located upstream of the structural *mxa* gene cluster (118). MxaY and MxaX are a protein histidine kinase and a DNA binding response regulator, re-

spectively, and form a set of proteins that belongs to the family of two-component regulators. MxaZ has no significant homology to any protein in the databases. As suggested above, the effector molecule for the sensor domain of MxaY is probably formaldehyde. The general principle for signal transduction via two-component regulatory systems is that binding of the effector molecule triggers the autophosphorylation of the kinase, after which the phosphate group is transferred to the response regulator. Once phosphorylated, the latter may bind to the promoter region of its target gene in order to facilitate transcription by the RNAP (220). Surprisingly, however, MxaY was shown to be dispensable in this cascade, suggesting that an alternative kinase takes over its role (339). The role of MxaZ in this cascade is still unclear. The MxaYX regulatory system appears to be specifically dedicated to the regulation of expression of the *mxa* genes; *mxaX* mutants showed an unimpaired expression of the *mau*, *flhA*, and *xox* genes (116, 118). A two-component system, MxcQE, with a function similar to that of MxaYX has now also been identified in *Methylobacterium organophilum* XX. The regulatory gene cluster is, however, separated by more than 40 kbp from its target, the *mxa* locus (330).

The methylamine gene cluster of *P. denitrificans* is regulated by MauR, which is a transcription activator that belongs to the family of one-component LysR-type regulators (313). The spatial organization of the functional domains involved in DNA binding, signal perception, and dimerization in the members of this family is conserved (256). Usually, the molecule that is oxidized by the proteins encoded by the target locus of this type of gene activator also serves as the trigger molecule for activation of the latter. This finding would be in agreement with the suggestion that methylamine is the molecule that triggers the expression of the *mau* gene cluster (313). The current view with respect to the mode of action of LysR-type regulators is that signal perception induces dimerization of the transcription activator, which is followed by binding to a target site upstream of the promoter. As a result, the dimer may come into contact with RNAP, after which transcription gets under way. In agreement with this view was the observation that MauR binds to the *mau* promoter region in a gel shift assay (65). The target site for LysR-type activators is usually a conserved T-N_{11}-A motif that harbors small palindromic sequences at its ends. Such a motif, however, has not been recognized in the *mau* promoter region, indicating that the target site of MauR is slightly different from the conserved one. The target site for *Pseudomonas putida* CatR, another member of the LysR-type activators, is G-N_{11}-A (221), suggesting that small deviations of the target sequence do occur. Promoter probe studies have further shown that *P. denitrificans mau* gene expression increases almost 1,600-fold during growth on methylamine compared to that during heterotrophic growth. This increase was not observed in the *P. denitrificans* MauR mutant, and this result confirms that MauR is a regulator of methylamine oxidation (65). The expression of the *mauR* gene itself is more or less constant, independent of the growth conditions and of MauR itself. Apparently, its expression is not autoregulated, a feature which is different from that of most other *lysR*-type genes. A counterpart of *mauR* has been described in *P. versutus* (130). Analysis of the corresponding region upstream of the *mau* gene cluster from *Methylobacterium extorquens* AM1 did not reveal a *mauR*-like gene (48).

Apart from their dedicated activators, the regulation of both *mxa* and *mau* gene clusters is subject to some kind of hierarchical control that suppresses transcription during heterotrophic growth. Indeed, it has been shown that the expression of the *mau* genes is virtually blocked when the bacterium grows in

the simultaneous presence of succinate and methylamine (213, 313). Further, both MADH and MDH are absent once succinate is added to the growth medium in addition to their corresponding substrates (119). The view that a global transcription activator is responsible for these phenomena has recently been corroborated by the analysis of a mutant disturbed in many aspects of C_1 metabolism (115). This mutant was unable to grow on methanol, methylamine, or choline. Moreover, the expression of the *flhA*, *mxa*, *mau*, and *xox* genes was completely blocked. These pleiotropic effects were explained by assuming that the mutated gene in this strain encodes a regulator that affects the expression of all these C_1 genes and gene clusters. Apparently, this regulator is not a repressor protein, since one would expect constitutive expression rather than repression in the knockout situation. Therefore, it has been suggested that this protein acts as a second activator in synergism with either of the two dedicated activators of *mxa* and *mau* gene expression. If this view is correct, one may expect that the presence and/or activity of this second activator would be suppressed during heterotrophic growth. The identification of a gene coding for this second activator has recently been facilitated by the isolation of a genomic locus that fully complements the mutation (116). A scheme of the regulatory pathways that control gene expression during C_1 metabolism is shown in Fig. 6.

Sulfur oxidation. The periplasmic enzymes specifically involved in the use of thiosulfate by *P. versutus* (enzyme A, enzyme B, cytochrome $c_{552.5}$, sulfite:cytochrome c oxidoreductase, cytochrome c_{551}, and cytochrome c_{552}) (Fig. 7) have also been called the thiosulfate-oxidizing multienzyme system (TOMES) (149). This system allows *P. versutus* to grow autotrophically with compounds such as thiosulfate or sulfite as the sole source of energy, and the system has been biochemically characterized (references 148 and 149 and references therein). *P. denitrificans* can also use thiosulfate as an energy source for autotrophic growth (86, 319), and *P. denitrificans* GB-17 (*P. pantotrophus*) was originally isolated as a facultative chemolithotroph (247). Strain GB-17 was found to be the most amenable strain for Tn5 mutagenesis and subsequent selection for sulfur oxidation (Sox) mutants (43).

One strain deficient in thiosulfate oxidation (43) was found to be missing one of the megaplasmids (that of 450 kbp) characteristic of *P. denitrificans* GB-17 strains (43, 190, 312). Sulfur oxidation cannot be definitively mapped exactly to this molecule, since the smaller (110-kbp) megaplasmid was also reduced in size. Another of the Sox mutants of *P. denitrificans* with a full complement of megaplasmids (TP19) was used to isolate a locus coding for some of the TOMES proteins (188). The 13-kbp region isolated hybridized with identical bands of *Eco*RI-restricted genomic DNA of type strains of *P. denitrificans*, but *P. versutus* showed a different hybridization pattern. Although the *sox* locus hybridized to *R. capsulatus* DNA, other thiosulfate-oxidizing bacteria, such as members of the "true" thiobacilli, did not appear to contain homologous sequences. The region in which the Tn5 had interrupted *sox* expression was named *soxB* (326); it coded for a periplasmic protein with similarity to the protein sequence of a sulfur oxidation enzyme purified from *P. versutus*. This enzyme (protein B) has been shown to play an essential part in thiosulfate oxidation (173).

Further sequencing of the *sox* locus (327) reveals further ORFs, possibly transcriptionally linked to *soxB*. The ORF named *soxC* has high identity to the nucleus-encoded sulfite oxidases of mammalian cells. Despite the differences in conserved residues between SoxC and other molybdopterin-containing proteins (327), the presence of an unusually long periplasmic targeting sequence containing a double arginine (20, 349) suggests that *P. denitrificans* GB-17 SoxC is a molyb-
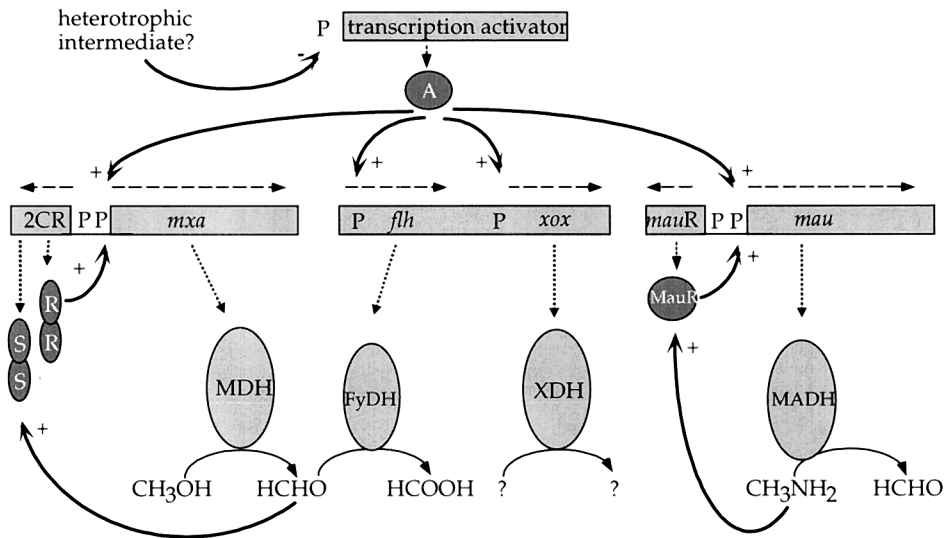
FIG. 6. Regulation of gene expression during C_1 metabolism in *P. denitrificans*. P, promoter; FyDH, formaldehyde dehydrogenase; XDH, *xox* gene products; Ami, amicyanin; SS, unknown signal sensor; RR, unknown response regulator; 2CR, two-component regulatory genes.

doprotein. The activity of SoxC as sulfite dehydrogenase was confirmed by the absence of activity in a *soxC* mutant (327). Of the remaining ORFs, *soxD* and *soxE* code for *c*-type cytochromes (perhaps corresponding to c_{551} and $c_{552.5}$, respectively) whereas *soxF* codes for a protein with high homology to the flavin domain of *Chromatium vinosum* flavocytochrome *c*. The role of this flavoprotein has yet to be integrated into the TOMES scheme proposed by Kelly et al. (149).

To summarize this section on the regulation of respiratory genes, it has emerged that the control of the changeover by *P. denitrificans* for aerobic growth involves an unanticipated complexity. Particularly intriguing is the existence of the Nnr protein as well as FnrP, when considered in the context that they can each differentiate between apparently identical DNA binding sequences. Hence, FnrP seemingly cannot substitute for Nnr and vice versa. The activation of Nnr by NO is also a remarkable finding. A related complexity applies to the regu-

lation of genes for C_1 metabolism. Here again, a toxic intermediate, in this case formaldehyde, appears to be the signalling molecule.

OTHER NONRESPIRATORY SYSTEMS

Members of the genus *Paracoccus* have not been studied solely with respect to their electron transport chains and unusual modes of metabolism. *P. denitrificans*, in particular, has proved to be a source of proteins (and their corresponding genes) with properties differing slightly from their better-known *E. coli* counterparts. In the remaining part of the review, a survey of these genes is presented. The only loci that are not discussed below are those of ribosomal protein S13, preprotein translocase (228), regulators of C_1 metabolism (241), and additional methanol oxidation genes (242). Although these loci have been sequenced and deposited in the
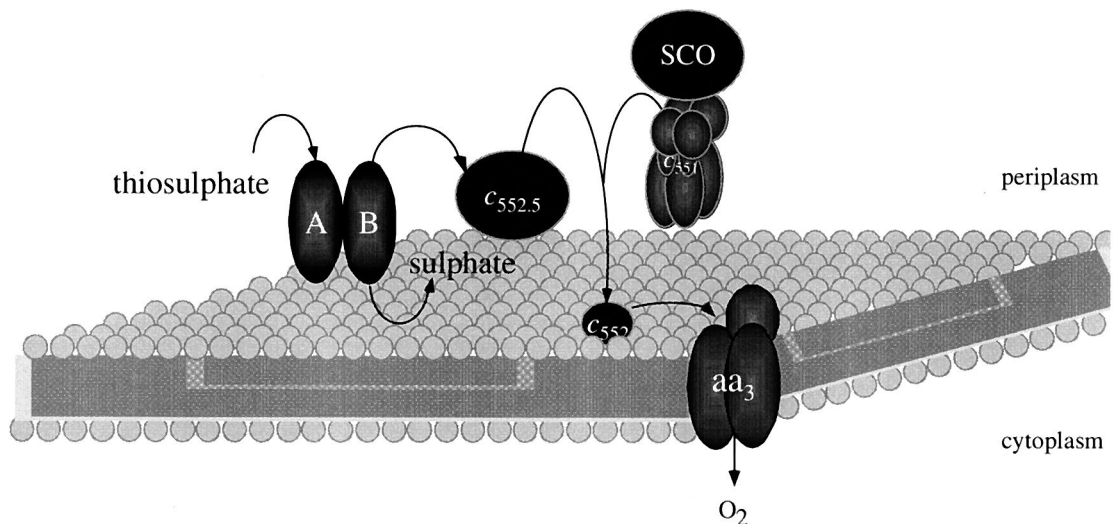

FIG. 7. Thiosulfate-oxidizing multienzyme system. The figure is derived from the scheme devised by Kelly (140) for *P. versutus*. SCO, sulfite:cytochrome *c* oxidoreductase; A, enzyme A (SoxA); B, enzyme B (SoxB).

```
Ec/aroF   TGTGTAAATAAAAATGTACGAAATATGGATTGAAAACTTTACTTT   EC 42
Ec/tyrP   ATTGTACATTTATATTTACACCATATGTAACGTCGGTTTGACGAA   EC 53
Ec/aroL   GGTGTATTGAGATTTTTCACTTTAAGTGGAATTTTTTCTTTACAAT   EC 53
Ec/aroP   GATGTAAACAAATTAATACAACAAACGGAATTGCAAACTTACACA   EC 47
Ec/aspC   TCTGTAACCATAATGGAACCTCGCATGTTTGAGAACATTACCGCC   EC 27
St/tyrB   TCTGTAAACCTGGAGAACCATCGCGTGTTTCAAAAAGTTGACGCC   ST 33
Ec/tyrB   CCCGTAAACCTGGAGAACCATCGCGTGTTTCAAAAAGTTGACGCC   EC 29
Pd/tyrB   CCTTAAAATCGCAACCGGAGACGCGTGATGCTGGGCAATCTGAAA   PD 33


          ++++++++++++++++++++++|++++++++++++++++++++
TyrR consensus  agTGTAAat---t-TTtACa-a|agTGTAAat---t-TTtACa-a
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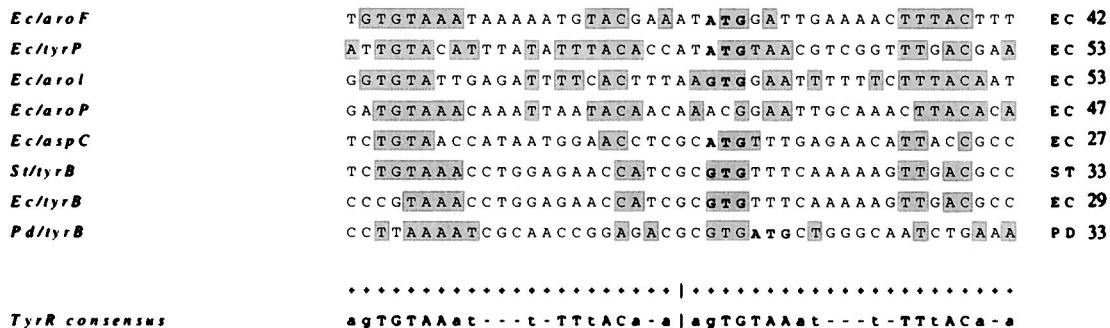
FIG. 8. Comparison of TyrR-dependent promoters in *E. coli* (*Ec*) and *S. typhimurium* (*St*) with the *tyrB* promoter region of *P. denitrificans* (*Pd*). The number at the end of the sequence denotes the percent identity of the TyrR box to the TyrR consensus.

database, there is no complementary biochemical information available.

Poly-β-Hydroxybutyrate Synthesis

P. denitrificans can produce a variety of polyhydroxyalkanoates, presumably as a reserve of carbon and energy (338). The homopolyester poly(3-hydroxybutyrate) is the predominant product during growth on methanol, but if *n*-pentanol is used, poly(3-hydroxyvalerate) is produced. The copolyester poly(3-hydroxybutyrate-co-3-hydroxyvalerate) is produced during growth on *n*-propanol (338). The genes responsible for this process (*phA* [β-ketothiolase], *phaB* [acetoacetyl coenzyme A reductase], and *phaC* [poly(3-hydroxyalkanoate) synthase]) have been identified in several bacteria, notably the closely related *R. sphaeroides* (274). The genes *phaA*, *phaB* (336), and *phaC* (298) have been identified in *P. denitrificans*. PhaA is 56% identical to PhaA of *R. sphaeroides*, and the 1,902-bp gene is possibly transcribed from its own promoter in *E. coli* (298), as judged from in vivo *lacZ* fusion studies. No detailed study of the promoter region has been made. Certainly, there is no obvious sequence corresponding to the *E. coli* σ[70] consensus, but the gene is followed by a Rho-independent terminator-like sequence. Reintroduction of the gene into *P. denitrificans* on a broad-host-range plasmid leads to a twofold increase in the amount of polyhydroxyalkanoate synthesized (298).

Aromatic Amino Acid Transferase

The gene *tyrB* codes for a multifunctional enzyme, aromatic amino acid transferase, involved in transamination reactions between amino acids (phenylalanine or tyrosine) and 2-oxoglutarate. In *E. coli*, the action of TyrB is complemented by a very similar enzyme, AspC, which transfers amino groups between aspartate and 2-oxoglutarate (83). The *tyrB* gene is subject to product repression in *E. coli*, with the concentration of tyrosine mediating expression of the gene via the TyrR repressor (reference 229 and references therein).

The DNA encoding TyrB from *P. denitrificans* has been sequenced, and the primary sequence of the protein showed high similarity to that of the *E. coli* protein (207). The restricted substrate specificity of the *P. denitrificans* enzyme implies that this protein can be better used as a model for a dual-substrate recognition than can the more catholic *E. coli* enzyme. Although active *P. denitrificans* TyrB can be expressed in appropriate mutants of *E. coli*, little interest has been shown in the regulation of *tyrB* in *P. denitrificans*. However, the putative *P. denitrificans* *tyrB* promoter/operator region has many elements in common with its enterobacterial counterparts. Sequences with significant identity to *E. coli* TyrR boxes (33% compared to the TyrR box consensus) can be found in a similar position relative to the *tyrB* promoters of *E. coli* (83) and *Salmonella typhimurium* (194) (Fig. 8). The TyrR boxes in the *tyrB* genes of *E. coli* and *S. typhimurium* have similarly low identity (27 and 33%, respectively) to the consensus. The optimal alignment is found if GTG is considered to be the start codon, rather than the ATG used in vivo (207).

The *tyrB* gene was characterized from an earlier clone of *P. denitrificans* aromatic amino acid transferase locus, isolated by Tagaki et al. (283). This sequence, although not yet in the databases, has a large region (>1,000 bp) of upstream DNA, which contains an ORF transcribed in the same direction as *tyrB* (5). This ORF is most likely to be the gene encoding thiosulfate sulfur transferase (see below). Transcriptional read-through from the putative sulfur transferase gene to *tyrB* is probably prevented by the rho-independent-like termination sequence found between the two genes (5).

Porin

Porin from *P. denitrificans* was first described by Zalman and Nikaido (344) as a dimer, but subsequent experiments (320) and its eventual crystallization (124) showed that porin is trimeric. The porin gene (*porG*) has had its transcript start site determined by S1 nuclease mapping (Table 5). Although an *E. coli* σ[70]-like hexamer can be seen at the −10 region, the presence of a −35 hexamer is not clear. The 3′ region of the *porG* locus includes a terminator-like stem-loop structure (254).

Adenylate Kinase

The adenylate kinase of *P. denitrificans* is a member of the nucleotide phosphate kinase family (272) and plays a role in phosphate group turnover, allowing the formation of ATP from ADP. Sequencing of the *adk* gene revealed that the protein from *P. denitrificans* is unusual in that it is more similar to the adenylate kinases from gram-positive bacteria than to those of its nearer relations, the gram-negative bacteria (66). Adenylate kinase from *P. denitrificans* is thought to contain zinc and iron, although the exact binding site of these ions has yet to be determined (66).

Thiosulfate Sulfur Transferase (Rhodanese)

A protein translated from DNA sequenced by Tagaki et al. (283), upstream of *tyrB* (see above), showed high homology to the thiosulfate sulfur transferases (rhodanese) of *E. coli*, (57% identity) and to a lesser extent to the mammalian isoenzymes from cows (41% identity) and humans (36% identity). The

identities were deduced after the published ORF had frame-shifts resolved to maximize identity (5). The function of this protein in either eubacterial or mammalian cells is not clear, but it has been implicated in the cysteine and methionine catabolic pathways and also in the detoxification of cyanide. ORFs similar to those coding for rhodanese have been found in all the eubacterial genomes sequenced so far. Although rhodanese is periplasmic in *P. versutus* (174), the signal sequence in the *P. denitrificans* protein is not clear.

It is difficult from the homology alone to propose a function for the rhodanese ORF. The *E. coli* genome (28) contains three rhodanese-like ORFs. Despite the name, this class of protein is not implicated in the oxidation of thiosulfate in *P. versutus* (174). Further upstream of the putative rhodanese gene is another ORF, probably coding for the polypeptide named "small protein b" (*sprB* [186]) in the *E. coli*, *Neisseria meningitidis*, *Haemophilus influenzae*, and *Enterococcus faecalis* genome-sequencing projects.

CONCLUDING REMARKS

The endowment of considerable metabolic versatility, especially with regard to respiration, requires *P. denitrificans* to have mechanisms for selecting which set or sets of respiratory genes to express. One prominent example is the switch from aerobic respiration with oxygen as a terminal electron acceptor to anaerobic respiration with an alternative terminal electron acceptor. In the latter situation, (i.e., denitrification), reductases for nitrate, nitrite, nitric oxide, or nitrous oxide have to be expressed along with any genes necessary for their biogenesis. It might have been expected, contrary to the experimental results, that a single global regulator would control denitrification because *P. denitrificans* and other denitrifying members of the genus *Paracoccus* have only two choices to sustain ATP production, aerobic or anaerobic respiration, there being no mode of fermentation.

The preference for aerobic respiration can be rationalized on the basis that passage of electrons to any oxidase of the respiratory system probably leads to a higher stoichiometry of proton translocation, and hence of ATP synthesis, than does electron transport to any of the reductases associated with the denitrification mode of respiration. It is emerging that the switch from aerobic to anaerobic respiration is controlled by a number of interacting factors rather than a single global factor. Clearly, there is a complex regulatory network. As the dissolved-oxygen tension declines, the transcriptional regulator FnrP is activated, thus stimulating the expression of the high-affinity oxidase (cytochrome cbb_3) as well as the respiratory nitrate reductase. It is likely, though not yet proven, that the expression of the latter will be further stimulated by the availability of nitrate, sensed by a NarL/NarX system analogous to that found in *E. coli*. However, the reductases for nitrite, nitric oxide, or nitrous oxide are not controlled, at least directly, by FnrP. Under natural conditions, it is possible that *P. denitrificans* encounters nitrous oxide alone, in which case switching on the entire denitrification pathway from the nitrate reductase onward would be wasteful. Nitrite and nitric oxide reductases must be coexpressed to stop the accumulation of toxic nitric oxide. They require for their expression the active form of Nnr, a second FNR-like transcription factor whose activation mechanism is at present unclear, despite the sequence similarity to FNR both in protein sequence and in the DNA binding sites upstream of the genes that the two proteins control. The evidence for the mechanism of action of Nnr suggests that the controlling signal is nitric oxide, in which case we are confronted with the relatively unusual situation in which the prod-uct of the nitrite reductase reaction activates expression of the enzyme responsible for its formation.

The denitrification system of respiratory proteins certainly has yet to reveal all the secrets of its regulatory mechanisms. For example, NirI, a protein of an unknown function, plays an undefined role in mechanisms of control for the expression of nitrous oxide reductase, which appears to be independent of both the FnrP and Nnr regulons. The reason for this complexity is not clear. To an extent, it may reflect the fact that the complete denitrification pathway comprises a set of proteins which individually or in some combination are also found in nondenitrifying bacteria (348). Thus, for example, nitrate reductase, with a similar gene organization and likely regulation to *P. denitrificans*, is found in *E. coli*. The occurrence of nitrite reductase genes and nitric oxide reductase genes in a cluster along with DNA coding for their regulatory factors on the genome of *P. denitrificans* could mean that these proteins were utilized by the bacterium later in its evolution, after nitrate reductase, and that the regulation of the complete denitrification pathway is overlaid on other existing sensory mechanisms.

The elucidation of the complex regulatory network that controls denitrification also requires identification of the types of RNAP that are involved. Our scrutiny of promoters in the alpha *Proteobacteria* shows that the previous consensus proposed for a σ^{70} polymerase binding site (5'-TCGGGN- $N_{(18 \pm 2)}$-GATNGS-3') (277), a binding site that is supposedly unique to *Rhodobacter* and *Paracoccus*, is not sufficient to define housekeeping promoters. Our analysis shows that *Paracoccus* and the alpha *Proteobacteria* have RNAP binding sites with some resemblance to the canonical *E. coli* sequence (5'-TT-GACA- $N_{(17 \pm 1)}$-TATAAT-3') (120), even though the data set is biased toward respiratory genes. The occurrence of canonical σ^{70} promoters is also indicated by the observation that counterparts of other *E. coli* DNA binding proteins recognize sequences similar to those described for *E. coli*. Clear examples are the binding sites for TyrR and FnrP in *P. denitrificans*. However, the definition of the fine structure of *Paracoccus* promoters requires the acquisition of more data, particularly from genes involved in the housekeeping functions of *Paracoccus*. However, preliminary results from the sequences of several promoter regions indicate that other sigma factors are involved in the transcription of denitrification genes. The discrimination between FnrP and Nnr activation could be on this basis.

Whereas the switch from aerobic respiration to denitrification involves a choice of electron acceptor, a choice of electron donor can also be made by *P. denitrificans*. Thus, if the bacterium encounters both a heterotrophic substrate (such as glucose, succinate or mannitol) and a C_1 substrate, like methanol or methylamine, its strategy is such that it will first metabolize the heterotrophic substrate and then metabolize the C_1 substrate. This strategy is sensible in that not only is the growth yield on heterotrophic substrates higher than that on C_1 substrates but also growth on the latter will present the cell with the toxic intermediate formaldehyde. The ability to choose the potential electron source is achieved by a very complex signal transduction network operating at the level of transcription. The current view envisages that all of the genes required for C_1 metabolism are silent during heterotrophic growth because a global transcriptional activator required for the transcription of all of them either is not synthesized or is inactive under these growth conditions. The concentration and/or activity of the global activator increases during depletion of the heterotrophic substrate and prepares the cell to switch metabolism. A dedicated signal sensor of a two-component regulatory system is activated by detecting formaldehyde, which is the reaction

product of methanol oxidation. The partner response regulator in turn acts synergistically with the global activator, and the *mxa* operon, encoding the enzymes required for methanol metabolism, is transcribed. In a similar sequence of events, enzymes required for methylamine metabolism are expressed. Signal transduction in this case, however, is mediated by a one-component transcriptional activator, MauR, which induces the *mau* operon encoding methylamine dehydrogenase upon binding of methylamine.

This survey of the molecular biology of *Paracoccus* has revealed that genes encoding respiratory proteins are scattered over both the replicons. It would appear that there is no need for a proximal location for these genes, a situation somewhat different from that found in clusters of genes encoding more defined metabolic pathways, such as the *mxa*, *mau*, *nir*, *nor*, and *nos* genes. These genomic loci encompass the genes encoding not only the structural protein but also the regulators of transcription, together with proteins required for activation, cofactor processing, and transport. In some instances, an electron transport donor or acceptor protein is also found on contiguous DNA (e.g., the *mxa* system). A coherent expression of all these proteins allows an efficient and rapid adaptation.

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