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CHAPTER 3.3.21

Nonmedical: *Pseudomonas*

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Introduction

Pseudomonas comprises a genus of species capable of utilizing a wide range of organic and inorganic compounds and of living under diverse environmental conditions. Consequently, they are ubiquitous in soil and water ecosystems and are important as plant, animal and human pathogens (Palleroni, 1992; Schroth et al., 1992). The genus *Pseudomonas* is well known for its metabolic versatility and genetic plasticity. The species of *Pseudomonas*, in general, grow rapidly and are particularly renowned for their ability to metabolize an extensive number of substrates, including toxic organic chemicals, such as aliphatic and aromatic hydrocarbons. Strains of *Pseudomonas* species are often resistant to antibiotics, disinfectants, detergents, heavy metals, and organic solvents. Some strains have been confirmed to produce metabolites that stimulate plant growth or inhibit plant pests.

Pseudomonas was already recognized as a complex collection of a large number of described species when the previous two editions of *The Prokaryotes* (Bergen, 1981; Schroth et al., 1981; Schroth et al., 1992; Stolp and Gadkari, 1981; Palleroni, 1992) were published. The heterogeneity of *Pseudomonas* has been well documented from comprehensive studies dating to more than 40 years ago. For example, the detailed survey by Stanier et al. (1966) of 267 strains of aerobic pseudomonads clearly exposed the catabolic diversity of the species comprising the genus and provided insight into the phylogenetic diversity that would become more apparent a few years later. Much of what can be written today about *Pseudomonas*, particularly related to the phenotypic, as well as metabolic characteristics, has already been described in detail by several different groups (Clark and Ornston, 1975; Clark and Richmond, 1975; Palleroni, 1975).

Wherever possible, the focus of this compilation on nonmedical aspects of the genus *Pseudomonas* is directed at information that has come to light since the last edition of *The Prokaryotes* in 1992. Much of microbial systematics has "evolved" in new directions within the last decade as a result of being able to access the phylogenetic relationships of microbial taxa. Elucidation of the "natural" relationships of bacteria has had a profound impact on the systematic reorganization of bacteria, in general, and on *Pseudomonas*, in particular. Many of the species that were described previously as *Pseudomonas* have been reclassified within new genera, and recognition of the phylogenetic heterogeneity of bacteria classified as *Pseudomonas* has initiated re-evaluations of the phenotypic characteristics, metabolic activities, genetics, ecology and other characteristics, in light of the inter- and intrageneric phylogenetic relationships. The early studies on the phylogenetic analyses of *Pseudomonas*, in fact, helped develop much of the methodology and technique that would be applied to the analyses of other prokaryotes in the years to come. Recently, advances in molecular biology, genome sequencing (the genomes of four species of *Pseudomonas* have been determined at the time of this writing), genomics and proteomics have presented a wealth of data for "pseudomonadologists" to access in trying to understand all facets of these bacteria. Researchers have only recently started to scale the mountain of information available.

Phylogeny

The phylogenetic heterogeneity of pseudomonads was recognized initially from work on the comparative biochemistry of the bacteria (Stanier, 1968), which established the groundwork upon which much of the later work was based. The ability to further differentiate *Pseudomonas* (sensu stricto) from phenotypically similar bacteria has been due, primarily, to the development and application of methods for analyzing bacteria at the molecular level. Palleroni and colleagues at the University of California at Berkeley (Palleroni et al., 1973) were able to discern distinct intrageneric

groupings among, what were, at that time, the species comprising the genus *Pseudomonas*, by the use of rRNA similarities. The group of De Ley, at the Universiteit Gent in Belgium, also applied the use of rRNA-DNA hybridization to an extensive number of studies on the pseudomonads. De Vos et al. (1989) proposed, and it is now generally accepted, that the genus *Pseudomonas* is limited to the species related to *P. aeruginosa* in the DNA-rRNA homology group I (Palleroni et al., 1973), within the γ-subclass of the Proteobacteria (Woese et al., 1985), currently reorganized as the class "Gammaproteobacteria" (Krieg and Garrity, 2001). The species comprising this grouping have been referred to as the "true *Pseudomonas*" or the "fluorescent pseudomonads" because of the notable fluorescent, water-soluble pigments produced by *P. aeruginosa*, *P. fluorescens* and some other well-known species of the genus. Nevertheless, to add yet another note of confusion to the complexity of *Pseudomonas*, not all species of the so-called "fluorescent pseudomonads" actually produce fluorescent pigments, e.g., *P. alcaligenes*, *P. mendocina*, *P. stutzeri*, to name but a few. To differentiate systematically *Pseudomonas* (sensu stricto) from other bacteria that were included previously in the genus or those that are newly isolated and characterized, analyses of ribosomal RNA (rRNA) similarities have proven applicable for inferring definitive systematic relationships to the genus level (in most cases) and to subgenus levels (in some cases).

The rRNA sequence similarities between *Pseudomonas* species were determined initially by hybridization of DNA to ribosomal rRNA, in the group of Roger Stanier at the University of California at Berkeley (Palleroni, et al., 1973). The hybridization methodology revealed internal subdivisions of five distinct rRNA "homology groups" that corresponded to the levels of differentiation observed between different genera or families. The observed subdivisions of *Pseudomonas* were confirmed repeatedly by various methodologies performed throughout the following years in many other laboratories (Byng et al., 1983; De Vos and De Ley, 1983; Oyaizu and Komagata, 1983; De Vos et al., 1985; De Vos et al., 1989; Stead, 1992).

Subsequent to the analyses using the DNA-rRNA hybridization methods, another approach targeting rRNAs was applied to the analysis of *Pseudomonas* in the laboratory of Carl Woese at the University of Illinois. Utilizing a protocol of sequence determination and profiling of rRNA oligonucleotides to produce taxa-specific "catalogues" (Fox et al., 1980), Woese et al. (1984) described the diversity of the pseudomonads along the same divisions that had been recognized previously by the Berkeley group. Essentially, some species that were classified as members of the genus *Pseudomonas* were observed to be less similar phylogenetically to other pseudomonads than they were to non-pseudomonad bacteria, e.g., enterics, phototrophs, nitrogen-fixing plant symbionts, etc. With such approaches, the "natural" system of bacterial classification that had been proposed as theoretically feasible by microbiologists such as Van Niel (1946), but which had remained so elusive practically, was acknowledged to offer significant possibilities for bacterial systematics.

While the DNA-rRNA hybridization and rRNA oligonucleotide cataloguing methods were able to provide overviews of the phylogenetic relationships of bacteria, the proponents of the methods acknowledged their respective limitations in being able to differentiate the closely related organisms (Lane et al., 1985; Woese, 1987). To this end, complete sequence determinations of the rRNAs were envisioned as the means for establishing a phylogeny-based bacterial systematics with the resolution to differentiate even the most closely related organisms. The polymerase chain reaction (PCR; Mullis and Faloona, 1987), combined with advances in DNA sequencing (Hunkapiller et al., 1991), has enabled the determination and comparison of rRNA gene sequences (i.e., rDNA) to become a practical methodology in most microbiology laboratories for rapidly analyzing large numbers of organisms (Edwards et al., 1989). Thus, the number of prokaryotic small subunit (i.e., 16S) rRNA/rDNA sequences compiled in databases has grown from less than 300 in 1990 (Neefs et al., 1991) to more than 35,000 (nearly full-length), as of June 2004 see the Ribosomal Database Project - II (http://rdp.cme.msu.edu) (Cole et al., 2003). Of these, only five pseudomonad 16S rRNA sequences were available in 1990. Currently, more than 1300 16S rRNA/rDNA sequences of strains of *Pseudomonas* species are available in the public databases.

The differentiation and inferred phylogenetic relationships of the various pseudomonad rRNA similarity groups, as well as of the species included in each one of the groups, by comparative analysis of 16S rRNA gene sequences has been described in detail in two comprehensive reviews (Kersters et al., 1996; Anzai et al., 2000). Not surprisingly, the phylogenetic relationships inferred from 16S rDNA sequence analyses corresponded with the earlier results determined by rRNA-DNA hybridization and rRNA cataloguing data. Thus, species retained within the genus *Pseudomonas* (sensu stricto) are observed to be those related to *P. aeruginosa*, in the Gammaproteobacteria, while other species are seen to cluster within genera of the Alphaproteobacteria

and Betaproteobacteria. The bacteria most closely related to the genus *Pseudomonas* include the species of the aerobic, free-living, nitrogen-fixing *Azotobacter-Azomonas* complex, cellulolytic species of the genus *Cellvibrio* and, somewhat more distantly related, marine bacteria of the genera *Microbulbifer* and *Marinobacterium*, endosymbiotic bacteria of the genus *Teredinibacter*, halophilic bacteria of the genera *Halomonas*, *Oceanospirillum* and *Marinomonas*, *Marinobacter* species isolated from marine environments, the Moraxellaceae family, and methylotrophs of the Methylococcaceae (Kersters et al., 1996; Anzai et al., 2000). These organisms exhibit quite diverse phenotypes and, on the basis of traditional analyses of phenotypic characteristics, probably would not be suspected as being the evolutionary "cousins" of *Pseudomonas*.

Taxonomy

The genus *Pseudomonas* comprises Genus I of the bacterial family Pseudomonadaceae. Five genera (*Pseudomonas*, *Azotobacter*, *Azomonas*, *Azorhizophilus* and *Cellvibrio*) are assigned to the family. Common to all constituent genera are certain physiological properties, such as aerobic, chemoorganotrophic metabolism, absence of fermentation, absence of photosynthesis, and capacity for growth at the expense of a large variety of organic substrates. There are a few exceptions to these general properties, but these phenotypic criteria are generally common to all members of the family.

The original creation of the genus *Pseudomonas* established a taxon based solely upon characteristics of cell morphology. At this point, it is appropriate to point out a discrepancy with respect to the publication date for the presentation of the genus. While the date for the publication of the genus *Pseudomonas* has been recognized as being 1894, Gunsalus (1996) and Zumft (1997) have pointed out previously that the genus was, in fact, presented for the first time in 1895 by Walter Migula at the Bacteriologischen Institut der Technischen Hochschule zu Karlsruhe in his publication of a seven-year effort, "Ueber ein neues System der Bakterien" (Migula, 1895), to describe and compare all known bacteria. The initial description of *Pseudomonas* by Migula was based solely upon morphological characteristics, as follows (translation):

Genus *Pseudomonas* nov. gen.

Cells with polar flagella. Endospore formation occurs in some species, but infrequently (e.g., *Pseudomonas violacea*).

This "succinct" description established the new genus within the family Bacteriaceae, accommodating bacteria on the basis of characteristic flagella type and the greenish or blue-green fluorescent pigment of the pus collected in bandages of hospital patients and wounded soldiers. A more comprehensive description of the genus followed in 1900 (Migula, 1900), including 75 species and registering *Pseudomonas aeruginosa*, previously described as "*Bacterium aeruginosum*" (Schroeter, 1872), as the type species of the genus.

Winslow et al. (1917) established the family Pseudomonadaceae, encompassing the genus *Pseudomonas* and a number of other genera, many of which have been reclassified throughout the following years. Many of the species of genera that were categorized within the family Pseudomonadaceae have come to be regarded as "pseudomonads" and, by association, related to *Pseudomonas*. This has proven often to be a source of confusion. Two terms should be defined at this point to clarify an important nomenclatural distinction that is often confusing. "*Pseudomonas*" (capitalized and written in italics) is the validly published (i.e., with nomenclatural standing) name of a bacterial genus comprising species of defined collective phenotypic characteristics. On the other hand, "pseudomonad" (not capitalized and not italicized) is a descriptive term (i.e., *Pseudomonas*-like), with no formal nomenclatural status, accorded to a nonexclusive collection of bacteria exhibiting various levels of similarity to species of the genus *Pseudomonas*. Pseudomonad bacteria previously characterized according to a limited selection of traits may have been observed to be similar to bacteria previously identified (correctly or incorrectly) as *Pseudomonas* species. Unfortunately, taxonomic convention, combined with a degree of uncertainty associated with the identifications of some pseudomonad bacteria, has led to the point that the terms "*Pseudomonas*" and "pseudomonad" are sometimes used interchangeably and incorrectly.

In the eighth edition of *Bergey's Manual of Determinative Bacteriology*, 29 well-characterized species of *Pseudomonas* were listed, with another 206 less well-described species included as addenda (Douderoff and Palleroni, 1974a). This was the first compilation of the Bergey's series that incorporated molecular data, i.e., the G+C content of genomic DNA (Marmur and Doty, 1962), in the descriptions of the bacterial species. However, the level of taxonomic resolution that genomic DNA mol% G+C could afford was limited and, at that time, the data were handled simply as an additional determinative characteristic without any systematic weight.

The Approved Lists of Bacterial Names published in the *International Journal of Systematic Bacteriology* in 1980 (Skerman et al., 1980), provided an inventory of bacterial species names described before 1980 that were recognized as having formal nomenclatural standing under the auspices of the International Code of Nomenclature of Bacteria (1976 Revision). The list included 87 *Pseudomonas* species, effectively reducing the number of *Pseudomonas* nomenspecies by not including names that were inconsistent with the rules of the Code.

In 1984, in the first edition of *Bergey's Manual of Systematic Bacteriology*, a listing of 94 *Pseudomonas* nomenspecies was presented (Palleroni, 1984b). Many of the species were included with the recognition that their taxonomic relationships were unclear but with the expectation that comprehesive analyses would conclusively define their taxonomic positions. Within the first edition of *Bergey's Manual of Systematic Bacteriology*, perhaps the most significant contribution to the taxonomy of *Pseudomonas* was the presentation of the subdivision of the genus on the basis of rRNA similarities and estimations of phylogenetic relatedness (Palleroni et al., 1973; Palleroni, 1984). The five distinct "rRNA homology" groups were: 1) rRNA group I—*P. aeruginosa*, *P. fluorescens*, *P. putida* and related species, also termed the "true" *Pseudomonas* (i.e., *Pseudomonas* sensu stricto); this group included species observed to cluster within Woese's γ-subdivision of the Proteobacteria (Woese et al., 1985; Stackebrandt et al., 1988); 2) rRNA group II—*P. cepacia*, *P. mallei* and related species (to be reclassified as *Burkholderia*) and *P. solanacearum*, *P. picketti* and related species (to be reclassified as *Burkholderia* and, subsequently, as *Ralstonia*); this group included species observed to cluster within Woese's β-subdivision of the Proteobacteria (Woese et al., 1984); 3) rRNA group III—*P. testosteroni* and related species (to be reclassified as *Comamonas*), *P. acidovorans* and related species (to be reclassified as *Comamonas* and, subsequently, as *Delftia*), *P. facilis* and related species (to be reclassified as *Acidovorax*), *P. palleronii* and related species (to be reclassified as *Hydrogenophaga*), and *P. saccharophila* (not reclassified, to date); this group included species also observed to cluster within Woese's β-subdivision of the Proteobacteria; 4) rRNA group IV—*P. diminuta* and *P. vesicularis* (to be reclassified as *Brevundimonas*); this group included species observed to cluster within Woese's α-subdivision of the Proteobacteria (Woese et al., 1984); and 5) rRNA group V—*P. maltophilia* (reclassified as *Xanthomonas* and, subsequently, as *Stenotrophomonas*); this group included species observed to cluster also within Woese's γ-subdivision of the Proteobacteria but clearly distinct from *Pseudomonas*.

These data effectively established the framework for the modern taxonomy of *Pseudomonas*, as well as other pseudomonads. Since then, many of the organisms described initially as species of *Pseudomonas* have been reclassified and new species have been added to the genus, largely because of the ability to recognize the phylogenetic relationships that have been determined through genotypic characterization. *Pseudomonas*, described in the second edition of *Bergey's Manual of Systematic Bacteriology* (Palleroni, 2004), comprises 61 species, representing the number of species of the genus that were available in the literature until the end of year 2000. Since the beginning of year 2001 until the time of this writing (June 2004), 30 new species of *Pseudomonas* have been described and the names validly published.

The taxonomic status of *Pseudomonas*, including the reclassifications of species formerly included within the genus, and the addition of new species, can be observed on-line through the internet at two principal sites: 1) the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) site for "Bacterial Nomenclature Up-to-Date" (http://www.dsmz.de/bactnom/bactname.htm); and 2) the (http://www.bacterio.cict.fr{site}) of the École Nationale Vétérinaire, "J.P. Euzéby "List of Bacterial Names with Standing in Nomenclature." These sites have compiled all the bacterial names of the "Approved Lists of Bacterial Names" (Skerman et al., 1980), as well as those that have been validly published since January 1, 1980.

Currently, *Pseudomonas* includes 156 species names that are recognized with nomenclatural standing, although 48 of these species have been reclassified and are now considered to be basonyms or synonyms of species placed in other genera (Table 1). Two species, *P. aureofaciens* and *P. perfectomarina*, are recognized as later heterotrophic synonyms of *P. chlororaphis* and *P. stutzeri* (genomovar 2), respectively. Another 10 species are recognized as not belonging to *Pseudomonas* (sensu stricto) but have not been reclassified as yet. At the time of this writing, 96 species have been described and recognized as belonging to the genus *Pseudomonas* (sensu stricto) and are listed with names of nomenclatural standing.

Habitat

Pseudomonas is a genus of truly ubiquitous organisms, which seems to be a consequence of their simple nutritional requirements, the range

Table 1. *Pseudomonas* species with validly published names and their current taxonomic status.

Species name[a]	Type strain	Re-classification[b]	16S rDNA accession no.[c]
P. abietaniphila	ATCC 700689		AJ011504
P. acidovorans[d]	DSM 39	*Delftia acidovorans*	
P. aeruginosa[e]	LMG 1242		Z76651
P. agarici	LMG 2112		Z76652
P. alcaligenes	LMG 1224		Z76653
P. alcaliphila	JCM 10630		AB030583
P. aminovorans[d]	DSM 7048	*Aminobacter aminovorans*	
P. amygdali	LMG 1223		Z76654
P. andropogonis[d]	DSM 9511	*Burkholderia andropogonis*	
P. anguilliseptica	NCIMB 1949		AB021376
P. antarctica	DSM 15318		AJ537601
P. antimicrobica[d]	LMG 18920	*Burkholderia gladioli*	
P. asplenii	LMG 2137		Z76655
P. aurantiaca	ATCC 33663		AB021412
P. aureofaciens[f]	DSM 6698	*P. chlororaphis*	Z76656
P. avellanae	DSM 11809		
P. avenae[d]	DSM 7227	*Acidovorax avenae*	
P. azotoformans	IAM 1603		D84009
P. balearica	DSM 6083		U26418
P. beijerinckii[d]	DSM 7218	(*Halobacter* sp.)	
P. beteli[d]	LMG 978	(*Stenotrophomonas* sp.)	
P. boreopolis[d]	LMG 979	(*Xanthomonas* sp.)	
P. brassicacearum	CFBP 11706		AJ293858
P. brenneri	CIP 106646		AF268968
P. cannabina	CFBP 2341		AJ492827
P. carboxydohydrogena[d]	DSM 1083		
P. caricapapayae	ATCC 33615		D84010
P. caryophylli[d]	JCM 9310	*Burkholderia caryophylli*	
P. cattleyae[d]	LMG 5286	*Acidovorax avenae*	
P. cedrina	CIP 105541		AF064461
P. cepacia[d]	LMG 1222	*Burkholderia cepacia*	
P. chloritidismutans	DSM 13592		AZ017341
P. chlororaphis	LMG 5004		Z76657
P. cichorii	LMG 2162		Z76658
P. cissicola[d]	LMG 2167	(*Xanthomonas* sp.)	
P. citronellolis	DSM 50332		Z76659
P. cocovenenans[d]	DSM 11318	*Burkholderia cocovenenans*	
P. congelans	DSM 14939		AJ492828
P. corrugata	ATCC 29736		D84012
P. costantinii	CFBP 5705		AF374472
P. cremoricolorata	IAM 1541		AB060137
P. delafieldii[d]	DSM 64	*Acidovorax delafieldii*	
P. denitrificans[d]	ATCC 19244	*nomen ambiguum*	
P. diminuta	DSM 7234	*Brevundimonas diminuta*	
P. doudoroffii[d]	DSM 7028	*Oceanimonas doudoroffii*	
P. echinoides[d]	DSM 50409	*Sphingomonas echinoides*	
P. elongata[d]	DSM 6810	*Microbulbifer elongatus*	
P. extremorientalis	LMG 19695		AF405328
Pseudomonas facilis[d]	DSM 649	*Acidovorax facilis*	
P. ficuserectae[f]	LMG 5694	(*P. amygdali*)	Z76661
P. flava[d]	DSM 619	*Hydrogenophaga flava*	
P. flavescens	ATCC 51555		U01916
P. flectens[d]	LMG 2187		
P. fluorescens	DSM 50090		Z76662
P. fragi	IFO 3458		AB021413
P. frederiksbergensis	DSM 13022		AJ249382
P. fulva	IAM 1529		D84015
P. fuscovaginae[f]	MAFF 301177	(*P. fuscivaginae*)	AB021381
P. gelidicola	IAM 1127		
P. geniculata[d]	LMG 2195	(*Stenotrophomonas* sp.)	
P. gessardii	CIP 105469		AF074384
P. gladioli[d]	DSM 4285	*Burkholderia gladioli*	

Table 1. *Continued*

Species name[a]	Type strain	Re-classification[b]	16S rDNA accession no.[c]
P. glathei[d]	DSM 50014	*Burkholderia glathei*	
P. glumae[d]	DSM 9512	*Burkholderia glumae*	
P. graminis	DSM 11363		Y11150
P. grimontii	CIP 106645		AF268029
P. halophila	DSM 3050		AB021383
P. hibiscicola[d]	LMG 980	(*Stenotrophomonas* sp.)	
P. huttiensis[d]	DSM 10281	(*Herbaspirillum* sp.)	
P. indica	DSM 14015		AF302795
P. indigofera[d]	DSM 3303	*Vogesella indigofera*	
P. iners[d]	CIP 106746	*Marinobacterium georgiense*	
P. jessenii	CIP 105274		AF06825
P. jinjuensis	LMG 21316		AF468448
P. kilonensis	DSM 13647		AJ292426
P. koreensis	LMG 21318		AF468452
P. lanceolata	ATCC 14669		AB021390
P. lemoignei[d]	DSM 7445	*Paucimonas lemoignei*	
P. libanensis	CIP 105460		AF057645
P. lini	ICMP 14138		AY035996
P. lundensis	ATCC 49968		AB021395
P. lutea	LMG 21974		AY364537
P. luteola	IAM 13000		D84002
P. mallei[d]	ATCC 23344	*Burkholderia mallei*	
P. maltophilia[d]	DSM 50170	*Stenotrophomonas maltophilia*	
P. mandelii	CIP 105273		AF058286
P. marginalis	LMG 2210		Z76663
P. marina[d]	DSM 4741	*Cobetia marina*	
P. mediterranea	CFBP 5447		AF386080
P. meliae[f]	LMG 2220	(*P. amygdali*)	AB021382
P. mendocina	LMG 1223		Z76664
P. mephitica	ATCC 33665		AB021388
P. meridiana	DSM 15319		AJ537602
P. mesophilica[d]	DSM 1708	*Methylobacterium mesophilicum*	
P. migulae	CIP 105470		AF074383
P. mixta[d]	DSM 4832	*Telluria mixta*	
P. monteilii	CIP 104883		AB021409
P. mosselii	CIP 105259		AF072688
P. mucidolens	IAM 12406		D84017
P. multiresinivorans	ATCC 700690		X96787
P. nautica[d]	DSM 50418	*Marinobacter hydrocarbonoclasticus*	
P. nitroreducens	IAM 1439		D84021
P. oleovorans	DSM 1045		Z76665
P. orientalis	CIP 105540		AF064457
P. oryzihabitans	IAM 1568		D84004
P. palleroniana	CFBP 4389		AY091527
P. palleronii[d]	DSM 63	*Hydrogenophaga palleronii*	
P. parafulva	JCM 11244		AB06013
P. paucimobilis[d]	DSM 1098	*Sphingomonas paucimobilis*	
P. perfectomarina[f]	ATCC 14405	*P. stutzeri*	
P. pertucinogena	IFO 14163		AB021380
P. phenazinium[d]	DSM 10684	*Burkholderia phenazinium*	
P. picketti[d]	DSM 6297	*Ralstonia pickettii*	
P. pictorum[d]	LMG 981	(*Stenotrophomonas* sp.)	
P. plantarii[d]	DSM 9509	*Burkholderia plantarii*	
P. plecoglossicida	DSM 15088		AB009457
P. poae	DSM 14936		AJ492829
P. proteolytica	DSM 15321		AJ537603
P. pseudoalcaligenes	LMG 1225		Z76666
P. pseudoflava[d]	DSM 1034	*Hydrogenophaga pseudoflava*	
P. pseudomallei[d]	ATCC 23343	*Burkholderia pseudomallei*	
P. psychrophila	JCM 10889		AB041885
P. putida	DSM 291		Z76667
P. pyrrocinia[d]	DSM 10685	*Burkholderia pyrrocinia*	
P. radiora[d]	DSM 1819	*Methylobacterium radiotolerans*	

Table 1. *Pseudomonas*

Species name[a]	Type strain	Re-classification[b]	16S rDNA accession no.[c]
P. resinovorans	LMG 2274		Z76668
P. rhizosphaerae	LMG 21640		AY152673
P. rhodesiae	CIP 104664		AB021410
P. rhodos[d]	DSM 2163	*Methylobacterium rhodinum*	
P. rubrilineans[d]	LMG 2281	*Acidovorax avenae*	
P. rubrisubalbicans[d]	DSM 11543	*Herbaspirillum rubrisubalbicans*	
P. saccharophila[d]	DSM 654		
P. salomonii	CFBP 2022		AY091528
P. savastanoi[f]	ATCC 13522	(*P. amygdali*)	AB021402
P. solanacearum[d]	DSM 9544	*Ralstonia solanacearum*	
P. spinosa	ATCC 14606		AB021387
P. stanieri[d]	DSM 7027	*Marinobacterium stanieri*	
P. straminea	IAM 1598		D84023
P. stutzeri	CCUG 11256		U26262
P. synxantha	IAM 12356		D84025
P. syringae	LMG 1247		Z76669
P. syzygii[d]	DSM 7385	*Ralstonia syzygii*	
P. taeniospiralis[d]	DSM 2082	*Hydrogenophaga taeniospiralis*	
P. taetrolens	IAM 1653		D84026
P. testosteroni[d]	DSM 50244	*Comamonas testosteroni*	
P. thermotolerans	DSM 14292		AJ311980
P. thivervalensis	CFBP 11261		AF100323
P. tolaasii	LMG 2342		Z76670
P. tremae	CFBP 6111		AJ492826
P. trivialis	DSM 14937		AJ492831
P. umsongensis	KACC 10847		AF468450
P. vancouverensis	ATCC 700688		AJ011507
P. veronii	CIP 104663		AB021411
P. vesicularis[d]	DSM 7226	*Brevundimonas vesicularis*	
P. viridiflava	LMG 2352		Z76671
P. woodsii[d]	LMG 2362	*Burkholderia andropogonis*	

Abbreviations: ATCC, American Type Culture Collection, Manassas, Virginia, United States; CFBP, Collection Francaise des Bacteries Phytopathogenes, Station de Pathologie Végétale et Phytobactériologie, Beaucouzé Dedex, France; CCUG, Culture Collection University of Göteborg, Göteborg, Sweden; CIP, Collection de l'Institut Pasteur, Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; IAM, Institute for Applied Microbiology, University of Tokyo, Tokyo, Japan; ICMP, International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; IFO, Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, The Institute of Physical and Chemical Research, Hirowawa, Wako-shi, Japan; KACC, Korean Agricultural Culture Collection, National Institute of Agricultural Biotechnology, Seodun-dong Gwonseon-gu, Suwon, Korea; LMG, Laboratorium voor Mikrobiologie, Universiteit Gent, Gent, Belgium; and NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom.

[a]Validly published names of species of the genus *Pseudomonas*, species names that have not been validly published are not included.

[b]Reclassification names in parentheses indicate that a reclassification has not been formally proposed.

[c]Accession numbers are given only for species of *Pseudomonas* (sensu stricto).

[d]Species that should not be included within the genus *Pseudomonas*.

[e]*P. aeruginosa* is the type species of the genus.

[f]*Pseudomonas* species that should be reclassified with a new species name.

of carbon compounds they utilize, and their genetic and metabolic adaptability. *Pseudomonas* species have a wealth of habitats available to it, ranging from various soil and water environments to plant and animal tissue. Essentially, any habitat with a temperature range of 4–42 C, a pH between 4 and 8 and containing simple or complex organic compounds is a potential habitat for *Pseudomonas. Pseudomonas* species are aerobes and the requirement for oxygen is apparently the major constraining factor for habitation by *Pseudomonas*.

In fact, *Pseudomonas* species are generally found in soils and water environments that have aerobic, mesophilic and neutral pH conditions. In nature, *Pseudomonas* species exist as saprophytes and parasites. In general, they are not prominent in anaerobic environments, and they do not occur in extreme thermophilic or acidophilic habitats. On the basis of the data

derived from cultivation-dependent analyses such as selective enrichments and isolations, *Pseudomonas* would appear to occupy a prominent position in nature.

The conditions under which *Pseudomonas* species survive in soil are also favorable for growth of aerobic actinomycetes of the genus *Streptomyces* (prominent in the degradation of complex organic compounds) as well as many other types of bacteria. Soil-dwelling *Pseudomonas* grow in association with the streptomycetes and other bacteria, which may be providing them with monomeric carbon sources for growth.

The phytopathogenic *Pseudomonas* generally can be found only on diseased plants, in which they appear as relatively homogeneous populations when the pathological lesions are young. The distribution of many of these pathogens outside their host plants is poorly known, although many seem to be able to exist as saprophytes, as well.

Pseudomonas species play important roles in the food industry, where spoilage of meats, poultry and fish occurs, even under refrigerated conditions (Barrett et al., 1986). Some species of *Pseudomonas* are found in tap water and in hospital saline solutions, which are presumably devoid of nutrients (Van der Krooij, 1977). *Pseudomonas* can be particularly problematic in the development of biofilms within pipelines. New species of *Pseudomonas* have recently been isolated from relatively oligotrophic mineral water sources (Elomari et al., 1996; Dabboussi et al., 1999; Verhille et al., 1999; Ivanova et al., 2002).

Bacteria, including *Pseudomonas* species, traditionally have been studied as "planktonic forms" in cultures of broth media. However, bacteria surviving in various natural ecosystems are observed to live in small or extensive biofilm environments (Tolker-Nielsen and Molin, 2004). Quorum-sensing appears to play important roles in the development of biofilms, through the sensing of population densities and the exertion of some control in the expression of genes involved in the various stages of biofilm development (Whiteley et al., 2001).

This section describes some of the various habitats and niches of *Pseudomonas* species. While this treatment is relatively general, recent reviews have presented much more detailed overviews of the soil, rhizosphere and phyllosphere habitats of *Pseudomonas* (Bailey, 2004; Lugtenberg and Bloemberg, 2004; Sørensen and Nybroe, 2004).

Pseudomonas in Soil Environments

The soil environment may comprise the most complex of environmental niches that are encountered by microorganisms. The heterogeneity of factors for growth in a soil presents the bacteria a challenging matrix of aggregate surfaces and pore structures. As the relationships between soil, plants and microbes are so intricately interwoven, it is difficult to obtain consistent overviews of the relevant microorganisms that are clearly demarcated between bulk soil and rhizosphere soil. *Pseudomonas* species, as well as species of many other pseudomonad genera, are clearly impacted by the rhizosphere influence.

Soil-dwelling *Pseudomonas* species are distributed throughout the world and, in general, may be observed to comprise similar rRNA sequence types and similar rRNA gene intergenic spacer (IS) types, as determined from a worldwide sampling of fluorescent species of *Pseudomonas* for assessing the inherent endemicity of microbial populations (Cho and Tiedje, 2000). However, at the level determined by genome fingerprinting (BOX-PCR) analyses, i.e., the strain level, *Pseudomonas* may be seen to comprise endemic populations that are determined by the specific transects of sampling sites.

A predominant question in microbial ecology is related to the actual presence and abundance of bacterial taxa within the respective ecosystems. Estimates suggest that cultivations are able to detect less than 1% of the total microbial populations in soil samples. Thus, while *Pseudomonas* species are able to be isolated from most soil samples, still unclear is whether the results obtained from enrichments and isolations reflect the situations in situ. In general, studies of various bulk soil ecosystems, employing cultivation-independent analyses of bacterial diversity, do not reflect the diversity or proportion of *Pseudomonas* species that are normally obtained by cultivation-dependent approaches. Even in xenobiotic-contaminated soils, which readily yield a diversity of isolates of *Pseudomonas* species, cultivation-independent approaches suggest that other bacterial taxa are numerically more predominant and that *Pseudomonas* may play only a minor role (Nogales et al., 1999; Nogales et al., 2001). However, such observations may not be contradicting the true nature of *Pseudomonas* species, as saprophytes and parasites. Even though *Pseudomonas*, as a group, may be observed to take advantage of its diverse metabolic potential and simple nutrient requirements, various *Pseudomonas* species, with their relatively rapid growth, may discover that they are less able to compete in bulk soil environments with other bacterial taxa that may be better adapted to dispersed levels of nutrients and that may possess various strategies for withstanding periods of "starvation". While the soil ecosystem may appear to offer a diversity of

nutrient resources for microorganisms to use, the bioavailability of those resources, which may be complexed with inorganic matrices, also play an important role in selecting for which bacterial taxa are able to thrive in any given soil ecosystem. A comparative study of the bacterial taxa within bulk soil samples and in earthworm (*Lumbricus rubellus*) casts demonstrated significant differences in the numbers of *Pseudomonas* species detected, which was believed to be related to the nutrients available to the microorganisms during passage through the gut (Furlong et al., 2002). Limited available resources that may be obtained within bulk soil environments may not actually afford *Pseudomonas* species their best options and they may be more adapted to occupy an ecological niche as opportunistic scavengers in more nutrient-rich environments.

Pseudomonas in Association with Plants

The rhizosphere is referred to as a "zone" influenced by plants (Hiltner, 1904). This zone around the roots of plants is one of intense microbial activity, due to the secretion of organic and amino acids by the plants. *Pseudomonas* species are among the most competent rhizosphere colonizers of soil (Lugtenberg et al., 2001). Lugtenberg and Bloemberg (2004) have described the traits necessary for successful rhizosphere colonization, i.e., motility and chemotaxis, specialized pili for attachment to surfaces, lipopolysacharide (LPS) and outer membrane integrity for efficient uptake of nutrients, ability to synthesize vitamins and macromolecule building blocks, ability to utilize specific exudate components, resistance to toxins, and other plant defenses. *Pseudomonas* species, in general, possess all of these traits, which provide them with a selective advantage for exploiting the resources in the rhizosphere. *Pseudomonas* species also effectively inhibit the colonization of plants by other microorganisms (Bianciotto et al., 1996). Thus, *Pseudomonas* is seen as an important agent for biocontrol of plant diseases.

In addition to the microbial communities below ground, an extensive diversity of microorganisms (epiphytes) populate and interact with the surfaces of plants (phyllosphere), primarily the leaves. Bacteria that have adapted to life in the phyllosphere must possess characteristics that protect them from exposure, as well as nutrient and water limitations (Bailey, 2004). Mercier and Lindow (2000) have demonstrated the availability of amino acids, carbohydrates and organic acids that leach from the interior of the leaf to the exterior, providing significant levels of nutrients to plant surface colonizing bacteria. Bacteria appear to exist on plant surfaces, predominantly, as biofilms (Morris et al., 1998).

Epiphytes, including *Pseudomonas* species, may affect plant productivity negatively, e.g., through induction of frost injury (Lindow, 1995), or positively, e.g., by production of phytohormones that enhance development (Brandi et al., 2001). Just as specific traits enable certain bacteria to colonize successfully the rhizosphere environment, traits have also been identified that are associated with epiphytic fitness. Some of these traits are correlated with locations on leaf surfaces of pathogenic (i.e., *P. syringae*) and nonpathogenic species, and with the ability of the pathogens to access and multiply in protected sites of the phyllosphere (Wilson et al., 1999).

The phytopathogenic species of *Pseudomonas* are diverse with respect to their genetics, ecology, and the diseases they cause. One species, *P. syringae*, includes more than 50 pathovars, most of which specifically colonize different plant hosts. Phytopathogenic *Pseudomonas* species are distributed worldwide, causing diseases of most major groups of higher plants. Besides the pathogenic associations, *Pseudomonas* species also exist in other types of associations with plants. Some species affect plant growth through their inhibition of fungal plant pathogens or by their effects on the roots of plants. *Pseudomonas* species may further colonize other plant parts, with no apparent damage or benefit to the plant (Lodewyx et al., 2002).

Isolation

Pseudomonas species, in general, have simple nutritional requirements and are readily isolated from a variety of environments. In the laboratory they grow well in media containing some organic matter in solution, at neutral pH, and at temperatures in the mesophilic range. The optimal growth temperature for *P. aeruginosa*, the most likely species to be encountered in medical specimens, is 37 C. However, species of *Pseudomonas* grow well at 28–30 C, which is more appropriate for most of the species.

The range of nutrient sources that are used by *Pseudomonas* is extensive and species of *Pseudomonas* tend to grow relatively rapidly, often outgrowing the species of other genera. Most species of *Pseudomonas* favor a rich medium, such as nutrient broth and agar or tryptic soy broth and agar and other media rich in peptides, although different numbers and diversity of *Pseudomonas* species may be obtained with different peptide concentrations in the media (Aagot et al., 2001). Saprophytic species of *Pseudomonas* can be isolated by streaking

samples on nutrient agar or tryptic soy agar plates. Denitrifying *Pseudomonas* species are be isolated by specific enrichment procedures in a medium containing nitrate (NO_3), under anaerobic conditions, at 30–40 C.

Most *Pseudomonas* species grow in chemically defined media without added growth factors. With the reorganizations of the taxonomy of *Pseudomonas*, species known to require special growth supplements are now recognized as belonging to separate and distinct evolutionary lineages. No particular minerals or vitamins are necessary for supporting growth of species of *Pseudomonas* (sensu stricto).

A medium described by Palleroni and Douderoff (1972), developed for autotrophic and heterotrophic enrichments, functions well for the cultivation of *Pseudomonas*. *Pseudomonas* species grow well also on mineral medium used for isolating *Arthrobacter* (Owens and Keddie, 1969) and on R2A mineral medium for oligotrophic growth (BBL Microbiology Systems, Cockeysville, MD). In general, good growth of *Pseudomonas* species is observed in media including organic compounds from 0.1–1.0% (w/v) as carbon and energy sources.

Cultivation media that have been used for selective enrichments of *Pseudomonas* species may be deficient in iron, enabling the detection of fluorescent *Pseudomonas* species. The fluorescence is due to increased production of siderophore pigments extruded into the media. Other media have employed antibiotics, such as penicillin G, novobiocin and cycloheximide, which do not inhibit fluorescent *Pseudomonas* species (Sands and Rovira, 1970).

Selective media used extensively for the detection and isolation of fluorescent *Pseudomonas* species are King's media A and B (King et al., 1954), which employ potassium and magnesium salts to enhance pyocyanin and pyoverdin pigment production. Gould's S1 medium contains sodium lauroyl sarcosine, which effectively inhibits the growth of Gram-positive bacteria. The antibiotic trimethoprim may be included in the medium to inhibit nonfluorescent pseudomonads (Fromin et al., 2001). Other selective-differential media that support *Pseudomonas* growth include Pseudosel agar medium (BBL Microbiology Systems), Cetrimide agar medium, *Pseudomonas* isolation agar medium and *Pseudomonas* agar F medium (Difco Laboratories, Detroit, MI) containing cetyltrimethylammonium bromide (Cetrimide or CTAB), 2,4,4-trichloro-2-hydroxydiphenyl ether (Irgasan), or similar compounds, although *Pseudomonas* species are isolated in lower numbers and diversity on the strongly selective media (Gilardi, 1985). The use of Cetrimide was recommended (Lowbury, 1951) for inhibiting the growth of accompanying microbial flora and minimizing interference with the growth of *P. aeruginosa*. The pigment production of *P. aeruginosa* is not inhibited when grown on this medium. Addition of nalidixic acid (Goto and Enomoto, 1970) improves further the inhibition of accompanying microbial flora.

As Schroth et al. (1992) have pointed out, the choice between a general or selective medium for isolation of *Pseudomonas* depends upon the samples to be analyzed. Selective media are recommended for samples from soil, water and organic materials that contain many other microorganisms, whereas general growth media will be more useful for obtaining larger numbers and diversity of isolates from sources exhibiting strong selective pressures already.

Recently, new species of *Pseudomonas* have been isolated from various soil types (Delorme et al., 2002; Kwon et al., 2003), rhizosphere (Achouak et al., 2000), phyllosphere (Behrendt et al., 1999), water (Elomari et al., 1996; Dabboussi et al., 1999; Verhille et al., 1999; Ivanova et al., 2002), and Antarctic cyanobacterial mats (Gundlapalli et al., 2004), as well as by the application of some uncommon selection protocols, including, the use of resin acids, which may be biologically toxic (Mohn et al., 1999), chlorate as a terminal electron acceptorand acetate as an electron donor (Wolterink et al., 2002), and cooking water from a cork-processing factory at 50 C (Manaia and Moore, 2002).

Identification

Straight or slightly curved rods but not helical, 0.5–1.0 m in diameter by 1.5–5.0 m in length. Most of the species do not accumulate granules of poly-β-hydroxybutyrate, but accumulation of poly-hydroxyalkanoates of monomer lengths higher than C_4 may occur when growing on alkanes or gluconate. Do not produce prosthecae and are not surrounded by sheaths. No resting stages are known. Gram-negative. Motile by one or several polar flagella; rarely nonmotile. In some species, lateral flagella of short wavelength may also be formed. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor; in some cases nitrate can be used as an alternate electron acceptor, allowing growth to occur anaerobically. Xanthomonadins are not produced. Most, if not all, species fail to grow under acid conditions (pH 4.5 or lower). Most species do not require organic growth factors. Oxidase-positive or negative. Catalase-positive. Chemoorganotrophic. Strains of the species include in their composition the hydroxylated fatty acids 3-OH 10:0 and 12:0, and 2-OH 12:0, and ubiquinone Q-9.

Widely distributed in nature. Some species are pathogenic for humans, animals or plants. The mol% G+C content of the DNA is 58–69. Type species: *Pseudomonas aeruginosa* (Schroeter 1872) Migula 1900, 884.

The above is the definition of *Pseudomonas*, as it appears in the second edition of *Bergey's Manual of Systematic Bacteriology* (Palleroni, 2005). Characterizations of bacteria, including *Pseudomonas*, must address at least two aspects for purposes of identification. The first has to do with the characterization of *Pseudomonas* (sensu stricto) and differentiation from other genera, including those species that were classified as *Pseudomonas* in the past. The second has to do with resolving ution the intrageneric subdivisions of *Pseudomonas* (sensu stricto) into species. A complicating factor for such analyses is that species of the genus *Pseudomonas* and other pseudomonads are well known for their "promiscuity" in exchanging to exchanging genetic material. Questions of whether observed traits are stable, reliable and truly characteristic of a given organism become relevant for taxonomic and identification purposes.

The "species" is the basic unit of classification in biology and, as well, has been applied in microbiology, albeit relatively inconsistently. The intrageneric units in microbiology have been accepted somewhat empirically and guidelines for determining what is necessary for describing a bacterial species in any systematic manner have only recently evolved. Rosselló-Mora and Amann (2001) have discussed the complexities of conceptualizing the prokaryotic species, and have proposed guidelines for categorizing what they have termed the "phylo-phenetic species," i.e., "a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property."

The genus *Pseudomonas* comprises a group of species for which the amount of information available is quite uneven. It is relatively vast in the case of the type species of the genus, *P. aeruginosa*, primarily because of its medical importance as an opportunistic pathogen. However, markedly less is known about other species of the genus, particularly those isolated recently from some relatively uncommon sources. Perhaps, owing to better recognition of the properties useful in determinative schemes, the isolation of bacteria, such as *P. mendocina*, *P. stutzeri* and other *Pseudomonas* species, are being reported with increasing frequency from clinical samples. Generally speaking, *Pseudomonas* has a well-deserved reputation for a variety of beneficial biotechnological properties. In the case of *P. fluorescens* and *P. putida*, catabolic properties have dominated other details of *Pseudomonas* biology, including the recently growing amount of information on the effect of these species on stimulating plant growth. *P. Fluorescens* and *P. putida* are represented in nature by several biovars, and the species names lack precise systematic definition. However, the genus also is notorious because of species that are opportunistic pathogens of humans as well as phytopathogens that significantly impact the agricultural and economic well-being worldwide.

While some species of the genus *Pseudomonas* (e.g., *P. aeruginosa*) are homogeneous taxonomic units that are relatively easily differentiated and identified, other species include complex subdivisions, such as the *P. fluorescens* and *P. putida* biovars, the *P. syringae* and *P. marginalis* pathovars, or the *P. stutzeri* genomovars. Such internal organization of the genus is a reflection of the heterogeneity of bacteria that have been studied extensively and whose characteristics have not conformed completely to recognized taxonomy. Discrepancies between phentotypic and genotypic properties have further hindered attempts to resolve the systematics. However, given these complications in bacterial taxonomy, the "species" remains the biological unit that purports to represent a systematic description of an organism's basic properties.

Traditional and more recent characteristics applicable to *Pseudomonas* identification at the genus and subgenus levels are described below.

Cell Morphology

Pseudomonas species are straight or slightly curved rod-shaped cells (maximum length ca. 4.0 m) that occur singly or in pairs or short chains. Their morphology may occasionally deviate from that in standard descriptions. The cells typically stain Gram-negative. Electron microscopy reveals cell walls and membranes typical of Gram-negative bacteria, although different species may exhibit different chemical compositions. The cells of many of the species lyse readily in EDTA solution, which correlates generally with the high phosphorous content of the outer membranes (Wilkenson, 1970). Many species of *Pseudomonas* have been observed to produce poly-β-hydroxybutyrate (PHB) granules, particularly when grown under nitrogen-limiting conditions. The traditional description of a short rod-shaped cell does not always fit the morphology of all strains of *Pseudomonas* species. The cells of some strains can be extremely short, while in others (*P. putida* and *P. syringae*) they may be unusually long. Particularly in the cases of older cultures, the cells may be of unusual shapes and sizes.

With few exceptions, flagellar motility is a common characteristic of *Pseudomonas* species. Motility allows the cells to respond to chemical stimuli (chemotaxis) and enhances the ability of the bacterium to locate organic substrates at low concentrations. The majority of *P. aeruginosa* cells have only one flagellum, although exceptional *P. aeruginosa* cells with two or three flagella have been observed. *P. alcaligenes*, *P. mendocina*, *P. pseudoalcaligenes* and *P. stutzeri* are also characterized by having a single flagellum per cell. Most other species possess more than one. However, variations are observed in the flagellation of strains of *Pseudomonas* species and nonmotile strains may be isolated occasionally. The flagellar number of *P. aeruginosa* is reported to be controlled by FleN, a putative ATP-GTP binding protein, and the disruption of the *fleN* gene results in multiflagellation of otherwise monoflagellated strains, as well as in chemotactic defects (Dasgupta et al., 2000). The flagella are inserted at the poles of cells, although some exceptions have been observed, e.g., in *P. stutzeri* (Palleroni et al., 1970). Lateral flagella are thought to participate in swarming activity of cells (Shinoda and Okamoto, 1977).

Pili are observed in many Gram-negative bacteria, functioning as appendages for attaching to cell surfaces (Buchanan and Pearce, 1979). Pili have been studied extensively in *P. aeruginosa*, primarily because of their involvement in pathogenesis (i.e., as components of virulence factors), although the structures of these cell appendages are not known for most other species. Pili have been observed also in strains of *P. alcaligenes*. On the other hand, no pili have been observed in cells of *P. chlororaphis*, *P. fluorescens*, *P. oleovorans* or *P. putida*.

The low G+C content of the pilin genes in *P. aeruginosa* suggests that they may have been transmitted by horizontal transfer and, as a consequence, have a different pattern of codon usage (West and Iglewski, 1988).

A common characteristic of the fluorescent pseudomonads is the production of pigments that fluoresce in short wavelength (254 nm) ultraviolet light, particularly after growth under iron limitation. Some of these pigments and their derivatives are known to act as siderophores in these bacterial iron uptake systems (Meyer et al., 2002). The production of pigments by *Pseudomonas* species is demonstrated by cultivating the bacteria in media such as King's medium B, which contains no added iron. The medium is also recommended for demonstrating the production of the nonfluorescent blue pigment, pyocyanin, characteristically produced by most strains of *P. aeruginosa.* While *P. aeruginosa* is the most notable species of *Pseudomonas* for the production of distinctive pigments, other species are known to produce soluble and insoluble pigments that may be diagnostic. Pyoverdine (yellow-green fluorescence) is represents the main siderophore type of *Pseudomonas* species. (Meyer and Hornsperger, 1998). The taxonomic value of siderophore-typing appears to lie in the species- and strain-specificities. The results of siderophore-typing of a large number of fluorescent and nonfluorescent strains correlated with the results of genotypic analyses, indicating that this approach is promising for differentiation at the species level (Fuchs, 2001; Meyer et al., 2002). Other pigments produced by species of *Pseudomonas* include pyocyanin (*P. aeruginosa*, blue color), pyorubin (*P. aeruginosa*, red color), oxychlororaphin (*P. aureofaciens*, orange color), chlororaphin (*P. chlororaphis*, green color), oxychlororaphin (*P. chlororaphis*, orange color), etc. (Hugh and Gilardi, 1980).

The basic morphological characteristics of *Pseudomonas* are common to many families of bacteria and so are of little value in the positive identification or diagnosis of members of the genus.

Nucleotide Base Composition of Genomic DNA

The first genotypic analysis applied in bacterial characterization (Lee et al., 1956), the nucleotide base ratio of genomic DNA is measured as the ratio of the amount of guanine and cytosine nucleotides to the total amount of nucleotide bases (i.e., % G+C). Among prokaryotes, the G+C content ranges between 22–74 mol% (Marmur et al., 1963) and has proven to be useful for differentiating some taxa that are phenotypically similar. Although no exact guidelines have been established, generally, the G+C content has been reported to vary no more than 10 mol% within a bacterial genus (Stackebrandt and Liesack, 1993). Among the *Pseudomonas* species (sensu stricto), the range of G+C content is ca. 59–68 mol%, while among species reclassified within new genera, the range of G+C is 65–69 mol% for *Burkholderia* species, 64–68 mol% for *Ralstonia* species, 62–70 mol% for *Acidovorax* species, 62–67 mol% for *Comamonas* species, 65–67 mol% for *Brevundimonas* species, etc. (Palleroni, 1984). Though markedly different genomic DNA G+C contents certainly reveal different bacterial taxa, similar G+C contents do not necessarily indicate genomic relatedness.

Ribosomal RNA Gene Sequences

During the last decade, a concerted movement in microbial taxonomy has adopted phylogenetic relationships inferred from genotypic analyses, particularly those derived from rRNA and

rRNA gene (rDNA) sequence analyses, as an important component of a taxonomic "framework". Analyses of rRNA-rDNA sequence similarities of bacteria have been essential for elucidating their intergeneric relationships, and sequence determinations and analyses of 16S rRNA genes are among the recommended minimal standards for descriptions of new species. However, the application of rRNA-rDNA sequence analysis for determining intrageneric relationships of bacteria has not proven to be as successful. Given the early optimism for applying a principle of "one rRNA sequence type = one bacterial species," the reality observed in the last decade has proven to be sobering in light of the conservative nature of the rRNAs, which have proven to be limited in resolving the fine bacterial phylogenetic structure below the genus level. Stackebrandt and Goebel (1994) assessed the relationships between genomic DNA-DNA similarity (determined by hybridization) and 16S rRNA gene sequence similarities, and they have described the levels of 16S rRNA gene sequence similarity (or dissimilarity) that indicates when a bacterium does not belong to a given species. However, a definition of bacterial species based upon 16S rRNA-rDNA sequence similarities still is not established.

The limitation in achieving definitive resolution of bacterial species does not suggest that 16S rRNA-rDNA cannot be useful for estimating intrageneric relationships. Suffice it to say that 16S rRNA gene sequences have been used to demonstrate considerable internal evolutionary structure within the spectrum of *Pseudomonas* species (sensu stricto; Moore et al., 1996; Anzai et al., 1997; Anzai et al., 2000). Moore et al. (1996) detected at least two distinct intrageneric divisions designated, for want of better labels, 1) the "*P. aeruginosa* intrageneric cluster" and 2) the "*P. fluorescens* intrageneric cluster." Most species of *Pseudomonas* (isolated and characterized to date) group within one of these two clusters. Furthermore, *Pseudomonas* species were observed to be linked through distinct "evolutionary lineages" within the primary dichotomy of the genus. The same overall topography of the two major clusters, as well as the relationships of species comprising the evolutionary lineages, with some exceptions, has been observed in subsequent phylogenetic reconstructions (Anzai et al., 2000). Admittedly, the *Pseudomonas* intrageneric clusters and evolutionary lineages mentioned here comprise inferred natural branching orders. However, the organization of the two major intrageneric clusters, as well as the species comprising the evolutionary lineages, have been observed also in nonribosomal RNA-based analyses, using fatty acid methyl ester (FAME) and phospholipid fatty acid profiling (Vancanneyt et al., 1996b), or sodium dodecylsulfate-polyacrylamide gel electrophoresis SDS-PAGEanalysis of whole cell proteins (Vancanneyt et al., 1996a). Questions exist as to whether *P. putida* and closely related species, as well as some newly isolated species, are included as evolutionary lineages within one of the two primary intrageneric clusters or comprise separate, distinct intrageneric clusters.

Pseudomonas pertucinogena and "*P. denitrificans*" are seen to branch clearly outside the phylogenetic spectrum of all other species of the genus (Anzai et al., 2000), raising the question as to their taxonomic placement. "*P. denitrificans*" was placed on the list of nomina rejicienda (rejected synonyms) as a nomen ambiguum, because of previous confusion regarding its taxonomic position (Douderoff et al., 1974b). Until comprehensive, polyphasic analyses of these two organisms has been carried out and their proper taxonomic positions confirmed, it is premature to include either species within the genus *Pseudomonas* (sensu stricto).

With the increase in *Pseudomonas* species. isolated and characterized by 16S rRNA gene sequence comparisons, the resolution between the individual sequences of the species has decreased and the branching orders have become less discernible. Such observations led to a significant problem in using rRNA-rDNA sequences for species identifications. Overall, 16S rRNA gene sequence similarities between species of *Pseudomonas* range from approximately 93% to 99.9% (given some unknown degree of error in sequencing results, 16S rRNA gene sequences of different species that may be identicalcannot be ruled out). The complete 16S rRNA gene sequences are approximately 1540 nucleotides long. However, less than 10% (148 nucleotide positions) of the gene of *Pseudomonas* species appear to be susceptible to nucleotide base change and most of the positions that vary between species are located within hypervariable regions. Given these observations, if it were conceivable to apply the "one rRNA sequence type = one species" principle, the number of possible *Pseudomonas* species would be $(4)^{148}$ (where 4 = the number of nucleotides that can exist at any given variable position, and 148 = the number of variable nucleotide positions). However, bacteria of different species have been observed to possess identical 16S rRNA gene sequences (Fox et al., 1992). With such an observation on record, one cannot assume that a unique 16S rRNA gene sequence will characterize a single bacterial species. This premise, of course, has important consequences for *Pseudomonas* taxonomy and the analyses of *Pseudomonas* in studies of microbial ecology and diversity.

Another potential genotypic target for resolving the species of the genus *Pseudomonas* are the 23S rRNA genes. In general, the limited number of sequences that have been determinedhave indicated that the larger (ca. 2500 nucleotides) bacterial 23S rRNA genes possess a higher degree of sequence variation than do the 16S rRNA genes (Olsen, 1988). Christensen et al. (1994) analyzed selected, variable target regions within the 23S rRNA genes of some *Pseudomonas* species. and the data failed to provide a reliable basis for identifying closely related species. However, the number of analyses on the 23S rRNA genes of bacteria, including species of the genus *Pseudomonas*, has remained limited, and, as a result, their value for resolving the fine intrageneric relationships is not yet established.

The similarities in rRNA gene sequences and the phylogenetic relationships may be used, in turn, for developing diagnostic tools for detecting and identifying bacteria at the genus and species levels in environmental or clinical samples. The high specificity of nucleic acid probes and the different regions of the rRNA molecule, containing variable numbers of conserved sites, provide ready targets for oligonucleotides labeled with an appropriate molecule for in vitro or in situ detection of bacteria at different taxonomic levels (Amann et al., 1995). Genus- and species-specific oligonucleotide probes have been designed (Schleifer et al., 1992; Amann et al., 1996) that enable the detection of combinations of pseudomonad species from the different proteobacterial classes. However, a single set of hybridization probes for differentiating all species of the various pseudomonad genera or all species of the genus *Pseudomonas* has yet to be successfully developed. While it may be theoretically possible to differentiate bacteria on the basis of a single base change in a probe target region, the sequence regions exhibiting the nucleotide differences (i.e., "signatures") between species of a genus are limited in number, as well as in their structural availability to probing. Thus, Amann et al. (1995) have applied the practice of using nested probes in a "top-to-bottom" approach, whereby combinations of oligonucleotides used to differentiate organisms at different taxonomic levels systematically refine the levels of identification that may be determined. Such a strategy, which requires a large number of probes for identifying an organism of a specific sequence type, becomes realistic when used with micro-array hybridization systems.

In the same way that rRNA-rDNA sequence data have provided the means for developing hybridization probes as tools for differentiating bacterial species, the sequence data has also been used for devising PCR-diagnostic assays of high specificity. Widmer et al. (1998) combined specifically targeted PCR-amplification assays with restriction fragment length polymorphism (RFLP) analyses to develop a rapid assay for detecting 16S rDNA sequence types that cluster within the phylogenetic spectrum of *Pseudomonas* species.

Genomic DNA Similarities

Genomic DNA-DNA reassociation similarities have been accepted for many years as the molecular standard by which bacteria are classified at the species level (Johnson, 1984). The "cut-off" levels of DNA-DNA similarity that are used to "define" strains of a species range from above approximately 60% similarity ($\delta T_{m(e)}$, 6–9 C to approximately 70% similarity ($\delta T_{m(e)}$, 5 C; Brenner et al., 2001).

The original DNA-DNA hybridization experiments were performed by McCarthy and Bolton (1963) and were applied at Berkeley to the analysis of the pseudomonads to complement the existing phenotypic data. The results further supported previous conclusions based upon phenotypic analyses pointing to a significant degree of heterogeneity and fundamental differences among the species assigned to *Pseudomonas*, as the genus was known at that time. Subsequent analyses supported the subdivision of the genus (Johnson and Palleroni, 1989).

rRNA Gene Intergenic Spacer (IS) Sequences

A potential target site for resolving intrageneric relationships of *Pseudomonas* may be found in the noncoding, intergenic spacer (IS) region between the 16S and 23S rRNA genes. This DNA is excised during the process of transcribing the ribosomal RNA during the synthesis of ribosomes and is, thus, exposed to less evolutionary conservation than the rRNA genes.

Tyler et al. (1995) were able to differentiate *P. aeruginosa* and *P. mendocina*, as well as different pseudomonad bacteria, to the species level by sequence comparisons of PCR-amplified 16S-23S rDNA IS regions. IS sequences from different strains of the same species did not exhibit sequence differences greater than 5% and, in most cases, were not more than 2% divergent. IS sequences of different species of the same genus exhibited significantly higher (25–20%) sequence dissimilarities.

Guasp et al. (2000) have used 16S-23S rDNA IS sequence characteristics to resolve the genomovars of *P. stutzeri*, developing assays using restriction digestions of PCR-amplified IS regions, and correlating the results with differentiations determined by DNA-DNA hybridization

analyses. Although the IS sequence data for other species of *Pseudomonas* is sparse, these studies, and others analyzing various bacterial taxa, suggest that the results of sequence analyses of these DNA regions correlate well with results of DNA-DNA hybridization analyses and may offer an alternative to the DNA-DNA hybridization technique for identifying *Pseudomonas* and other bacterial species.

Housekeeping Gene Sequences

The use of conserved protein-coding gene sequences as molecular chronometers has met with varied success. In general, the rates of change are significantly greater than those observed for rRNA genes and the sequences have been proposed as alternatives for estimating close (i.e., intrageneric) phylogenetic relationships (Santos and Ochman, 2004). Probably the most comprehensive studies employing housekeeping gene sequence targets in *Pseudomonas* have come from analyses of the *gyrB* gene that encodes the β-subunit protein of DNA gyrase (topoisomerase type II; Yamamoto and Harayami, 1998; Yamamoto et al., 2000). The base substitution rates of *gyrB* within species of the genus *Pseudomonas* were observed to be greater than those of the 16S rRNA genes of the same organisms, presumably because of the number of sites available for neutral base substitutions. Interestingly, while the branching order of the most closely related bacteria, such as different strains of *P. putida*, was clearly much more defined by *gyrB* gene sequence comparisons than could be achieved with 16S rDNA sequences (Yamamoto et al., 2000), the overall branching order of the range of different species of *Pseudomonas* was observed to be essentially the same.

Studies targeting other genes with purported evolutionary chronometer characteristics and applied to *Pseudomonas* include the *rpoD* gene for the σ^{70} factor of the RNA polymerase (Yamamoto and Harayama, 1998) and the *narG* and *nosZ* genes for nitrate reductase and nitrous oxide reductase, respectively (Delorme et al., 2003). However, the number of species and strains analyzed in these studies has been too limited to be able to determine whether these genes provide reliable reconstructions of *Pseudomonas* phylogeny.

Santos and Ochman (2004) have argued that problems associated with relying upon a single gene, such as the 16S rRNA gene, with its inherent limitations, to estimate organismal phylogenies may be circumvented by typing multiple single-copy gene loci of selected "chronometer" proteins. While such multilocus sequence typing (MLST) analyses have been carried out on some bacterial taxa, this approach has yet to be applied to the analysis of *Pseudomonas*.

Cell Lipid Compositions

The lipid composition of cell walls has been recognized to reflect the "natural relationships" between bacteriafor nearly 40 years (Ikawa, 1967). Fatty acid, polar lipid, lipoquinone and polyamine profiles are regarded as valuable phenotypic markers of taxonomic value (Ratledge and Wilkinson, 1988; Tindall, 1994), although limited in their ability to resolve close relationships. Whole-cell fatty acid methyl ester (FAME) profile databases are now available as commercial products (e.g., the Microbial Identification System from Microbial ID, Inc. [Newark, DE]) and are used to identify bacteria at the genus level, in most cases, and subgenus levels in many cases.

In several laboratories, the compositions of cellular lipids of pseudomonads and other taxa have been described (Wilkinson et al., 1972; Moss and Dees, 1976; Ikemoto et al., 1978; Collins and Jones, 1981; Oyaizu and Komagata, 1983; Vancanneyt et al., 1996b). Whole-cell fatty acids of 16:0 and isomers of 16:1 and 18:1 appear to be typical of most Proteobacteria and are of limited diagnostic value in differentiating *Pseudomonas* (sensu stricto) from other genera (Vancanneyt et al., 1996b). *Pseudomonas* species have 3-OH 10:0, 3-OH 12:0 and 12:0, in addition to 16:1 ω9c, 16:0 and 18:1ω7c, as their cellular fatty acids, as well as the Q-9 ubiquinone. Most species also have the saturated 12:0 2-OH component and no significant levels of 3-OH 14:0 (Vancanneyt et al., 1996b). A small number of species may also produce the 14:0 fatty acid. Although few analyses have been carried out on the polar lipid compositions of *Pseudomonas*, phosphatidyl glycerol, phosphatidyl ethanolamine, and diphosphatidyl glycerol (cardiolipin) have been observed as the major cell components, with a number of uncharacterized minor components also present. These comprise the typical cellular chemical compositions of *Pseudomonas* spp. (sensu stricto). The differential value of the lipid components of bacteria is found in the overall combinations of the lipid species, not necessarily in the presence of a single lipid biomarker. Furthermore, quantitative and qualitative differences in some lipid components (i.e., cellular fatty acids) also are valuable taxonomic markers for *Pseudomonas* species.

Alginate Production

Alginate is an exopolysaccharide (EPS) comprised of a linear β-1,4-linked polymer of D-

mannuronic acid and L-gluronic acid. Alginate is produced by some bacteria, most notably by *P. aeruginosa* infecting the lungs of cystic fibrosis patients and causing severe respiratory problems (Govan, 1988), as well as by phytopathogenic or plant-associated fluorescent *Pseudomonas* species (Fett et al., 1986).

The genes involved in alginate synthesis in *P. aeruginosa* have been cloned and analyzed. Screening of bacteria for alginate genes, using DNA probes that target four genes involved in alginate production, detected DNA sequences homologous to all *P. aeruginosa* alginate genes in the chromosomal DNA of many species of *Pseudomonas* (sensu stricto), with the exception of *P. stutzeri*, which reacted to three of the four probes (Fialho et al., 1990). All of the alginate genes analyzed were detected in *Pseudomonas* species and the *Azotobacter-Azomonas* lineage, but fewer numbers of the genes were found in the other pseudomonad species and enteric genera.

In a related study, the *trans*-activation of gene *algU*, which controls the conversion of *P. aeruginosa* to a mucoid phenotype, was observed to be caused by a mutation in gene *algN*, at an adjacent locus, the product of which, presumably, inhibits the regulation of alginate production (Goldberg et al., 1993). *Pseudomonas* species. were screened by hybridizing homologous DNA fragments to a probe for *algU* from *P. aeruginosa* (Dubnau et al., 1965). Homologous sequences were detected in the DNA of rRNA homology group I *Pseudomonas* species (*P. fluorescens*, *P. putida*, *P. mendocina* and *P. stutzeri*) but not in *Comamonas acidovorans* (rRNA group III), *Brevundimonas diminuta* (rRNA group IV), or *Stenotrophomonas maltophilia* (rRNA group V).

These data suggest that alginate production may be a useful indicator for differentiating *Pseudomonas* (sensu stricto) from other bacteria, including other phenotypically similar pseudomonads.

Cellular Proteins

The comparison of amino acid sequences of selected homologous proteins offers the potential for assessing relationships among different bacterial genera (Schwartz and Dayhoff, 1978). However, protein sequence determinations are not a routine procedure. Thus, methods for determining the antigenic divergence of heterologous proteins, using antisera raised against reference proteins, can be used to identify amino acid sequence differences.

Glutamine synthetase, an important enzyme involved in nitrogen metabolism, has been the object of comparative immunological studies of pseudomonads. The results of these studies were identical to those of the nucleic acid hybridization experiments (Baumann and Baumann, 1978). Other immunological studies of selected enzymes (such as muconolactone isomerase [Stanier et al., 1970], aliphatic amidases [Clarke, 1972], and histidine ammonia-lyases [Rokosu, 1983]) have determined the degrees of similarity among homologous proteins of pseudomonads. These studies have provided useful determinative applications and further confirmation of the internal subdivisions of *Pseudomonas* (Palleroni, 1975; Palleroni, 1993), although, in an immunological study of the relatedness of histidine ammonia-lyases (histidases) of *Pseudomonas* species, the anti-P antibody appeared to be diagnostic for fluorescent species of *Pseudomonas* but not for the nonfluorescent species (Robert-Gero et al., 1969).

Outer membrane proteins include three or four predominant proteins that can be discerned by electrophoresis and are conserved among species of rRNA homology group I *Pseudomonas* (Kragelund et al., 1996). An antibody raised against one of these proteins from a strain of *P. fluorescens* (OprF, 37 kDa) was used in Western blot screenings of isolates from rhizosphere samples, and specificity for rRNA homology group I *Pseudomonas* was confirmed.

Tesar et al. (1996) described another approach exploiting the immunological differentiation of characteristic cellular proteins in which whole cell protein electrophoresis patterns, reacted with antibodies of appropriate specificity in a Western blot, enable identifications over a wide phylogenetic range. Monoclonal antibodies generated against *Pseudomonas*-specific epitopes of outer membrane proteins were able to differentiate *Pseudomonas* species (sensu stricto) from species of closely related genera.

Such analyses appear to be applicable to different taxonomic levels, such as the differentiation of the genus *Pseudomonas* (sensu stricto), although the immunological approaches may be most useful for intrageneric identification, because of the potential for extremely high antibody specificity.

Substrate Utilization

Substrate utilization has been an essential criterion for fine-resolution differentiation of species (and strains) of *Pseudomonas*. The lists of substrates utilized by *Pseudomonas* species are extensive, and the pathways for metabolism have been reviewed comprehensively (Stanier et al., 1966; Palleroni and Douderoff, 1972; Palleroni, 1984a; Palleroni, 1984b; Palleroni, 1993). Nutritional screenings of *Pseudomonas* species have been used to assess the capacity for growth at the

expense of a variety of carbon compounds (Stanier et al., 1966) and the findings have demonstrated the metabolic versatility that has made the genus renowned since the beginning of the twentieth century (den Dooren de Jong, 1926). In one case, a single strain of *P. putida* was observed to utilize 77 of 200 compounds tested, including carbohydrates, alcohols, saturated and unsaturated fatty acids, amino acids, amides and amines (den Dooren de Jong, 1926). In a separate study (Palleroni et al., 1973), another strain of *P. putida* grew on 80 of 150 compounds tested.

Many aromatic compounds are used by *Pseudomonas* species as growth substrates. During the early analyses at Berkeley, these compounds were observed to be typically metabolized to a common intermediate, β-ketoadipate (3-oxoadipate), by the fluorescent species. Species associated with rRNA homology groups II-V used different pathways for the metabolism of various aromatic compounds, although the β-ketoadipate pathway is also followed by pseudomonads and other bacteria unrelated to *Pseudomonas* species. (sensu stricto; Stanier, 1968). The mechanisms of aromatic ring cleavage appear to be characteristic for the different pseudomonad rRNA homology groups. By using antibodies against crystalline preparations of two enzymes of the β-ketoadipate pathway, muconate lactonizing enzyme (muconate cycloisomerase) and muconolactone isomerase isolated from *P. putida*, heterologous reactions were observed in preparations from *P. putida* biovars, *P. aeruginosa*, all biovars of *P. fluorescens* and *P. stutzeri* but not in preparations from *B. cepacia* or species of the *Comamonas* group (Stanier et al., 1970).

Metabolism of Amino Acids

Different pathways leading to L-phenylalanine or L-tyrosine are conserved in bacterial taxa (Byng et al., 1982) and analysis of key enzymatic features in these pathways, such as 2,4-diamino-6-hydroxypyrimidine (DAPH) synthase, prephenate dehydrogenase, arogenate dehydrogenase, prephenate dehydratase and arogenate dehydratase, have been used successfully to differentiate the members of pseudomonad rRNA homology groups (Byng et al., 1983). The multibranched pathway of aromatic amino acid synthesis offers not only a degree of variation in biochemical details not exhibited in the pathway of aliphatic amino acid synthesis, but also a richer source of regulatory information. For these reasons, Jensen and his collaborators exploited the interconnected pathways of aromatic amino acid synthesis as a model for the study of the phylogenetic relationships among the groups of aerobic pseudomonads (Byng et al., 1980; Byng et al., 1982; Whitaker et al., 1981). The results of this work were in overall agreement with the subdivision of *Pseudomonas* into five distinct rRNA similarity groups.

Several pathways for the degradation of arginine by different species of the genus *Pseudomonas* are known. In one of the pathways, known as the arginine dihydrolase or arginine deiminase pathway, arginine is converted to citrulline and this, in turn, into ornithine and ATP, which allows the cells to maintain their motility for an extended time under anaerobiosis (Hills, 1940; Slade et al., 1954; Sherris et al., 1959). The presence of arginine deiminase is assayed by the disappearance of arginine or, more simply, an increase in the pH of the medium, owing to ammonia liberation (Thornley, 1960).

Pathways of arginine degradation in *Pseudomonas* are further characterized by key reactions: arginine dehydrogenase (oxidase) and arginine succinyl transferase in fluorescent species, as well as in species of other pseudomonad genera (Stalon and Mercenier, 1984; Stalon et al., 1987, Jann et al., 1988). The arginine dihydrolase system may no longer be considered to be characteristic of *Pseudomonas*, since it is detected also in species of other genera, and some *Pseudomonas* species (such as *P. stutzeri*) give a negative reaction. However, analysis of arginine dihydrolase, in combination with other tests, still can be of diagnostic value.

Arginine metabolism in *Pseudomonas* species with multiple catabolic pathways for its utilization as carbon and nitrogen sources is of particular importance for the control of metabolic integration in cells. Transcriptome analyses identified genes controlled by the arginine regulatory protein ArgR in *P. aeruginosa* (Lu et al., 2004). Ten putative transcriptional units of 28 genes were observed to be inducible by ArgR and arginine, indicating that ArgR is important in the control of arginine and glutamate metabolism and that arginine and ArgR may have redundant effects in the induction of uptake systems of certain compounds.

Physiology

Metabolism and Metabolic Pathways

The metabolism of *Pseudomonas* (sensu stricto) is traditionally recognized to be strictly respiratory, although the arginine deiminase pathway yields energy and is termed “arginine fermentation” and a form of pyruvate fermentation may be carried out by some species. All species respire aerobically, using oxygen as the terminal electron acceptor for oxidative phosphorylation.

Some species also have a supplementary anaerobic respiratory system, working concurrently with the aerobic pathway. The supplementary pathway, which has been a determinative characteristic for some species of *Pseudomonas*, uses nitrate (NO_3) as the final electron acceptor (nitrate respiration). Denitrification via nitrate respiration is an energy-yielding catabolic process. Assimilatory denitrification of nitrate as a source of nitrogen for growth occurs through its reduction to ammonia.

The cytochromes present in the electron transport chains of some species of *Pseudomonas* have been characterized (Stanier et al., 1966), with absorption spectra detecting *a*-, *b*- and *c*-type cytochromes in most species, although some phytopathogenic species (e.g., *P. syringae*, *P. viridiflava*, *P. savastanoi*, etc.) lack the *c*-type cytochrome (Sands et al., 1967). These observations correlate with analyses of oxidase assays of *Pseudomonas* species, in which the *c*-type cytrochrome is necessary for positive reactions (Jurtshuk and McQuitty, 1976).

The first step in the oxidation of many organic substrate compounds by *Pseudomonas* species is carried out by the action of oxygenases, incorporating molecular oxygen into the chemical structure of the compound. Oxygen is a requirement for these reactions. The oxygenases form a diverse class of enzymes (mono- and dioxygenases), differing in structure, specificity and mechanism, and catalyze the oxidation of an extensive number of compounds, including hydrocarbon contaminants. Within the University of Minnesota Biocatalysis-Biodegradation Database (http://umbbd.ahc.umn.edu), more than 30% of the total number of enzymes catalogued are oxygenases, many derived from *Pseudomonas* species (Wacket, 2003).

As mentioned previously, some species of *Pseudomonas* are able to use nitrate as an alternative electron acceptor. Nitrate respiration is dependent upon the activities of nitrate and nitrite, reductases, which are induced in the presence of nitrate under anaerobic conditions and may be repressed by oxygen. Thus, levels of oxygen may play an important role in determining the success of denitrifying populations of *Pseudomonas* species in various habitats, for example, in well-aerated soils.

Additionally, a new species of *Pseudomonas* was described recently, *P. chloritidismutans*, which is able to utilize chlorate (ClO_3^-) as an alternative energy-yielding electron acceptor, in addition to oxygen (Wolterink et al., 2002). The ability to consume and oxidize nitric oxide (NO) to nitrate under oxic conditions has been observed in one *Pseudomonas* species. The mechanism of oxidation remains unclear, although it appears to be associated to detoxification or co-oxidation rather than an energy-yielding process. Although *Pseudomonas* has been studied for more than a century, new catabolic pathways are being detected in new isolates. The tricarboxylic acid (TCA) cycle in *Pseudomonas* is central in the regulation of cell catabolism and biosynthesis in all species. Species of *Pseudomonas* tend to utilize organic acids in preference to more complex organic compounds and this, in turn, represses many inducible peripheral catabolic enzymes. All or most of *Pseudomonas* species have incomplete glycolytic pathways, lacking 6-phosphofructokinase. *Pseudomonas* species dissimilate sugars and organic acids preferentially through the Entner-Doudoroff pathway. Hexoses and related compounds are converted to glyceraldehyde-3-phosphate and pyruvate via the Entner-Doudoroff pathway and various peripheral pathways, in which 6-phosphogluconate is a key intermediate (Eisenberg et al., 1974). All genes for the pentose phosphate pathway, the TCA cycle, the glyoxylate shunt, as well those for the oxidative and electron transport chain, are present in the genomes of the species of *Pseudomonas* whose genomes have been sequenced.

Pseudomonas species are able to use amino acids as carbon and nitrogen sources. When amino acids are present, the cell activates a specific membrane permease, which provides the transport mechanism for the amino acids to cross into the cytoplasmic space. Using amino acids as nutrient sources saves the cell energy, as the amino acids are immediately usable, requiring little or no modification to to be incorporated directly into the synthesis of cell biomass.

Some defining physiological characteristics have not been critically tested in all members of the genus, and as a consequence, occasional reports of exceptional strains have been noted. Thus, nitrogen fixation has been reported to occur in *P. stutzeri*, and *P. aeruginosa* may be capable of growing anaerobically, albeit slowly, with arginine and small amounts of yeast extract.

Genetics

Almost 10% of the genes in *P. aeruginosa*, *P. putida*, *P. syringae* and *P. fluorescens* genomes encode products involved in gene regulation, which reflects the evolutionary emphasis of this genus in monitoring and responding to a large number of environmental signals. The major regulatory control in bacteria of this genus is at the level of transcription, although an increasing number of posttranscriptional control systems are also becoming evident in this genus, to fine-tune the levels of expression of certain proteins.

This section summarizes some of the features involved in the transcriptional machinery and general regulatory circuits in *Pseudomonas*.

SIGMA FACTORS IN *PSEUDOMONAS* In *Pseudomonas*, core RNA polymerase can choose among a large number of sigma factors to transcribe the extensive variety and number of genes that species of this genus possess. *Pseudomonas* has a major sigma factor, σ^{70}, that recognizes promoters controlling the expression of housekeeping genes. Domínguez-Cuevas and Marqués (2004) compiled 149 σ^{70}-dependent *Pseudomonas* promoters, in which the transcriptional start point had been determined experimentally. They found that these promoter regions are significantly richer in AT pair content (approximately 50%) than the genomes from which they are derived (33.4% to 38.4%). The pattern resembles the consensus described for *Escherichia coli*, i.e., two conserved hexamers centered at positions –10 and –35 relative to the transcription start site. The –10 element in *P. aeruginosa* is TAtAAT and slightly different from that in *P. putida* (TATAcT), whereas the –35 element in both species is TTGACC. The spacer sequence between the –10 and –35 elements has an average length of 17 bp, although a 1-bp deviation of this length occurs with some frequency.

Multiple open reading frames (ORFs) coding for putative extracytoplasmic function (ECF) sigma factors that constitute a phylogenetically and functionally distinct subgroup within the σ^{70} family have been found in the genomes of *Pseudomonas* (Martínez-Bueno et al., 2002). Among these sigma factors are RpoS (sigma-38), RpoH (sigma-32), FliA (sigma-27), AlgU (sigma-22, homologous to RpoE in *E. coli*), PvdS and others. They control several iron uptake pathways, alginate biosynthesis, expression of virulence factors, tolerance to several stresses, expression of outer-membrane porins, etc. (Venturi et al., 1995; Brinkman et al., 1999; Burger et al., 2000; Rowen and Deretic, 2000; Schnider-Keel et al., 2001; Martínez-Bueno et al., 2002; Visca et al., 2002; Beare et al., 2003). Two ECF systems have been well characterized in *P. aeruginosa*: 1) AlgU, involved in the regulation of alginate biosynthesis, conferring a mucoid phenotype (Govan and Deretic, 1996), and directing the expression of a gene encoding the major heat shock sigma factor σ (thus, it likely plays a role in global gene regulation; Schurr and Deretic, 1997) and 2) σ^{PvdS}, a sigma factor involved in the regulation of pyoverdine siderophore biosynthesis.

The σ^{E} ECF factor of *P. aeruginosa* is encoded by the *algU* gene, which is part of the *algU-mucA-mucB-mucC* gene cluster. σ^{E} activity is controlled by the membrane-localized MucA protein, which appears to be stabilized by the periplasmic protein MucB, although the precise function of MucC is still unknown (Boucher et al., 2000). In addition to the alginate biosynthesis genes, transcriptional microarray profiles of σ^{E}-dependent expression in *P. aeruginosa* revealed that the following genes are part of the σ^{E} circuit, namely: *pfpI*, encoding a putative protease; *osmE*, encoding an osmotically inducible lipoprotein; genes encoding several membrane proteins; genes encoding metabolic proteins; and genes encoding proteins involved in adhesion and in drug resistance (e.g., efflux pumps). σ^{E} has also been reported in a plant growth-promoting *P. fluorescens* strain and shown to be important for tolerance to osmotic and desiccation stress, although, unlike *P. aeruginosa*, it did not play a role in protection against heat damage (Schnider-Keel et al., 2001). The σ^{E}-regulated promoters exhibit the following consensus sequence [(–35) GAACTT—N16/17—(–10) TctgA].

Several ECF sigma factors are involved in iron uptake, a function that appears to be crucial for the ecological fitness of all *Pseudomonas* (Martínez-Bueno et al., 2002; Venturi et al., 2004). The best characterized of these iron uptake sigma factors is PvdS, which initiates transcription of the genes required for the biosynthesis of pyoverdine (Lamont et al., 2002; Beare et al., 2003). The signaling pathway leading to σ^{PvdS} activation involves the ferri-pyoverdine outer membrane receptor, FpvA, which, upon interaction with ferric-pyoverdine, transmits a signal to the anti-σ^{PvdS} factor, FpvR, releasing the suppression of σ^{PvdS} and resulting in initiation of the transcription of pyoverdine genes and the *toxA* and *prpL* genes in *P. aeruginosa* (Lamont et al., 2002). A three-component system similar to FpvA/FpvR/σ^{PvdS} has been found in *P. putida* KT2440. Orthologues of σ^{PvdS} have also been identified to regulate siderophore production in *P. fluorescens* (Sexton et al., 1995). The *P. aeruginosa* PvdS protein has been shown to bind to several *pvd* promoters in which a DNA sequence at the –35 region, designated the "IS box" (G/C G/C TAAAT T/A C/G), is important for proper promoter functioning through specific recognition by this sigma factor (Wilson et al., 2001).

All *Pseudomonas* genomes have an *rpoN* gene encoding the σ^{54} factor that constitutes a group of its own, with characteristics that differ greatly from the σ^{70} family (Valls et al., 2004). As in other Gram-negative bacteria, promoters of this kind typically span DNA segments of approximately 150–200 bp. The most distinctive feature of the σ^{54}-systems is the presence of sequences that are characteristic for binding of the σ^{54}-containing RNAP, the so-called "–12/–24 motifs." These

include GG and GC doublets at positions –24 and –12, respectively, instead of the typical –10 and –35 hexamers of the σ^{70}-promoters. The second major functional motif in σ^{54}-promoters is the binding site(s) for the cognate activators. These sites may be located at various distances (50–150 bp) upstream from the –12/–24 motif. In addition to the –12/–24 and the upstream activating sequence (UAS), many (though not all) promoters have an integration host factor (IHF)-binding site at the intervening region. IHF binding to DNA sharply bends the target sequence, an event that has multiple consequences. Activation of this type of promoter is mediated mainly by members of the NtrC-family of regulators (see below). A feature to highlight is that the number and functions of the gene products of the genes transcribed by σ^{54} vary tremendously among the different *Pseudomonas* strains. However, a common characteristic is that the *rpoN* mutants of *P. aeruginosa* (Totten et al., 1990) and *P. putida* (Kohler et al., 1989) lack glutamine synthetase and fail to produce urease. *P. syringae* and *P. aeruginosa rpoN* mutants are less virulent than the corresponding wildtype strains. In the case of the plant pathogen, this is because it fails to synthesize the phytotoxin coronatine, and in *P. aeruginosa*, it is because the RpoN mutant fails to synthesize a number of virulence factors (Hendrickson et al., 2001). Last but not least, *rpoN* is required for the production and assembly of the flagellum.

Families of Regulators The LysR transcriptional regulator family is the largest paralogous group in *Pseudomonas* genomes, comprising more than 100 members, which is a remarkably high value for prokaryotes (normally ranging between 2 and 12) and comparable only to the situations in *Sinorhizobium meliloti* (86), *Agrobacterium tumefaciens* (75), and *Escherichia coli* strains (45–60). LysR-type regulators are associated with the regulation of many diverse functions, and play a central role in the activation of the expression of enzymes and proteins involved in aromatic metabolism, such as in protocatechuate (PcaQ) and catechol catabolism (CatR), and of other soil- and plant-related functions. AraC transcriptional regulators control processes such as carbon metabolism, stress response, and pathogenesis and, with more than 30 members, are also highly represented in the genomes of *Pseudomonas*. Members of the NtrC family that work with σ^{54} are also abundant in all *Pseudomonas* (around 25 members) and work in many different regulatory circuits (Valls et al., 2004). In *P. aeruginosa*, these regulatory circuits include the PhhR regulator for phenylalanine metabolism (Song and Jensen, 1996), and the regulator of the *lipA* gene, encoding a powerful lipase (Jaeger et al., 1996). In different species of the genus *Pseudomonas*, a number of catabolic pathways for the metabolism of aromatic compounds, i.e., phenol, toluene/*m*-xylene, *o*-xylene and 2-hydroxybiphenyl, are under the control of σ^{54} regulators such as DmpR, XylR, TouR and HbpR (Ramos et al., 1997; Arenghi et al., 2001; Jaspers et al., 2001; Shingler, 2004; Valls et al., 2004).

The genomes of *Pseudomonas* often contain a diversity of other regulatory gene families, such as repressors of the TetR and IclR family, which often regulate the expression of resistance to antibiotics, detergents and solvents. In all *Pseudomonas* genomes, members of the following families have been found: *asnC*, *gntR*, *lacI*, *luxR*, *Cro/cI*, *merR*, *marR* and *fis*.

Two-component Phosphorelay Systems Involved in Sensing Nutrients and Starvations All strains of *Pseudomonas* species contain a large number of two-component phosphorelay systems (TCSs). These systems consist of a histidine-kinase sensor and a response regulator (RR). Variations on the histidine-kinase (HK) sensor system have been reviewed recently by Filloux et al. (2004). In short, the sensor autophosphorylates in response to environmental stimuli and then transduces the signal to a response regulator, belonging to one of the above families, which is, in turn, activated upon phosphorylation. Examples of TCSs include the FleS/FleR pair that regulates motility and adhesion to mucins in *P. aeruginosa* (Richtings et al., 1995) and the PilS and its RR PilR that regulate transcription of the pilin gene *pilA* (Hobbs et al., 1993). CrbA is a classical HK which functions in unison with CrbB (Nishijyo et al., 2001) to control several specific metabolic pathways, and modulates the catabolism of various natural substrates in response to different carbon-nitrogen (C/N) ratios. Nitrogen nutrient deprivation also involves the TCS system NtrB–NtrC, while PhoR–PhoB is involved in phosphate assimilation. As proof of the complexity of iron metabolism in *Pseudomonas*, two TCSs have been shown to play an important role in iron acquisition in *P. aeruginosa*. The PfeS-PfeR pair is required for the enterobactin-inducible production of the ferric enterobactin receptor PfeA (Dean and Poole, 1993), and the PirR-PirS system is used as a second low-affinity ferri-enterobactin uptake system (Vasil and Ochsner, 1999). The GacS sensor is involved in the regulation of virulence in *P. aeruginosa* and works with the RR GacA (Reimmann et al., 1997). Some relevant issues of this system are described below.

Catabolite Repression in Pseudomonas When strains of *Pseudomonas* species are exposed to a mixture of potential carbon sources, they often assimilate them in an orderly fashion. In fact, most *Pseudomonas* species metabolize many organic acids or amino acids in preference to sugars, i.e., when *Pseudomonas* is confronted with succinate and glucose, the enzymes for glucose metabolism are not induced until succinate is exhausted (Collier et al., 1996). Nevertheless, glucose is known to repress the metabolism of mannitol and histidine. In contrast to other bacteria, catabolite repression is not mediated by cAMP but rather by the integration of several signals (Rojo and Dinamarca, 2004). Although the molecular basis for catabolite repression is, as yet, not well understood, evidence gathered in different laboratories points towards a series of proteins involved in signal integration. For instance, the Crc protein is involved in the repression of the genes involved in metabolism of sugar, amino acid, and nitrogenated compounds in *P. aeruginosa* and *P. putida*. Crc controls the metabolism of branched-chained amino acids and alkanes (MacGregor et al., 1996; Canosa et al., 2000; Yuste and Rojo, 2001). This protein is also said to be involved in biofilm development in *Pseudomonas* (O'Toole et al., 2000). A surprising finding is that catabolite repression of phenol metabolism (Petruschka et al., 2001) in *P. putida* strain H, and alkane metabolism in *P. putida* strain GPo1 is alleviated by the inactivation of the cytrochrome *o* ubiquinol oxidase (encoded by *cyoABCDEF*). Cells are believed to sense the redox state of the respiratory chains, triggering catabolite repression processes.

Sigma-54-regulated Pu and Po promoters for the metabolism of toluene and xylenes and phenol encoded by plasmid pWW0 and pVI150 (Powlowski and Shingler, 1994; Ramos et al., 1997) are also under catabolite repression. Indeed, these promoters are not expressed during the exponential growth phase in rich media in the presence of pathway substrates (Marqués et al., 1994; Cases et al., 1996; Sze et al., 1996). Both promoters respond to the alarmone (p)ppGpp, although Po is more sensitive than Pu (Sze and Shingler, 1999; Carmona et al., 2000). The level of the alarmone is low in the exponential phase and increases in the stationary phase. The Pu promoter is also down-modulated in response to glucose in a process that involves the activities of the PtsN and PtsO proteins (Cases et al., 1999; Cases et al., 2001). Inactivation of *ptsN* makes Pu unresponsive to repression by glucose, although glucose metabolism is not affected in the mutant. On the contrary, inactivation of *ptsO* inhibits Pu activity regardless of the presence of glucose (Cases et al., 2001). The Po promoter is not significantly influenced by PtsN-mediated glucose repression (Sze et al., 1996; Sze et al., 2002).

Quorum Sensing In many strains of *Pseudomonas* species, production of secondary metabolites, rhamnolipids, extracellular enzymes and virulence factors is controlled by a cell-cell signaling system that is generally described as "quorum sensing" (QS) or "density-dependent gene regulation" (Fuqua et al., 1994; Fuqua et al., 1996; Salmond et al., 1995; Sitnikov et al., 1995). The basis of this system is that the bacterium produces autoinducer or signal molecules such as *N*-acyl homoserine lactones (HSLs) that accumulate in the growth medium and trigger the expression of target genes when a threshold concentration is reached.

In *P. aeruginosa*, the QS circuit is composed of the *lasRI* and the *rhlRI* systems (Van Delden and Iglewski, 1998). The *lasRI* system is composed of *lasI*, the autoinducer synthase gene responsible for the synthesis of 3-oxo-C_{12}-HSL (*N*-[3-oxododecanoyl]-L-homoserine lactone), and *lasR* which encodes the transcriptional regulator LasR (Gambello and Iglewski, 1991; Passador et al., 1993; Pearson et al., 1994). The *rhlRI* system is composed of *rhlI*, the C_4-HSL (*N*-butyrylhomoserine lactone) autoinducer synthase gene, and *rhlR*, which encode the transcriptional activator RhlR (Ochsner et al., 1994a; Ochsner et al., 1994b; Latifi et al., 1995; Pearson et al., 1995; Winson et al., 1995). At low cell density, the autoinducers 3-oxo- C_{12}-HSL and C_4-HSL are synthesized at basal levels and diffuse, or are transported into the surrounding media where they become diluted; as a consequence, no gene transcription occurs (Pearson et al., 1999). With increasing cell density, the two autoinducers accumulate until their intracellular concentration reaches a threshold level. At this critical concentration, they bind to their corresponding regulatory protein (Fuqua et al., 1996). The regulator protein-autoinducer complex binds to specific DNA sequences upstream of the target genes enhancing their transcription (Stevens et al., 1994; Stevens and Greenberg, 1997). These systems, therefore, allow bacteria to communicate with each other (cell-to-cell signaling), to sense their own density (quorum sensing), and to behave in a coordinated manner, expressing specific genes as a population rather than as individual cells (Van Delden and Iglewski, 1998). The *lasRI* system regulates *lasB* expression and is required for optimal production of other extracellular virulence factors such as LasA protease and exotoxin A (Gambello et al., 1993). This system has also been shown to induce the transcription of the *xcpP* and *xcpR* genes that encode

proteins of the *P. aeruginosa* secretory pathway (Chapon et al., 1997).

The *rhlRI* system regulates the expression of the *rhlAB* operon that encodes a rhamnosyltransferase required for rhamnolipid production (Ochsner et al., 1994a), and is involved in the optimal production of LasB elastase, LasA protease, pyocyanin, cyanide, and alkaline protease (Brint and Ohman, 1995; Latifi et al., 1995; Pearson et al., 1997; Reimmann et al., 1997).

Both QS systems are highly specific in the sense that the respective autoinducers are unable to activate the transcriptional activator protein of the other system (Latifi et al., 1995; Pearson et al., 1997). However, both quorum-sensing systems are not completely independent of each other. The LasR/3-oxo-C_{12}-HSL complex activates the expression of *rhlR* placing the *lasRI* system in a QS hierarchy above the *rhlRI* system (Latifi et al., 1996; Pesci et al., 1997).

Transcriptome analysis has shown that QS controls not only virulence in *P. aeruginosa* but also many other genes involved in basic cellular processes, such as DNA replication, RNA transcription and translation, cell division, and amino acid biosynthesis. Another set of genes under QS control are those of chemotaxis and biofilm formation (Davies et al., 1998; De Kievit et al., 2001). Rhamnolipids, which depend on the *rhlRI* system, are required for the maintenance of biofilm architecture (Davey et al., 2003). The timing of QS activation is a continuum. Some genes are activated during early growth phase, whereas others are activated during exponential growth, or even during the stationary phase. This timing is not dependent on the concentration of autoinducer but on the availability of the transcriptional regulator proteins (LasR and RhlR; Schuster et al., 2003; Vasil, 2003).

The regulation of the QS circuit itself is complex and involves several other transcriptional regulators. The *lasRI* system is regulated both positively and negatively at several levels. It has been shown to be positively controlled by the global response regulator GacA (which together with the sensor kinase GacS forms a two-component system; Reimmann et al., 1997), as well as by Vfr (a homologue of the global transcriptional regulator CRP), which is required for the transcription of *lasRI*. The RsaL inhibitor represses the transcription of *lasI*. QscR has been shown to inhibit the premature activation of *lasI* expression at low cell density (Chugani et al., 2001). Expression of the QS system is influenced by the RpoS and RpoN sigma factors, the global posttranscriptional regulator, RsmA, and the alarmone (ppGpp; Latifi et al., 1996; Whiteley et al., 2000; Pessi et al., 2001; Van Delden et al., 2001). Adding to this complexity is the finding of a *Pseudomonas* quinolone signal (PQS) that depends on the QS circuit for its production, and which positively regulates *rhlI*, *rhlR* and *lasR* expression and induces *lasB* transcription (Pesci et al., 1999; McKnight et al., 2000; Calfee et al., 2001; Gallagher et al., 2002).

Phenazine biosynthesis is regulated by QS in *P. aureofaciens*, *P. chlororaphis* and *P. aeruginosa* (Pierson et al., 1994; Brint and Ohman, 1995; Wood and Pierson, 1996; Chin-A-Woeng et al., 2001). Expression of the phenazine biosynthetic operon in *P. aureofaciens* is controlled by PhzI and PhzR, which are members of the family of QS regulators (Wood and Pierson, 1996). Target regulatory boxes are located upstream of both the *phzI* gene and the *phz* biosynthetic operon, enabling activation of transcription in response to high concentration of the autoinducer C_6-HSL. The central importance of QS for this strain is shown by the data that phenazine production is greatly reduced in a *phzI* mutant and is restored by addition of C_6-HSL (Wood et al., 1997). GacS/GacA system regulates phenazine production (Haas et al., 2000) via transcriptional regulation of the *phzI* gene (Chancey et al., 1999). The level of *phzI* expression in a *gacA* or a *gacS* mutant is very low.

Posttranscriptional Control in *P. fluorescens* In the plant-beneficial strain *P. fluorescens* CHA0, the *hcnABC* operon, which encodes hydrogen cyanide synthase, is regulated at the posttranscriptional level, and involves the translational regulatory proteins RsmA and RsmE (Blumer et al., 1999; Haas et al., 2002; Heeb et al., 2002; Haas and Keel, 2003). Evidence for this mechanism comes from measurements of the expression of transcriptional and translational studies in different genetic backgrounds. The current model proposes that GacS/GacA activates expression of target genes at the posttranscriptional level by inducing the transcription of at least two small, untranslated regulatory RNAs, termed "RsmY" and "RsmZ," which alleviate the repressive effects of RsmA (Heeb et al., 2002; Valverde et al., 2003) and of RsmE (Pessi and Haas, 2004). RsmA and RsmE seem to bind to the mRNAs of target genes blocking their translation (Blumer et al., 1999). Mutational analysis of the *hcnA* untranslated mRNA leader region suggests that about ten nucleotides surrounding the Shine–Dalgarno sequence (GGA) are the target recognized by RsmA.

Genomics

As of June 2004, the genomes of four species of *Pseudomonas* have been sequenced and analyzed (Stover et al., 2000; Nelson et al.,

2002; Buell et al., 2003). At the time of this writing, the sequence for *P. fluorescens* was not published (see http://www.sanger.ac.uk/Projects/P_fluorescens/{The Wellcome Trust: Sanger Institute Web site}). The four species (*P. aeruginosa* strain PAO1, *P. putida* strain KT2440, *P. syringae* pv. tomato strain DC3000, and *P. fluorescens* strain SBW25) are derived from distinct phylogenetic lineages within the genus and include, respectively, an important opportunistic human pathogen, a strain used as a model system for biodegradation and other biotechnological applications, an important agricultural phytopathogen, and a plant growth-promoting strain colonizing roots and leaves.

The genomes of *Pseudomonas* species have approximately 6 Mbp (*P. aeruginosa*, 6,264,403 bp; *P. putida*, 6,181,863 bp; *P. syringae*, 6,397,126 bp; and *P. fluorescens*, approximately 6,700,000 bp), and approximately 5500 putative genes have been detected. These genomes are among the largest of the bacterial genomes that have been sequenced to date and the G+C contents of these genomes are among the highest: *P. aeruginosa*, 66.6%; *P. putida*, 61.5%; *P. syringae*, 58.4%; and *P. fluorescens*, 60.0%. Interestingly, the G+C contents determined by sequencing match almost exactly the G+C contents estimated previously for these species (Palleroni, 1984).

Detailed comparisons of the genomes of the four species of *Pseudomonas* have been compiled by Jensen et al. (2004). As this chapter is devoted to nonpathogenic aspects of *Pseudomonas*, the focus will be centered on data derived from the *P. putida* genome-sequencing project (Martins dos Santos et al., 2004; also see {TIGR Comprehensive Microbial Resource}).

Pseudomonas putid a is one of the best studied species of the genus, although information on genetic organization, gene transfer, metabolism and regulation, catabolic abilities, etc., is fragmented and dispersed, providing a restricted and incomplete view of the metabolic properties and capabilities that determine its lifestyle and niche specificity. The biology of *Pseudomonas* species, their metabolic versatility, behavior under varied environmental conditions, genetic variability, and interactions with other organisms and the environment can be assessed and appreciated only within the biological context in the diverse ecological niches where they reside. *P. putida* strain KT2440 is a paradigm of the metabolically versatile species and is used worldwide as a model system for genetic and physiological studies, and for the development of biotechnological applications. *P. putida* KT2440 has been certified by the Recombinant DNA Advisory Committee of the United States National Institutes of Health as the host strain of a host-vector biosafety (HV1) system for gene cloning (Federal Register, 1982).

Genome Features and Genetic Plasticity

The 6.2-Mb genome of *P. putida* KT2440 contains 894 paralogous gene families, which is considerably higher than that of the 6.3-Mb genome of *P. aeruginosa* strain PAO1, which has 809 putative paralogs. This large number of paralogous families, some 50% more than so far found in other large bacterial genomes is indicative of the high degree of functional versatility of *P. putida*. The most abundant of the putative genes include the LysR transcriptional regulator family with 110 members, the response regulator receiver domain with 94 members, and the ABC transporter family with 93 members. Interestingly, the 27 families represented by more than 20 members are related to proteins involved in transcription regulation (LysR, AraC, TetR, Sigma-54, etc.), transport (ABC, major facilitator family [MFS], binding-protein-dependent transport system, and porins), environmental signaling (response regulator receiver domains, histidine kinases, PAS domain S-boxes, TonB receptors, among others) or catabolism (dehydrogenases, hydrolases, transferases, oxygenases, etc.). These features reflect the emphasis in *P. putida* on powerful cellular mechanisms that enable it to thrive in diverse environments and to compete successfully with other organisms. Of the remaining putative gene families, 770 of them (86%) have 7 or fewer members, and 537 (60%) have only 3 (171) or 2 (513) members, which is similar to other large bacterial genomes. This suggests that selection for environmental versatility has favored expansion of genetic capability through the development of numerous small paralogous families, whose members encode distinct functions, rather than by increasing the number of family members (Stover et al., 2000).

The *P. putida* KT2440 genome contains other features that reflect high functional diversity. For instance, 508 ORFs were identified as putative duplications that arose after *P. putida* diverged from a common evolutionary lineage with *P. aeruginosa*, with which it shares approximately 85% of its genome (Nelson et al., 2002). As in other studies, comparison of the *P. putida* and *P. aeruginosa* genomes suggests that most lineage-specific genes are expansions of paralogous gene families. Of the duplications found in *P. putida* KT2440, 111 (21.8%) are conserved hypothetical proteins, and 110 (21.6%) are hypothetical proteins that probably relate to the unique biology of this organism. A large family of these duplications includes 22 transposase genes resembling members of the IS4 and IS110 gene families (see

below). Lineage-specific gene duplications may reveal species-specific adaptations to habitat, since they are often accompanied by functional diversification and divergence (Tettelin et al., 2001). Indeed, of the 42 transposase and maturase genes that are present in KT2440, 24 (57%) are flanked by genes involved in energy metabolism, implying an association between these mobile elements and the metabolic capabilities of *P. putida* KT2440. This association may serve to mediate enhanced horizontal transfer or altered expression of the corresponding metabolic genes, or both.

Insertion sequence (*IS*) elements are frequently associated with genes and operons encoding accessory (i.e., non-housekeeping) functions, such as catabolic enzymes and pathways (Mae and Heinaru, 1994; Wyndham et al., 1994; Tan, 1999), pathogenicity factors (Weinel et al., 2002), and protective functions against noxious agents, such as antibiotics, biocides, detergents, etc. (Wery et al., 2001). Moreover, *IS* elements mediate gene rearrangements, and facilitate the dissemination by horizontal transfer of such gene clusters among bacterial populations (Mahillon and Chandler, 1998). The *P. putida* KT2440 genome contains a large number of *IS* elements belonging to several families, although, of the previously described transposable elements from plasmids of *Pseudomonas* species, only Tn*4652*, which was previously shown to be responsible for inserting plasmid genes into the *P. putida* chromosome (Mae and Heinaru, 1994), could be identified within the genome sequence. The abundance and diversity of *IS* and other transposable elements in the *P. putida* KT2440 genome underscores the importance of the accessory functions of this organism and is indicative of its genetic plasticity.

Many other atypical regions, most containing mobile elements and determinants of catabolic pathways, have been identified in the *P. putida* KT2440 genome. Codon composition analysis revealed 39 regions of the genome greater than 10 kb in length that have atypical composition, the majority of which also have a G+C content that is significantly different from that of the rest of the genome (Nelson et al., 2002). Many of these regions encode bacteriophage genes, transposable elements, or determinants for proteins involved in the metabolism of aromatic compounds. In total, 90 transposon and 92 prophage determinants have been detected.

Inspection of the *P. putida* KT2440 genome reveals that a 35-bp sequence, having the structure of an imperfect palindrome, is repeated more than 800 times throughout the genome, which has not previously been reported for prokaryotes other than those of the Enterobacteriaceae family (Aranda-Olmedo et al., 2002). More than 80% of these sequences are extragenic and have thus been designated "Repetitive Extragenic Palindromic" ("REP") sequences. Of these, 82% are situated within 100 bp (and many within 30 bp) of the end of a neighboring gene. Given the positions and distribution of REP sequences in the *P. putida* genome, Aranda-Olmedo et al. (2002) suggested that they may serve to allow DNA gyrase to bind and relax DNA when excessive supercoiling is generated, thereby playing an important role in the regulation of gene expression.

Habitat-specific Functions

The genome of *P. putida* KT2440 is a complex mosaic structure with numerous and varied mobile elements, exhibiting features characteristic of terrestrial, rhizosphere and aquatic bacteria and suggests that acquisition of the combination of such features has equipped *P. putida* with its ability to thrive in diverse, often inhospitable environments, either free-living or in close association with plants.

DILUTE ENVIRONMENTS. An important characteristic of bacteria adapted to nutrient-poor aquatic lifestyles is the possession of high affinity transporters for substrates and nutrients. In addition to the relatively large number of high affinity transporters (most with an unknown substrate specificity), *P. putida* KT2440 has at least two ferric siderophore-transport systems and three ABC iron transporters, as well as 18 outer membrane ferric and ferric-related siderophore-receptors, three phosphate and two sulfonate ABC transport systems (in addition to a periplasmic sulfonate protein), as well as various transporters for sulfate, nitrate, common oligoelements, and metals. The existence of multiple transport systems for iron and phosphate is consistent with the need to overcome phosphate and iron limitation in dilute environments and the capacity of *P. putida* to deal with the diversity of micronutrients in heterogeneous terrestrial and aquatic systems.

P putida KT2440 specifies a large number of energy-gated outer membrane channels for substrates coupled to lower affinity cytoplasmic membrane transporters, a combination related to the ability of Proteobacteria to thrive in dilute aquatic environments (Niermman et al., 2001). *P. putida* KT2440 has 29 genes that share a TonB-dependent receptor C-terminal region, whereas bacteria such as species of Enterobacteria, which inhabit nutrient-rich niches, usually will have less than 10 (*Caulobacter crescentus* strain CB15 has 46), and a large number of high affinity transporters. The large majority of these TonB-

dependent receptors seem to be associated with iron uptake.

In addition to these diverse transport systems, *P. putida* KT2440 possesses a complex repertoire of chemosensory systems for detecting and responding to environmental signals (two-component sensors, sensor histidine kinases, chemotaxis, flagellum-related genes, etc.), and 17 extracytoplasmic function (ECF) sigma factors, 13 of which are associated with subfamily protein signature. Other "environmental" bacteria also have large numbers of ECF sigma factors: *P. aeruginosa* strain PAO has 17, *C. crescentus* 13, *Mesorhizobium loti* 19, *Sinorhizobium meliloti* 11, and *Agrobacterium tumefaciens* 11, although most genomes sequenced to date have far fewer. Furthermore, like *P. aeruginosa* strain PAO1, *P. putida* KT2440 has 22 σ^{54}-dependent transcriptional regulators, which is more than what is observed in most bacterial genomes thus far sequenced, including those of plant-associated bacteria.

SOIL AND PLANT ENVIRONMENTS. Genome features associated with a soil lifestyle include a 1-acyl-*sn*-glycerol-3-phosphate-synthase that would form N-(3-hydroxy-7-*cis*-tetradecenoyl) homoserine lactone, a quorum-sensing molecule, and a large battery of stress response systems appropriate to the fluctuating and adverse environmental conditions of terrestrial habitats. These include active efflux systems for metals (arsenate, arsenite, copper, cadmium, chromate, cyanate, etc.), organic solvents (toluene), paraquat (two paralogous sets), antibiotics (e.g., penicillin) and, interestingly, the export of fusaric acid, a toxin produced by common fungal phytopathogens such as *Fusarium oxysporum* (Schnider-Keel et al., 2000), and several export systems for biopolymers and antibiotics. These characteristics underscore the potential of *P. putida* for biocontrol activity against fungal pathogens of plants. At least, one ABC efflux transporter shows similarities to ABC pumps associated with the efflux of organic solvents (Kim et al., 1998).

Interestingly, the *P. putida* KT2440 genome possesses the determinants for resistance against many heavy metals, as this capacity has not been recognized to any great extent in *Pseudomonas*. Additionally, not less than 13 putative glutathione-S-transferase genes were identified in the *P. putida* KT2440 genome. This number is similar to those in other *Pseudomonas* (i.e., *P. aeruginosa* [15] and *P. fluorescens* [10]), or in *C. crescentus* (19), *M. loti* (10) and *S. meliloti* (16), although it is much larger than those of most sequenced genomes. Glutathione-S-transferases generally act on synthetic chemicals (Vuilleumier, 2001), although these proteins are also known to play a significant role in the degradation or inactivation of electrophilic compounds, such as most xenobiotics and heavy metals (Fraser et al., 2002).

A major stress factor in soils is water deprivation, and several genes (including those for flagellin, flagellar hook, two-component sensor, outer membrane protein, sugar binding proteins, peptidyl-prolyl *cis-trans* isomerases, two-component regulators, etc.) have been observed to be involved in water deprivation stress in *P. putida* KT2440. In fact, the response of *P. putida* KT2440 to water stress seems to involve the differential expression of membrane proteins, flagella related proteins, enzymes with action on membrane composition, or heat shock proteins. *P. putida* KT2440 has a large number (10) of genes coding for universal stress proteins, which is similar to that of *Halobacter* (11) and *P. aeruginosa* (8), but higher than in most other bacterial genomes sequenced thus far. Furthermore, *P. putida* KT2440 has genes for 7 cold shock proteins, similar to *P. aeruginosa* (6) and *M. loti* (7), and more than most other bacteria. *P. putida* KT2440 has determinants for 5 heat shock proteins, *M. loti* has 7, whereas *P. aeruginosa* and *C. crescentus* each have 2. *P. putida* KT2440 possesses a number of stringent starvation-related proteins (15), most of which (13) are glutathione-S-transferases (*P. aeruginosa* has 18, *M. loti* has 10, and *C. crescentus* has 19), whereas in most other genomes, the numbers of starvation-related proteins are substantially lower.

RHIZOSPHERE AND BIOFILM FITNESS *P. putida* KT2440 has been shown to be a versatile, aggressive colonizer, being able to establish itself and persist within the rhizosphere and in bulk soils at high cell densities (Molina et al., 2000). Features that contribute to rhizosphere and soil fitness include adhesion and colonization abilities, antibiotic production, resistance to (multiple) antibiotics, capability to use seed and root exudates, production and utilization of siderophores, and ability to cope with oxidative stresses.

ADHESION. *Pseudomonas putida* KT2440, other *P. putida* strains, as well as *P. fluorescens* and other *Pseudomonas* species, colonize and quickly proliferate in the rhizosphere of a number of agriculturally important plants. The *P. putida* KT2440 genome has three relatively large ORFs, encoding the largest protein in the genome (8682 amino acids), which has been demonstrated to be essential for seed colonization in *P. putida* (Espinosa-Urgel et al., 2000). The presence of an operon for the biosynthesis of cellulose (absent in *P. aeruginosa*), as in *A. tumefaciens* and in rhizobia suggests that cellulose may facilitate the

adhesion of *P. putida* KT2440 to root tissue (Mathysse et al., 1983).

Two previously uncharacterized polysaccharide biosynthesis and export gene clusters were also identified in *P. putida* KT2440. The first cluster, absent from the genome of *P. aeruginosa*, includes four putative glycosyl transferases, a putative serine *O*-acetyltransferase, and a VirK homologue, a factor essential for intercellular spreading of *Shigella*. The second cluster included, as well, four putative glycosyl transferases, two putative lipopolysaccharide core biosynthesis proteins, and a deoxy-D-manno-octulosonic-acid (KDO) transferase. Polysaccharides are likely to play a role in the attachment of *P. putida* to plant roots. Other surface polysaccharides and lipopolysaccharides and cell-envelope components, as well, are important for adhesion to plant surfaces, as shown by Sauer and Camper (2001), who detected upregulation of genes involved in the biosynthesis of exo- and lipopolysaccharides (EPS and LPS, respectively) as well as in membrane protein genes in *P. putida* following attachment.

In the *P. putida* KT2440 genome, 23 of the 24 genes for alginate biosynthesis of regulation have been identified. The omission of the gene, the fine-tuning transcriptional regulator AlgM/MucC, is likely to hamper alginate biosynthesis (Ohman et al., 1996). Previous studies suggest that alginate biosynthesis in *P. putida* may be relatively common and it cannot be ruled out that *P. putida* KT2440 is not able to produce alginate under appropriate conditions, for instance upon attachment to plants.

COLONIZATION AND DEFENSE. Effective colonization requires the specific action and coordination of certain enzymes and proteins. Gene clusters in *P. putida* KT2440, encoding several proteins specifically involved in plant-rhizosphere interactions are absent in *P. aeruginosa* PAO1, for example, a site-specific recombinase that is essential for competitive root colonization in *P. fluorescens* (Dekkers et al., 1998). Interestingly, *P. putida* KT2440 not only colonizes roots easily, but it actually senses metabolites in the neighborhood of the rhizosphere and moves quickly towards the roots (Espinosa-Urgel et al., 2002). In addition to a complex chemosensory and chemotaxic machinery (Nelson et al., 2002), *P. putida* KT2440 has a type IV fimbrial biogenesis gene set, a large operon for the biosysnthesis of flagella as well as the genes coding for type I pili, curli fiber, and two chaperone/usher gene clusters, all of which are likely to contribute to the attachment capacity of *P. putida* KT2440. Consistent with this, Sauer and Camper (2001) reported that various fimbrial biosynthesis and flagellar genes in *P. putida* were differentially expressed following attachment onto surfaces. Several other studies have demonstrated the key role of motility-related proteins in the attachment and colonization of plant or inert surfaces by *P. putida* (Turnbull et al., 2001). Other proteins related to the general type II secretion pathway, such as those encoded by the genes involved in the transport and assembly of type IV pili, also have been shown to be important for adhesion of *P. putida* (Sauer et al., 2001).

The genome of *P. putida* KT2440 includes ORFs with similarities to genes for the synthesis of the lipodepsinonapeptide class phytotoxins, such as syringomycin, as well as a cluster containing genes for polyketide biosynthesis that are typically found in antibiotic-producing strains of *Pseudomonas* species. *P. aeruginosa* pyocins (bacteriocins) have been shown to have bactericidal activity towards related pseudomonads, as well as other bacteria. Note that *P. putida* KT2440 has a large number of pyocin-related genes, 24 of them orthologues to those in *P. aeruginosa* PAO1 (Nelson et al., 2002). Additionally, *P. putida* KT2440 and other *P. putida* strains are protected from other bacterial strains or a plant host by extruding toxic compounds through their many multidrug efflux systems and by synthesis of enzymes that include penicillin resistance proteins, (metallo)-β-lactamases, cardiolipin proteases, phospholipase D, endolysin, etc. In many cases, this response is surface induced (Espinosa-Urgel et al., 2000; Sauer and Camper, 2001).

P. putida KT2440 exhibits extensive transport capabilities, with approximately 350 cytoplasmic membrane transport systems, 15% more than *P. aeruginosa* POA1. The genes encoding for these systems form approximately 12% of the whole genome. The largest family corresponds to an ABC transporter (94 paralogous members), of which a significant proportion is predicted to be devoted to amino acid uptake. This is consistent with the ability for *P. putida* KT2440 to colonize plant roots, since root exudates are rich in amino acids, and reflects its physiological emphasis on the metabolism of amino acids and their derivatives to compete successfully in the rhizosphere. *P. putida* KT2440 also has the determinants for 11 LysE family amino acid efflux transporters (*P. aeruginosa* PAO1 has only one) that, presumably, play a key role in preventing the accumulation of inhibitory levels of amino acids or their analogues into the cell. Also consistent with its ability to colonize plant roots, *P. putida* KT2440 has a predicted ABC family opine transporter (previously been described for other rhizosphere bacteria; Lyi et al., 1999) and enzymes for the metabolism of opines, suggesting that it is capable of exploiting plant-produced opines induced in the rhizosphere by other bacterial species.

Opines are amino acids found in hairy-root hyperplasia and crown-gall tumors (Dessaux et al., 1993), and are important C- and N-sources for promoting rhizosphere-associated growth of Gram-negative bacteria.

In addition to these transport systems, the genome contains the determinants for the import of sugars (e.g., glucose, ribose and fructose), organic acids (aromatic, dicarboxylic and tricarboxylic), oligopeptides, anions (taurine, phosphate, phosphonate, sulfate, sulfonate and nitrate) and cations (ammonium, magnesium, copper, zinc, nickel, cobalt, potassium, sodium, heme and iron). Interestingly, *P. putida* KT2440 has only one phosphotransferase system (PTS) sugar transporter and a PTS-like system for nitrogen. Another large (i.e., the second largest) class of transporters is that of the Major Facilitator Superfamily, for which 48 determinants were found in the genome of *P. putida* KT2440. Most of the members are predicted to be involved in the efflux of toxic compounds although, in many cases, the substrate is unknown. The same applies to another large class, the resistance-nodulation-cell division (RND) efflux transporters.

IRON UPTAKE. A major phytoprotective effect of fluorescent pseudomonads is thought to be due to the production of siderophores under iron-limiting conditions, as well as to the ability to capture iron more efficiently than do plant pathogens (O'Sullivan and O'Gara, 1992; Walsh et al., 2001). Accordingly, *P. putida* KT2440 produces a siderophore, pyoverdine, which is a complex polypeptide produced by most fluorescent species of *Pseudomonas* and is strain-specific (Meyer et al., 2002). The pyoverdine genes in *P. putida* KT2440 are clustered in three groups and the organization is similar to that in the phytoprotectant bacteria *P. fluorescens* strain SBW25 and *P. putida* strain WCS358. The main pyoverdine transcriptional activator gene, *prf*I, is located next to the pyoverdine synthetase gene and is divergently transcribed. The gene in *P. putida* KT2440 has 93% identity with *prf*I experimentally characterized in *P. putida* WCS358 (Venturi et al., 1995) and 85% identity with *pvd*S, which is involved in the regulation of various pyoverdine biosynthesis genes in *P. aeruginosa* (Visca et al., 2002). The expression of the genes for pyoverdine production and siderophore uptake in the rhizosphere is influenced by other bacteria that coexist with *P. putida* (Loper and Henkels, 1999). Consistent with this, *P. putida* KT2440 has 29 genes whose products are predicted to be TonB-dependent outer-membrane siderophore receptors, most of which are within a gene cluster involving a transmembrane sensor, an ECF σ^{70} factor, and a transcriptional regulator, as predicted by Visca et al. (2002) for various prokaryotes. In some cases, the siderophore receptors are clustered next to a siderophore transport system or siderophore biosynthesis genes, as for pyoverdine biosynthesis. Not surprisingly, the number of siderophore receptors in *P. putida* KT2440 is similar to that in the phytoprotectant *P. fluorescens* SBW25 but much larger than that in plant pathogens *P. syringae*, *Xylella fastidiosa*, *Ralstonia solanacearum* or *Agrobacterium tumefaciens*.

OXYGEN METABOLISM. *P. putida* KT2440, similar to plant symbionts *S. melliloti* and *M. loti* and the phytoprotectant saprophyte, *P. fluorescens*, has two complete sets of cytochrome *c* oxidase of the type *cbb3*, which has a high affinity for oxygen. Plant pathogens such as *P. syringae* or *A. tumefaciens* have only one set, and *X. fastidiosa* and *R. solanacearum* have none. Additionally, both *P. putida* KT2440 and *S. meliloti* have a second (*S. meliloti* has a third) formate dehydrogenase, which are important in fermentative processes. These two observations are consistent with the need of these bacteria to survive in the low-oxygen conditions of the rhizosphere or plant nodules.

To be able to thrive in their hosts, plant-associated microorganisms (both pathogens and symbionts or mutualists) must cope with oxidative stress, which is the first line of defense by plants against intruders. Not surprisingly and similar to other plant-associated microorganisms, *P. putida* KT2440 has many genes for dealing with oxidative stresses, including superoxide dismutases, catalases, (betaine-) aldehyde dehydrogenases, etc. In some cases, the regulation of these genes is iron-dependent and coupled to that of iron-binding proteins such as bacterioferritin.

Other relevant findings in *P. putida* KT2440 are the determinants for a bacteriophytochrome photoreceptor system that, in related prokaryotes, binds biliverdin, a breakdown product of heme metabolism (Bhoo et al., 2001). Although their precise functions are not fully known, it has been suggested such bacteriophytochromes play a role in signal transduction under oxygen and iron limitation. Interestingly, in prokaryotes, these phytochromes have been found only in microorganisms whose main habitats are soils and plants, which may be indicative of a habitat-specific function.

POTENTIAL PATHOGENICITY FACTORS. In addition to the genes described above, *P. putida* KT2440 has a number of genes that have been considered to be involved in animal or plant pathogenesis. ORFs coding for gacA, a cognate response regulator of gacS (sensor kinase) and a global regulator of secondary metabolites in Gram-negative bacteria are found also in *P. putida* KT2440. These genes are

involved in the regulation of the syringomicin production in *P. syringae* and of virulence functions in *P. aeruginosa* PAO1 (Rhame et al., 2001). Also of interest is the presence of determinants for several thiol:disulfide bond-forming enzymes, of which some have been shown to be involved in pathogenesis of both human and plant pathogens (Cao et al., 2001).

P. putida KT2440 possesses two orthologues to the htrA serine protease family, which have been shown to be involved in oxidative stress responses in pathogenic strains of *Salmonella* and *Yersinia* species. Additionally, one ORF encodes for a marR family regulator similar to rovA of *Y. pestis* and *Y. pseudotuberculosis*, which, in these bacteria, mediates the regulation of invasion in response to environmental signals.

Membrane lipopolysaccharides (LPS), particularly the B-band of O antigen, are often associated with virulence by many pathogens, including *P. aeruginosa* (Belanger et al., 1999). *P. putida* KT2440 has a large number of genes coding for surface polysaccharides and lipopolysaccharides, including those for the synthesis of the B-band of O antigen. However, the roles of these genes in pathogenicity are questionable.

Finally, several other ORFs, which seem to encode reported pathogenic factors have been detected: 1 ankyrin domain protein, 1 hemolysin-type calcium-binding bacteriocin, 7 leucine-rich domains, 1 endoglucanase, 3 phospholipase Ds, 7 lytic transglycosylases, β-metallo-lactamases, and VGR proteins (i.e., proteins with repetitions of Val-Gly). Interestingly, in the genome of *P. putida* KT2440, 23 paralogs of the hlyD (hemolysin) family secretion protein are found. However, still unclear is whether these proteins are expressed or, if so, whether they are associated with pathogenesis or simply have a habitat-specific function.

Central Metabolism Like many other pseudomonads and rhizosymbionts, *P. putida* KT2440 has an incomplete glycolytic pathway, lacking 6-phosphofructokinase, and uses a complete Entner-Doudoroff route for utilizing glucose and other hexoses. However, it does have the genes coding for a fructose-1,6-biphosphatase and for glucose-6-phosphate isomerase, which are involved in early stages of gluconeogenesis, as well as in EPS biosynthesis. Also, like *P. aeruginosa* PAO1, *P. putida* KT2440 lacks the aldose-1-epimerase and glucose-1-phosphatase. Glucose is converted to glyceraldehyde-3-phosphate and pyruvate via the Entner-Doudoroff pathway, in which 6-phosphogluconate is a key intermediate. There are two main routes for converting glucose to 6-phosphogluconate: 1) by direct oxidation of glucose into gluconate or 2-ketogluconate, both of which are transported to the cytoplasm, converted to 6-phosphogluconate and then further oxidized in the Entner-Doudoroff pathway; and 2) transport of glucose into the cytoplasm (via a sugar ABC-transporter), followed by phosphorylation of glucose to glucose-6-phosphate and then to 6-phosphogluconate. These two routes are cooperative, with the operation of one able to compensate for the loss of the other, although one or the other prevails, depending upon the physiological conditions. The direct oxidative pathway seems to prevail under aerobic conditions or higher substrate availability, whereas the phosphorylative pathway predominates under oxygen or glucose-limited conditions (Lessie and Phibbs, 1984).

The determinant for the membrane-associated pyrroloquinoline quinone (PQQ) glucose dehydrogenase is clustered with those for a porin B and for a TonB-dependent outer membrane receptor. The gluconokinase gene is just next to that for gluconate transport. Similarly, the genes encoding 2-ketogluconate metabolism (2-ketogluconate kinase, 2-ketogluconate 6-phosphate reductase, and epimerase) are clustered with those for a 2-ketogluconate transporter and a transcriptional regulator. Interestingly, this cluster is separated from that of the membrane-associated gluconate dehydrogenase complex, whose protein products convert gluconate to 2-ketogluconate by a transposase reading in the same direction and a potential terminator.

A patchy configuration of the various clusters coding for the enzymes of the initial stages of glucose utilization and the presence of different regulators in these gene clusters suggests a complex interplay of regulatory mechanisms with various control loops. Many of these regulatory mechanisms have been experimentally elucidated in various species of *Pseudomonas* (Clarke and Ornston, 1975), although a more comprehensive view of the gene organization within a genomic context has only became possible with the genome sequence information.

As in other *Pseudomonas* species, fructose metabolism in *P. putida* KT2440 occurs via a phosphoenolpyruvate phosphotransferase system (PTS). The genes are clustered with the 1-phosphofructokinase and the fructose transport system repressor. Other PTS-like systems encode proteins participating in a phosphorylation cascade and play an essential role in the regulation of nitrogen and carbon metabolism (Reizer et al., 1999).

P. putida KT2440 has also the glutathione-dependent glyoxalase I and II genes (as well as one for a D-lactate dehydrogenase) that code for the enzymes in the catabolism of methylglyoxal

to pyruvate. Several determinants for biosynthesis and catabolism of glycogen are clustered together within the genome. The phosphoglucomutase gene is found elsewhere in the genome and forms a cluster with an outer membrane ferric siderophore receptor, a transmembrane sensor, and an ECF σ^{70}, which indicates an iron-dependent regulatory link between environmental signals and glycogen metabolism. This is consistent with sensitive control of glycogen synthesis or for glycogen degradation, depending upon changing nutritional conditions.

Additionally, scattered throughout the genome are the genes for a periplasmic β-glucosidase, as well as those for β-(1-6)-glucan synthase, ß-(1,3)-glucosyl transferase, and another copy of a glycosyl hydrolase. The β-(1-6)-glucan synthase and β-(1-3)-glucosyl transferase are similar to those in *Bradyrhizobium japonicum*, where they are involved in the synthesis of β-cyclic glucan, which is thought to be important for symbiotic interactions and to play a role in hypoosmotic adaptation (Bhagwat et al., 1996). The presence of genes coding for enzymes involved in the biosynthesis and degradation of other polysaccharides suggests a tight coupling to other "metabolic modules" and suggests a dependency upon prevailing environmental conditions.

All genes for the pentose phosphate pathway, the TCA cycle, the glyoxylate shunt, as well those for the oxidative and electron transport chain, are present in the genome of *P. putida* KT2440. ATP synthesis is driven by the resulting chemiosmotic gradient and occurs via an F-type ATP synthase. Analysis of the genome suggests that nitrogen and energy are derived from the catabolism of amino acids alanine, arginine, aspartate, asparagine, glutamine, glutamate, glycine, histidine, leucine, isoleucine, methionine, phenylalanine, serine, valine, lysine, proline, and hydroxyproline, and from the metabolism of a range of carbon compounds, including acetoin, fructose, butyrate, betaine, glucose, gluconate, glutarate, glycerol, hydantoin, lactate, malate, mannose, ribose, sorbate and sucrose, among others. Growth on these compounds has been confirmed experimentally by growing *P. putida* KT2440 on minimal media supplemented with these compounds.

Biosynthesis The genes for the synthesis of all 20 amino acids have been identified in the genome of *P. putida* KT2440. The first step in methionine biosynthesis from homoserine in *P. putida* KT2440 and other species of *Pseudomonas* is catalyzed by gene products with similarities to several known and putative homoserine *O*-acetyltransferases (Andersen et al., 1998; Alaminos and Ramos, 2001). The presence of homologues to methionine biosynthesis genes suggests that *P. putida* KT2440 is able to generate homocysteine from *O*-succinylhomoserine through transsulfuration (via cystathionine) or by direct sulfhydrylation. Conversion of homocysteine to methionine is mediated by either of the two methionine synthetases present. The pathway configuration, which combines two different pathways, renders a greater flexibility to methionine biosynthesis and is consistent with studies demonstrating that methionine metabolism plays a crucial role in plant-microbe interactions and in bacterial fitness under stressful environmental conditions (Andersen et al., 1998; Tate et al., 1999). Genome comparisons reveal that this combination of pathways is conserved in soil or plant-associated Gram-negative bacteria *P. syringae*, *P. fluorescens*, *M. loti*, *B. fungorum*, *R. solanacearum* and *R. metallidurans*) and *X. fastidiosa*.

The pathways for the synthesis of purines, pyrimidines and nucleotides are all complete in the genome of *P. putida* KT2440. *P. putida* KT2440 can synthesize and elongate fatty acids from acetate. The genes for at least two β-oxidation pathways are present. According to its genome content, *P. putida* KT2440 should be able to synthesize an extensive variety of cofactors and prosthetic groups, including biotin, folic acid, ubiquinone, pyochelin, pantothenate, coenzyme A, ubiquinone, glutathione, thioredoxin, riboflavin, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD), NAD phosphate (NADP), porphyrin, thiamine, cobalamin, pyridoxal 5′-phosphate, tetrahydrofolate, and lipoate. In most cases, the genes encoding for the enzymes involved are scattered throughout the genome and do not aggregate in gene clusters. Although *P. putida* KT2440 is not known to grow under anaerobic conditions nor to denitrify, it has several determinants characteristic of metabolism under low oxygen tension, such as two oxygen-independent coproporphyrinogen III oxidase genes, a nitrite reductase complex, and several determinants typical of fermentative metabolism such as those for D-lactate dehydrogenase, phosphotransacetylase, formaldehyde dehydrogenase, and an acetoin gene cluster.

Surface Polymers More than 330 genes were found in the genome of *P. putida* KT2440 that are presumed to encode for proteins related to surface components and the biosynthesis and degradation of key cell-surface components. Apart from being an integral part of the cell structure, cell surface components are crucial for *P. putida* in determining motility and sensing and

in resistance and promoting attachment. The genes for the murein sacculus are grouped in three clusters scattered throughout the chromosome. Interestingly, two of these clusters include a penicillin-binding protein gene. The two other penicillin-binding protein genes are, as well, clustered with lipoproteins. The murein cluster includes also a group of genes for the synthesis of lipoic acid. Cell-division protein determinants are adjacent to a peptidoglycan synthesis gene and clustered with a σ^{32} factor. These patterns are conserved in *P. aeruginosa*, *E. coli*, *C. crescentus*, *R. solanacearum* and *A. tumefaciens*, and may reflect the close link between cell division and murein sacculus biosynthesis.

The LPS layer of the outer membrane in Gram-negative bacteria consists of a hydrophobic lipid A region anchoring the molecule in the outer leaflet, an oligosaccharide core, and an O-antigen (O polysaccharide; Raetz and Whitfield, 2002). The genes for lipid A synthesis are clustered together, and include genes involved in phospholipid biosynthesis and a gene coding for an outer membrane protein of the bacterial surface antigen family. This cluster-like structure is conserved in *P. aeruginosa*, *E. coli*, *C. crescentus* and *S. meliloti*, which agrees with the general observation that the A-lipid part of the LPS is more or less conserved within the Proteobacteria, whereas the O-antigen region (the immunogenic part in pathogens) tends to be variable, even within strains of the same species (Raymond et al., 2002).

The oligosaccharide core can be divided, conceptually, into two regions: inner core (lipid A proximal) and an outer core. As in *P. aeruginosa* and *E. coli*, several of the inner core genes in *P. putida* KT2440 are clustered together in close proximity and include the determinants for the heptose kinases, the heptosyltransferases I and II, and two lipopolysaccharide core biosynthesis proteins. However, unlike *P. aeruginosa* PAO1, the 3-deoxy-D-manno-octulosonic-acid transferase gene and the ABC lipid transporter that exports lipid A and phospholipids into the outer membrane are not within this cluster but, instead, cluster together in a region containing other genes related to the LPS-EPS core biosynthesis. This cluster includes a toluene tolerance protein as well as other genes related to oligosaccharide core biosynthesis in an arrangement similar to that of *P. aeruginosa* PAO1.

The O-antigen region in strains of *P. aeruginosa* is the most variable part of the LPS and is responsible for the different immunogenic responses in eukaryotic hosts. It generally contains an A- and a B-band. Junker et al. (2001) have determined that *P. putida* KT2440 lacks the A-band, although the genes encoding proteins for its biosynthesis are within the genome. Some of these genes, which code for various glycosyltransferases involved in the assembly of the A-band, are contained within a gene cluster that contains also the determinants for GDP-D-rhamnose biosynthesis from GDP-D-mannose and the determinants for an ABC transport system. This structure resembles in part that of *E. coli*, *Salmonella* or *P. aeruginosa* (Rochetta et al., 1998). However, this gene cluster in *P. putida* KT2440 lacks the gene encoding an essential rhamnosyl transferase and other genes that encode for proteins involved in B-band biosynthesis. Furthermore, the ABC system of *P. putida* KT2440 does not encode for the system responsible for the transport of O-repeat units, as in *P. aeruginosa* PAO1, but rather encodes for what seems to be a protein efflux system.

Adjacent to this A-band/B-band gene cluster, although reading in the opposite direction, is a cluster containing a cytidylyl-CMP-*N*-acetylneuraminic acid synthetase gene next to a hydrolase of the haloacid-dehalogenase family plus two glycosyl transferases that are likely to be involved in O-antigen synthesis. Adjacent to this group is a cluster containing the genes encoding the enzymes for the synthesis of dTDP-L-rhamnose, a sugar nucleotide precursor essential for the assemblage of the A-band of the O-antigen, as well as for alginate formation (Rocchetta et al., 1999). This cluster includes, as well, genes encoding a glycosyl transferase and an *O*-acetyltransferase involved in the B-band biosynthesis. Next to this gene cluster is another one that contains several genes encoding proteins possibly involved in the synthesis and transport of the A-lipid. These genes include the determinants for a mannosyl transferase, a phosphomannose isomerase, a phosphomannomutase gene, a lipopolysaccharide ABC-export system similar to the system for export of the individual and PP-linked O units (Raetz and Whitfield, 2002), a putative penicillin-binding protein (domain), a metallo-β-lactamase protein, and a β-subunit of an integration host factor. Like other plant- and soil-associated bacteria, *P. putida* KT2440 is observed to lack the pathway for transport of the O-units across the membrane. *P. aeruginosa*, on the other hand, and similar to *E. coli* and *Salmonella*, has two pathways. Possibly, these differences may account for the avirulence of *P. putida*, as compared to *P. aeruginosa*, in which the O-antigen of the various serotypes is considered an important virulence factor. The G+C content of this gene cluster (54.3%) is lower than that for the overall genome and is likely to have been acquired by gene transfer. All of these clusters with genes encoding proteins involved in the production of LPS-EPS components have a G+C content atypically low for *P. putida* and different from that of neighboring genes not involved in

biosynthesis of lipopolysaccharides. This suggests that acquisition of the LPS-EPS genes may have occurred independently, perhaps in clusters, as has been suggested for *X. campestris* (Vorhölter et al., 2001). The complete set of genes, as well as the organization of the synthesis of the O-antigen part of the LPS is different from that in *P. aeruginosa* in its various serotypes (Rocchetta et al., 1999; Raymond et al., 2002). Rather, the set of *P. putida* KT2440 resembles those in the genomes of other soil and plant associated bacteria, such as *R. solanacearum*, *X. campestris*, *M. loti* or *S. meliloti*, perhaps reflecting the common habitat and evolutionary conditions of these bacteria.

Sulfur Metabolism The operon enabling growth on aromatic and aliphatic sulfonates is present in *P. putida* KT2440. The genome contains the genes encoding the metabolism of alkylsulfonates, alkansulfonate-thiosulfate and thiosulfate. Furthermore, it has three genes coding for proteins similar to desulfurization enzymes although it lacks DszB monoxygenase for full desulfurization (Gallardo et al., 1996). However, the two specialized pathways encoded by asfRABC and atsSRBCA, described in other *P. putida* strains (Kahnert et al., 2000), are missing.

Peripheral Metabolism Strains of *P. putida* metabolize a variety of unusual and sometimes toxic compounds, particularly chemically stable aromatic organic compounds, and are frequently the predominant microbes isolated from polluted environments. A number of the corresponding catabolic pathways, for example, those for toluene and xylenes (Worsey and Williams, 1975; Williams and Worsey, 1976), phenols (Wong et al., 1976), naphthalene (Dunn, 1973), camphor (Rheinwald, 1973), biphenyls (Starovoitov, 1985), and chloroaromatics (Reineke, 1998) tend to be encoded by transmissible, broad host range catabolic plasmids that readily transfer between bacteria, and by catabolic transposons that readily transfer within the cell from plasmids to chromosomal locations. The location of catabolic determinants on mobile elements that can move horizontally among *Pseudomonas* species and related Proteobacteria provides such bacteria with considerable evolutionary potential, in terms of the metabolic capacities they can acquire for exploiting novel nutritional opportunities (Harayama et al., 1993).

P. putida KT2440 lacks catabolic plasmids but is known to have a chromosomal pathway for the degradation of benzoate, via catechol and 3-oxoadipate. Genome sequence analysis of *P. putida* KT2440 has revealed genetic determinants for putative enzymes able to transform a variety of other aromatic compounds. Several of these (ferulate, coniferyl- and coumaryl alcohols, aldehydes and acids, vanillate, *p*-coumarate, *p*-hydroxybenzoate and protocatechuate) are related to lignin, generated during decomposition of plant materials, and abundant in the rhizosphere. These compounds are, therefore, likely to constitute a carbon pool for rhizosphere associated microorganisms. Furthermore, these compounds have been shown to be important for signaling in gene expression between plants and some bacteria (Venturi et al., 1998). The general strategy exploited by *Pseudomonas* species for the degradation of different aromatic compounds is to modify their diverse structures to common intermediates that can be funneled into a limited number of central pathways (Dagley, 1971). In *P. putida* KT2440, for example, initial steps in the metabolism of ferrulate, *p*-coumarate, mandelate and benzoate would be mediated by different enzymes (upper pathways), although all routes ultimately converge via protocatechuate (ferrulate and *p*-coumarate) or catechol (mandelate and benzoate) to the common 3-oxoadipate (also known as β-ketoadipate) pathway. Interestingly, the 3-oxoadipate pathway has been found almost exclusively in soil- and plant-associated bacteria (Harwood and Parales, 1996) and is, presumably, evolved in response to the large number of phenolic compounds synthesized by plants.

Applications

Pseudomonas species are valuable agents for biotechnological applications, primarily because of the maleable genomes of the species, supporting a diverse range of metabolisms, and a propensity to accept genetic modifications. As a group, strains of various species of *Pseudomonas* are able to extract energy from a wide range of relatively inert compounds. Applications for *Pseudomonas* range from xenobiotic waste treatment and oil spill management (Timmis et al., 1994; Dejonghe et al., 2000), treatment of fossil fuels for improved quality (Bos et al., 1996; Foght et al., 1996), biocatalysis for the synthesis of various compounds (Zeyer et al., 1985; Olivera et al., 2001), and as agents of plant growth (Lugtenberg and Weger, 1992) and protection (Voisard et al., 1994; Walsh et al., 2001). Furthermore, because of the heterogeneity of the natural habitat of *Pseudomonas*, special requirements for growth and metabolic activity may not be necessary during biotechnological applications.

Bioremediation: Degradation of Chloroaromatics by Pseudomonas Chlorinated hydrocarbons comprise a spectrum of compounds that are

of industrial and economic importance. The introduction of chlorine atom(s) into a hydrocarbon significantly influences its physicochemical and biochemical properties and the tendency for bioaccumulation and environmental persistence. *Pseudomonas* species are well known for their widespread occurrence in natural habitats and their ability to utilize a variety of organic compounds, including haloaromatics.

THE CHLOROCATECHOL PATHWAY OF PSEUDOMONAS SPECIES. The first observations of microorganisms degrading chloroaromatic hydrocarbons were made in the 1960s by the groups of Alexander and Evans (Bollag et al., 1968; Tiedje et al., 1969; Gaunt and Evans, 1971; Evans et al., 1981). The elucidation of a dominant degradation pathway for chloroaromatics was carried out using one of the first bacterial strains observed to be able to degrade chloroaromatics, *Pseudomonas* sp. strain B13 (Dorn et al., 1974). *Pseudomonas* sp. B13 is capable of mineralizing 3-chlorobenzoate via 3-chloro- and 4-chlorocatechol as central intermediates. Degradation is initiated by a chromosomally encoded benzoate dioxygenase, forming 3-chloro- and 5-chlorodihydrodihydroxybenzoate in a 2:1 ratio (Reineke and Knackmuss, 1978a), followed by dehydrogenation resulting in 3-chloro- and 4-chlorocatechol (Reineke and Knackmuss, 1978b). Chlorocatechols are further degraded by a specialized set of enzymes, the chlorocatechol *ortho*-cleavage pathway (Dorn and Knackmuss, 1978a; Dorn and Knackmuss, 1978b; Schmidt and Knackmuss, 1980a; Schmidt et al., 1980b; Kaschabek and Reineke, 1992). Ring-cleavage of chlorocatechol is performed by a broad specificity chlorocatechol 1,2-dioxygenase to produce the corresponding chloro-*cis*, *cis*-muconates (Tiedje et al., 1969; Dorn and Knackmuss, 1978a; Dorn and Knackmuss, 1978b; Schmidt et al., 1980b; Broderick and O'Halloran, 1991). The elimination of the chlorine substituent has been assumed to occur spontaneously after 2-chloro- and 3-chloro-*cis*,*cis*-muconate are converted by chloromuconate cycloisomerases to 5-chloro- and 4-chloromuconolactone, respectively (Schmidt and Knackmuss, 1980a). The dienelactones (*cis*-dienelactone formed from 3-chloro- and *trans*-dienelactone from 2-chloro-*cis*,*cis*-muconate) are converted by a dienelactone hydrolase into maleylacetate (Schmidt and Knackmuss, 1980a), and a maleylacetate reductase catalyzes the reduction of the double bond to form 3-oxoadipate, the common metabolite of the 3-oxoadipate and the chlorocatechol *ortho*-cleavage pathway (Kaschabek and Reineke, 1992).

The genetic background for chlorocatechol degradation in *Pseudomonas* has been elucidated using an independently isolated 3-chlorobenzoate degrading *P. putida* strain AC858 (Chatterjee et al., 1981), and genes encoding chlorocatechol pathway enzymes were localized on a plasmid. Similarly, chlorocatechol genes were observed to be located on plasmids in various other chloroaromatic degrading isolates (Don, 1981), including *Pseudomonas* species (Van der Meer 1991). However, in the case of *Pseudomonas* sp. B13, the chlorocatechol genes were observed to be located on a 105-kb genomic island, named "the *clc* element," present in the chromosome in two separate locations (Ravatn et al., 1998). The *clc* element integrates into the 3′-end of a target glycine tRNA gene and obviously employs a phage-type integrase to achieve site-specific integrations (Ravatn et al., 1998; Van der Meer et al., 2001). With a certain frequency the element excises, resulting in a circular intermediate. In contrast to many pathogenicity islands, the *clc* element is self-transmissible to other recipient bacteria (Springael et al., 2002). The fact that chlorocatechol genes are localized on mobile genetic elements suggests that they are transferred readily and spread in the environment.

The genes encoding the chlorocatechol pathway usually form clusters, and only a few variations in the structures of the operons have been observed, in spite of the geographically distinct origins or differences in phylogenetic positions of the bacteria. Regulatory genes usually precede the structural genes and are orientated in the opposite direction. The gene products of the regulatory genes, which are members of the LysR family, act as positive regulators. 2-Chloro-*cis*, *cis*-muconate was demonstrated to be the inducer of the *clc* operon (McFall et al., 1997). However, not only is the structure of the operons of the chlorocatechol genes similar, but also isofunctional enzymes of the chlorocatechol pathway have been shown to be homologous and more similar to each other than to the corresponding enzymes of catechol pathways, indicating that the proteobacterial chlorocatechol pathways are derived from a common origin (Schlömann, 1994).

Like *Pseudomonas* sp. strain B13, various other *Pseudomonas* species have been described that degrade 3-chlorobenzoate via a chromosomally encoded benzoate dioxygenase/dehydrogenase system and chlorocatechol genes. Benzoate dioxygenase genes have been localized in *P. putida*, *P. aeruginosa* and *P. fluorescens*, and homologous genes seem to be spread among *Pseudomonas* species. In an early study, the capability to degrade benzoate was observed to be ubiquitously present in *P. aeruginosa*, *P. chlororaphis* and *P. putida*, but few *P. stutzeri* and *P. fluorescens* strains harbored that metabolic activity (Stanier et al., 1966). The substrate specificity

of benzoate dioxygenases is restricted usually to benzoate and 3-chloro-/3-methylbenzoate, as shown for chromosomally encoded enzymes from *Pseudomonas* sp. B13 (Reineke and Knackmuss, 1978a). Thus, strains, which have been obtained from enrichment cultures using 3-chlorobenzoate as the growth substrate, are usually the product of patchwork assembly, i.e., they harbor pathway segments from different origins obtained through horizontal natural gene transfer.

In contrast to benzoate dioxygenase, the toluate 1,2-dioxygenase (XylXYZ) of *P. putida* mt-2, whose natural function is the conversion of *m*- and *p*-toluate, transforms also 4-chlorobenzoate (Reineke and Knackmuss, 1978a). 4-Chlorobenzoate is a good substrate because of its structural analogy to *p*-toluate (4-methylbenzoate). Presumably, pathways evolved for the degradation of naturally occurring methyl-substituted growth substrates can often deal with substrates bearing chlorine substituents, since both substituents are of similar size.

Thus, it was reasoned that a combination of a toluate pathway with a chlorocatechol pathway should result in derivative strains capable of mineralizing 4-chlorobenzoate. *Pseudomonas* sp. B13 acquired the ability to utilize novel substrates like 4-chloro- and 3,5-dichlorobenzoate after transfer of the TOL plasmid of *P. putida* mt-2, harboring the toluate dioxygenase genes (Reineke and Knackmuss, 1979). Similarly, transfer of the *clc* element into *P. putida* strain mt-2 resulted in 4-chlorobenzoate-degrading derivatives (Reineke et al., 1982).

Additionally, various other enzyme systems, usually involved in the degradation of naturally occurring aromatics such as toluene, phenol, salicylate or aniline are rather unspecific and tolerate additional substituents on the substrate molecule. Thus, by the judicious combination of such peripheral enzyme systems and central chlorocatechol pathway segments in a suitable host organism, complete hybrid pathways have been produced for various mono- and disubstituted chloroaromatics (Pieper and Reineke, 2000), such as chlorinated benzenes (Oltmanns et al., 1988), phenols (Schwien and Schmidt, 1982), salicylates (Rubio et al., 1986), anilines (Latorre et al., 1984) and toluenes (Abril et al., 1989; Brinkmann and Reineke, 1992).

One of the best-described enzyme systems for the activation of benzene derivatives is the broad substrate specificity toluene dioxygenase of *P. putida* strain F1, which belongs to the toluene/biphenyl family of Rieske non-heme iron dioxygenases (Gibson and Parales, 2000). The enzyme system comprises an electron transport chain of ferredoxin reductase and ferredoxin, which channels electrons from NADH onto the catalytically active oxygenase consisting of α- and β-subunits (Yeh et al., 1977), with the α-subunit being predominantly responsible for the substrate specificity (Beil et al., 1998). Toluene is oxidized to the respective *cis*-dihydrodiol (Gibson et al., 1970), which is subsequently dehydrogenated to 3-methylcatechol. This enzyme system also accepts chlorobenzene, *p*-chloro- or *p*-bromotoluene as substrates, which are oxidized to their dihydrodiols and dehydrogenated to the respective catechols (for a review of toluene dioxygenase-catalyzed conversions, see Hudlicky et al., 1999). Similarly, 1,4-dichlorobenzene degraders can easily be evolved by acquisition of chlorocatechol genes in toluene degrading strains (Oltmanns et al., 1988). Only a few microorganisms have been isolated thus far on the basis of their abilities to degrade 1,2-dichloro- (Haigler et al., 1988), 1,3-dichloro- (De Bont et al., 1986), or 1,4-dichlorobenzene (Schraa et al., 1986; Oltmanns et al., 1988). Obviously, their enzymes are adapted specifically for transformation of their respective growth substrate. *Pseudomonas* sp. strain P51, isolated on the basis of its ability to degrade 1,2,4-trichlorobenzene (Van der Meer et al., 1987), contains a broad-spectrum chlorobenzene dioxygenase capable of transforming all dichloro- and even 1,2,4-trichlorobenzene. The respective *tcb* genes (Werlen et al., 1996) are very similar to the respective *tod* genes involved in toluene degradation by F1 (e.g., 89% identity in amino acid sequence of the respective α-subunits), indicating that small changes in amino acid sequence have a significant influence on substrate specificity.

The first enzymes of the chlorobenzene degradation pathway of strain P51, the chlorobenzene dioxygenase and the *cis*-chlorobenzene dihydrodiol dehydrogenase, are encoded on a plasmid-located transposon (Werlen et al., 1996), with the chlorocatechol genes located on the same plasmid. High similarity suggested that the chlorobenzene dioxygenase and dehydrogenase originated from a toluene or benzene degradation pathway, probably by horizontal gene transfer, to form a functional chlorobenzene degradative pathway in combination with the chlorocatechol genes.

The dioxygenation of toluene is only one of five different aerobic pathways for initializing toluene degradation. The TOL plasmid pWW0 is known to encode not only the enzymes for the degradation of *m*- and *p*-toluate (see above) but the complete set of enzymes necessary for the degradation of *m*- and *p*-xylene via the respective methylbenzoates and of toluene via benzoate as intermediates (Worsey and Williams, 1975). The degree of transformation of chloro-

toluenes by xylene monooxygenase (XylMA) depends on the position of the chlorine substituent. The substrate analogs 3-chloro- and 4-chlorotoluene are transformed at high rates, whereas no or only low activities have been detected with other chlorotoluenes. Substituents in the *ortho* position impair substrate binding (Brinkmann and Reineke, 1992). Transfer of the TOL plasmid into strain B13 allowed the isolation of chlorotoluene-degrading *Pseudomonas* species (Abril et al., 1989; Brinkmann and Reineke, 1992).

Toluene monooxygenases that hydroxylate the aromatic nucleus at all three possible positions, producing 2-methyl-, 3-methyl-, or 4-methylphenol have been described (Schields et al., 1989; Whited and Gibson, 1991; Olsen et al., 1994) and include the xylene monooxygenase systems of *P. stutzeri* OX1 (Bertoni et al., 1996) and *P. mendocina* KR1 (Whited and Gibson, 1991). These enzymes belong to an evolutionarily related family of soluble di-iron hydroxylases, including also phenol hydroxylases and methane monooxygenases. The enzyme complexes consist of an electron transport system comprising a reductase (and in some cases a ferredoxin), and a catalytic effector protein (Powlowski et al., 1997). Only limited information is available on the transformation of chlorophenols formed by monooxygenation by multicomponent toluene hydroxylases.

Salicylate hydroxylase is a flavoprotein monooxygenase that catalyzes the conversion of salicylate to catechol. The enzyme was first purified from *P. putida* S1 (Yamamoto et al., 1965), and later from various other *Pseudomonas* and pseudomonad species, and cloned and sequenced from various sources (You et al., 1991; Lee et al., 1995; Bosch et al., 1999a; Jones et al., 2000), predominantly *Pseudomonas* species. Usually, salicylate hydroxylase is included in the naphthalene pathway, and the gene encoding salicylate hydroxylase is followed by genes encoding a *meta*-cleavage pathway. Different so-called "NAH" plasmids harboring those genes have been described from *Pseudomonas* strains (Cane and Williams, 1982; Yen and Gunsalus, 1982), but in contrast, *P. stutzeri* AN10 harbors chromosomally located *nah* genes (Rosselló-Mora et al., 1994). Moreover, variations in gene organization have been observed. *P. stutzeri* AN10, besides containing *nahG* encoding salicylate hydroxylase located in one transcriptional unit with the *meta*-cleavage pathway genes, contains a second gene encoding salicylate hydroxylase, *nahW*, which is situated outside, but in close proximity to, this transcriptional unit. Both, the *nahG* and *nahW* genes of *P. stutzeri* AN10 are induced and expressed upon incubation with salicylate. Such a gene organization seems to be common in naphthalene-degrading *P. stutzeri* strains (Bosch et al., 1999b). Despite differences in gene organization and partially low similarity (NahW shares 23–25% amino acid sequence identity to other salicylate hydroxylases), most salicylate hydroxylases described thus far exhibit similar substrate ranges with significant activities against 4-chloro- and 5-chlorosalicylate, and a lower activity against 3-chlorosalicylate (Lehrbach et al., 1984; Rubio et al., 1986; Bosch et al., 1999b; Jones et al., 2000). However, recently a new group of three component salicylate 1-hydroxylases has been described, which consist of a hydroxylase component, a ferredoxin, and a ferredoxin reductase (Pinyakong et al., 2003), differing significantly in substrate specificity from previously analyzed single component salicylate 1-hydroxylases. Given the broad substrate-specificity of salicylate 1-hydroxylases, it is not astonishing that chlorosalicylate-mineralizing strains of *Pseudomonas* species could easily be obtained by combining a salicylate hydroxylase with a functioning chlorocatechol pathway (Lehrbach et al., 1984; Rubio et al., 1986). Genes similar to those encoding salicylate hydroxylase (ca. 25–30% amino acid sequence identity) have been localized in the genomes of *P. aeruginosa* strain PAO1 and *P. putida* strain KT2440, although *P. putida* strain KT2440 is reported not to grow on salicylate (Jimenez et al., 2002). Analyses have shown that only a few strains of *Pseudomonas* species are capable of mineralizing salicylate, whereas such a capability seems to be common in *Burkholderia cepacia* (Stanier et al., 1966).

Oxygenolytic Dehalogenation Usually, dioxygenases activating the aromatic ring attack either at two unsubstituted carbon atoms, as observed for benzenetoluene dioxygenase, or at an unsubstituted and a carboxylated carbon atom, such as demonstrated by benzoatetoluate dioxygenases, giving rise to *cis*-dihydrodiols that are transformed further by dehydrogenases to give 1,2-diphenols. Some ring-activating dioxygenases can bring about the dehalogenation of haloaromatic compounds. Benzoate dioxygenase of strain B13 attacks 2-fluorobenzoate predominantly in a 1,6-fashion, giving rise to a dihydrodiol, which is rearomatized to 3-fluorocatechol. As the ring-cleavage product 2-fluoromuconate is not a substrate for proteobacterial (chloro)muconate cycloisomerases, strain B13 cannot grow on 2-fluorobenzoate. However, prolonged adaptation resulted in derivatives that grow on this substrate. Obviously, spontaneous mutants of the benzoate dioxygenase arose, which performed a 1,2-dioxygenation (Engesser et al., 1980), such that one of the *vic*-hydroxyl groups in the

cis-dihydrodiol is bound to the same carbon as the fluoro-substituent. From such an unstable *vic*-dihydrodiol, the fluoro-substituent will be spontaneously eliminated to give catechol. However, 2-chlorobenzoate cannot be used as a growth substrate by B13-derivatives, indicating that proteobacterial benzoate dioxygenases cannot accommodate such a voluminous substituent in the *ortho*-position.

Since the 1980s, bacteria capable of degrading 2-chlorobenzoate have been reported and, thus far, only *Pseudomonas* and *Burkholderia* species have been observed to possess such a capability. The isolates could be grouped into those capable of degrading 2-chlorobenzoate, such as *P. putida* strain CLB250 (Engesser and Schulte, 1989) and *P. aeruginosa* strain 2-BBZA (Higson and Focht, 1990), and those capable of degrading also 2,4-dichloro- or 2,5-dichlorobenzoate, such as *P. stutzeri* strain KS25 (Kozlovsky et al., 1993), *P. putida* strain P111 (Hernandez et al., 1991), *P. aeruginosa* strain JB2 (Hickey and Focht, 1990), and *P. aeruginosa* strain 142 (Romanov et al., 1993). All of these organisms catalyze a 1,2-dioxygenation of 2-chlorobenzoate, thereby forming catechol, whereas 4-chlorocatechol is produced by the dioxygenation of 2,4-dichloro- and 2,5-dichlorobenzoate. Inasmuch as the degradation of 4-chlorocatechol requires a chlorocatechol degradative pathway, presumably organisms capable of degrading 2-chlorobenzoate only, and those capable of degrading also 2,4-dichloro- or 2,5-dichlorobenzoate, differ by the presence of a chlorocatechol pathway. However, two distinct 2-chlorobenzoate degrading dioxygenase systems have been described. The two-component 2-halobenzoate 1,2-dioxygenases (oxygenase consisting of α- and β-subunits and a reductase) are similar (56–59% in α-subunits amino acid sequence identity) to a two-component toluate 1,2-dioxygenase and to benzoate and toluate 1,2-dioxygenases (Harayama et al., 1991; Cowles et al., 2000) and belong to the family of benzoate dioxygenases (Gibson and Parales, 2000). These 2-halobenzoate dioxygenases are characterized by their high activity against 2-chlorobenzoate (Fetzner et al. 1992; Suzuki et al., 2001) but negligible activity against 2,4-dichloro- or 2,5-dichlorobenzoate. In contrast, the 2-chlorobenzoate dioxygenase system of *P. aeruginosa* strain 142 is a three-component dioxygenase system (an oxygenase consisting of α- and β-subunits, ferredoxin and reductase; Romanov and Hausinger, 1994). Moreover, the α-subunits of 2-chlorobenzoate dioxygenases of *P. aeruginosa* strains JB2 and 142 (Tsoi et al., 1999) exhibit only 22% amino acid sequence identity with that of *B. cepacia* strain 2CBS but higher levels of sequence similarity (42%) with the salicylate 5-hydroxylase from *Pseudomonas* sp. strain U2 (Fuenmayor et al., 1998). Thus, 2-chlorobenzoate dioxygenases are functionally similar but represent two different lineages with distinct activities.

Metabolism of Chloroaromatics by 3-Oxoadipate Pathway Enzymes The aerobic degradation of aromatic compounds usually involves the successive activation and modification such that they are channeled toward a few dihydroxylated intermediates, such as catechol, gentisate or protocatechuate, which are then subjected to ring cleavage. A variety of the enzyme systems capable of activating aromatic compounds are characterized by broad substrate specificities and transform chlorinated substrate analogues, often resulting in the formation of chlorinated catechols (see above). However, only a small fraction of bacteria able to transform chloroaromatics is also capable of mineralizing them, as this usually requires the chlorocatechol pathway. Chlorocatechols are regarded as environmentally important intermediates and their metabolic fate can be of environmental significance. The chromosomally encoded 3-oxoadipate pathway is one such pathway that is widely distributed in soil bacteria and fungi (Harwood and Parales, 1996).

Marked differences have recently been demonstrated between reactions catalyzed by the chlorocatechol and the 3-oxoadipate pathway enzymes. In both cases, chlorocatechols were subject to intradiol cleavage producing the corresponding *cis*,*cis*-muconates (Blasco et al., 1995). However, muconate and chloromuconate cycloisomerases perform distinct reactions. Whereas chloromuconate cycloisomerases catalyze a dehalogenation of 3-chloro-*cis*,*cis*-muconate to form *cis*-dienelactone, muconate cycloisomerases catalyze a dehalogenation and decarboxylation to form protoanemonin, a compound of high toxicity (Seegal and Holden, 1945). Protoanemonin formation was observed to be a common reaction performed by proteobacterial muconate cycloisomerases, including those from strains of *Pseudomonas* species (Vollmer et al., 1998). Protoanemonin formation (due to channeling of intermediary chlorobenzoate into the 3-oxoadipate pathway), in turn, was assumed to be the reason for the poor survival of organisms cometabolizing polychlorinated biphenyls in soil microcosms (Blasco et al., 1997). Even chloroprotoanemonin was reported to be formed from 2,4-dichloromuconate by *P. putida* muconate cycloisomerase (Kaulmann et al., 2001). Also in the case of 2-chloromuconate turnover, muconate and chloromuconate cycloisomerases were shown to catalyze different reactions. Whereas chloromuconate

cycloisomerase catalyzes dehalogenation to form *trans*-dienelactone, via 5-chloromuconolactone as an intermediate (Vollmer and Schlömann, 1995), muconate cycloisomerases catalyze cycloisomerization only, to form 2-chloro- and 5chloromuconolactone as stable products (Vollmer et al., 1994). Only chloromuconate cycloisomerase, but not muconate cycloisomerase, catalyzes the dehalogenation of 5-chloromuconolactone (Vollmer et al., 1994; Vollmer and Schlömann, 1995). However, 5-chloromuconolactone has been shown to be a substrate of proteobacterial muconolactone isomerases (Prucha et al., 1996a; Prucha et al., 1996b) of the 3-oxoadipate pathway. Muconolactone isomerase catalyzes a dehalogenation of 5-chloromuconolactone to form predominantly *cis*-dienelactone. The proposed mechanism was via abstraction of the C4 proton followed by spontaneous chloride elimination. Like 5-chloromuconolactone, 2-chloromuconolactone also harbors a proton at C4, which can be abstracted by muconolactone isomerase, and protoanemonin was shown to be the reaction product (Skiba et al., 2002).

The Alternative Pathway of 4-Halocatechol Degradation in *Pseudomonas* Muconate cycloisomerases, as described above, form protoanemonin as a toxic dead-end product (Blasco et al., 1995). Hence, transformation of 4-chlorocatechol by enzymes of the 3-oxoadipate pathway constitutes a problematic catabolic misrouting in bacterial communities (Blasco et al., 1997). No effective activity against protoanemonin has been characterized thus far, although a degradative pathway with protoanemonin as an intermediate was proposed for 4-chlorocatechol degradation by *Pseudomonas* sp. strain RW10 (Wittich et al., 1999). This organism was shown to have a 3-oxoadipate pathway but no chlorocatechol pathway, thus forming protoanemonin from 3-chloromuconate. *Pseudomonas* sp. strain MT1, the most predominant organism in a 4-membered community isolated by continuous culture enrichment on 4-chlorosalicylate as sole carbon source (Pelz et al., 1999), was similarly proposed to have a new 4-chlorocatechol degradative pathway, which was thought to involve protoanemonin as intermediate. Both strains do not express enzymes of the chlorocatechol pathway but contain a high level of a *trans*-dienelactone hydrolase when grown on chloroaromatics. This enzyme was shown to be the key enzyme of a novel 4-chlorocatechol degradative pathway operating in *Pseudomonas* sp. strain MT1 (Nikodem et al., 2003), and probably also in *Pseudomonas* sp. strain RW10. 4-Chlorocatechol was subject to ring-cleavage by a catechol 1,2-dioxygenase to yield 3-chloromuconate. 3-Chloromuconate was transformed to protoanemonin as the dominant reaction product by muconate cycloisomerase of strain MT1. Formation of protoanemonin is obviously a dead-end product of the pathway. Even though *trans*-dienelactone hydrolase does not act on 3-chloromuconate nor on protoanemonin, the simultaneous presence of muconate cycloisomerase and *trans*-dienelactone hydrolase yields considerably smaller protoanemonin concentrations, although higher amounts of maleylacetate from 3-chloromuconate, than muconate cycloisomerase alone (Nikodem et al., 2003). It is suggested that this enzyme acts on 4-chloromuconolactone as an intermediate in the muconate cycloisomerase-catalyzed transformation of 3-chloromuconate, thus preventing protoanemonin formation in favor of maleylacetate formation. The formed maleylacetate is reduced subsequently by maleylacetate reductase. Chlorocatechol degradation in strain MT1 thus occurs by a new pathway consisting of a patchwork of reactions recognized from the 3-oxoadipate pathway (catechol 1,2-dioxygenase and muconate cycloisomerase), the chlorocatechol pathway (maleylacetate reductase), and a *trans*-dienelactone hydrolase.

Alternative Central Pathways for Chlorinated 1,2-Diphenolic Intermediates For quite a long time, the presence of a catechol *meta*-cleavage pathway was assumed to interfere significantly with degradation of chloroaromatics. Catechol *meta*-cleavage routes are widespread in *Pseudomonas* species and are usually involved in the degradation of methyl-substituted compounds, such as toluene or methylphenols (Bayly et al., 1966; Worsey and Williams, 1975; Zylstra et al., 1988; Schingler et al., 1992). One of the reasons for interference was assumed to be the formation of a suicide product, reactive acyl chloride, e.g., from 3-chlorocatechol by the catechol 2,3-dioxygenase of *P. putida* mt-2 (Bartels et al., 1984). The formation of acyl chloride results in irreversible inactivation of the ring cleavage enzyme. Other enzymes such as the catechol 2,3-dioxygenase of *P. putida* F1 were reported to be reversibly inactivated by 3-chlorocatechol. Inactivation has been attributed to the potential of the substrate to chelate the active site ferrous ion (Klecka and Gibson, 1981). In other cases, reversible inactivation was shown to be due to a rapid oxidation of the active site ferrous iron into its ferric form with concomitant loss of activity (Vaillancourt et al., 2002).

Recently, *P. putida* strain GJ31 was observed to degrade chlorobenzene rapidly via 3-chlorocatechol and using a *meta*-cleavage

pathway (Mars et al., 1997). In contrast to other catechol 2,3-dioxygenases, which are subject to inactivation, the chlorocatechol 2,3-dioxygenase of strain GJ31 converts 3-chlorocatechol (Kaschabek et al., 1998; Mars et al., 1999). Stoichiometric displacement of chloride during substrate turnover leads to the production of 2-hydroxymuconate, which is further converted through the *meta*-cleavage pathway.

In contrast to 3-chlorocatechol, 4-chlorocatechol is a moderate substrate for various catechol 2,3-dioxygenases (Murray et al., 1972; Bartels et al., 1984; Kitayama et al., 1996), among them catechol 2,3-dioxygenases of family I.2.A (Eltis and Bolin, 1996), which are involved in the degradation of methylaromatics by *Pseudomonas* species. Some chlorinated compounds, degraded via 4-chlorocatechol, have been postulated to be mineralized via a catechol-degrading *meta*-cleavage pathway (Janke and Fritsche, 1979; McCullar et al., 1994; Arensdorf and Focht, 1995; Hollender et al., 1997), although the manner in which the products are dechlorinated is unknown.

Protocatechuate is a central intermediate in the degradation of various carboxylated aromatic compounds. Three modes of further degradation of protocatechuate have been reported. The intradiol cleavage by a protocatechuate 3,4-dioxygenase seems to be widespread in *Pseudomonas* species (all strains of a collection of more than 100 strains of *Pseudomonas* species displayed such an activity; Stanier et al., 1966), and the locations of the respective *pca* gene clusters have been determined within the genomes of *P. aeruginosa* strain PAO1, *P. putida* strain KT2440, *P. fluorescens* strain Pfo-1, and *P. syringae* pv. tomato DC3000 (Jimenez et al., 2002). However, this pathway seems to be unsuited for the degradation of chloroprotocatechuate.

Whereas a protocatechuate 2,3-dioxygenase has so far been described only in an isolate of a *Bacillus* species (Wolgel et al., 1993), protocatechuate 4,5-dioxygenase seems to be widespread. Strains of *Delftia acidovorans*, *Comamonas testosteroni* and *Sphingomonas paucimobilis* usually exhibit such an activity (Stanier et al., 1966; Noda et al., 1990), whereas isolates of *Pseudomonas* species do not (Stanier et al., 1966).

However, genes similar to those encoding the protocatechuate 4,5-dioxygenase pathway were observed in the genome of *P. putida* strain KT2440, although the functioning of this pathway has not been proven yet (Jimenez et al., 2002). Only the protocatechuate 4,5-dioxygenase pathway has been shown to be functional for the degradation of chloroprotocatechuate, and the formation of 2-pyrone-4,6-dicarboxylate by nucleophilic displacement of a halide ion from protocatechuates substituted with a halogen at the C5 of the nucleus was shown (Kersten et al., 1982; Kersten et al., 1985). This indicated that cyclization, entailing nucleophilic displacement of the halogen, provides an effective alternative to the enzyme suicide inactivation that occurs when a nucleophilic group of the dioxygenase undergoes acylation. Hydrolysis of the pyrone is followed by degradation through the *meta*-cleavage pathway.

THE 4-CHLOROBENZOATE HYDROLYTIC DEHALOGENATION The degradation of 4-chlorobenzoate by a pathway distinct from that involving chlorocatechol, but involving an early dehalogenation, was indicated as early as 1976 (Ruisinger et al., 1976). *Pseudomonas* sp. strain CBS3 can be regarded as the archetypal organism from which the metabolic route and the mechanism of hydrolytic dehalogenation have been elucidated in detail (Müller et al., 1984). The 4-chlorobenzoate dehalogenase system from *Pseudomonas* sp. strain CBS3 has been shown to be a three-component enzyme complex (Elsner et al., 1991; Löffler et al., 1991) and the role of each component has been clarified (Scholten et al., 1991; Savard et al., 1992). The activation of the substrate is carried out by an ATP-dependent 4-halobenzoate-coenzyme A ligase (Löffler and Müller, 1991; Chang et al., 1992). Identification of 4-chlorobenzoyl-coenzyme A, as an intermediate in the dehalogenation, is catalyzed by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp CBS3 (Löffler et al., 1992). This is followed by dehalogenation, catalyzed by a 4-halobenzoyl-CoA dehalogenase, forming 4-hydroxybenzoyl-CoA (Liang et al., 1993; Löffler et al., 1995) in a hydrolytic substitution reaction. This enzyme was reported to share ancestry with the 2-enoyl-CoA hydratase family (Babbitt et al., 1992; Xiang et al., 1999). The last step in the reaction forming 4-hydroxybenzoate is carried out by the 4-hydroxybenzoate:coenzyme A thioesterase.

The genes encoding the 4-chlorobenzoate dehalogenase system were reported to be chromosomally localized and organized in an operon in the order: *fcbB* (dehalogenase), *fcbA* (ligase), and *fcbC* (thioesterase; Babbitt et al., 1992). The gene organization in a second 4-chlorobenzoate-degrading *Pseudomonas* sp. strain DJ-12 was similar, although, with an additional three new genes, localized between *fcbA* and C and, supposedly, responsible for 4-chlorobenzoate transport (Chae et al., 2000).

METABOLISM OF CHLOROBIPHENYLS Among chlorinated aromatics, the degradation of bicyclic compounds such as polychlorobiphenyls (PCBs)

or chlorinated dioxins has received special attention. A number of biphenyldegrading organisms have been isolated and they are commonly capable of transforming PCB congeners. These organisms belong to Gram-negative and Gram-positive genera and include various isolates of *Pseudomonas* species, such as *P. pseudoalcaligenes* strain KF707, *P. putida* strain KF715, *P. putida* strain OU83, and catabolize biphenyl to benzoate and 2-hydroxypenta-2,4-dienoate via the so-called "upper pathway," consisting of four enzymes, i.e., biphenyl 2,3-dioxygenase (BphA), 2,3-dihydro-2,3-dihydroxybiphenyl 2,3-dehydrogenase (BphB), 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD) (Boyle et al., 1992).

Biphenyl degradation genes usually are organized in gene clusters and have been localized on bacterial chromosomes (Furukawa and Miyazaki, 1986; Erikson and Mondello, 1992), plasmids (Hooper et al., 1989) and transposons (Springael et al., 1993). Organisms such as *P. putida* strain KF717 contain a *bphABCD* gene cluster. In *Burkholderia* sp. strain LB400, genes that encode enzymes involved in the transformation of the C-5 carbon released during hydrolysis of the ring-cleavage product to form benzoate and a glutathione S-transferase are located between *bphC* and *bphD* (Hofer et al., 1994). A detailed analysis of various biphenyl degrading isolates revealed that such a genetic organization is not unique to *Burkholderia* sp. strain LB400 (Bartels et al., 1999) but occurs also in *P. pseudoalcaligenes* strain KF707 and various strains of *P. agarici*, *P. oleovorans*, *P. balearica* and *P. putida*, and seems to be widespread in *Pseudomonas*.

To a significant extent, the spectrum of PCB congeners that can be transformed by an organism is determined by the specificity of the biphenyl 2,3-dioxygenase, the enzyme which catalyzes the first step in the upper pathway and which belongs to the toluene/biphenyl family of Rieske type non-heme iron dioxygenases. Studies on various biphenyl 2,3-dioxygenases have revealed considerable differences in their congener selectivity patterns, as well as their preference of the attacked ring (Erikson and Mondello, 1993; Kimura et al., 1997; McKay et al., 1997; Seeger et al., 1999; Zielinski et al., 2002). The different substrate specificities in bacterial strains were due to relatively few differences in *bphA*, the gene coding the large subunit of the terminal biphenyl dioxygenase.

Although certain PCBs may serve as substrates for biphenyl dioxygenases, PCB-degrading organisms usually do not use PCBs as an energy source but rather cometabolize the substrates. Not surprisingly, metabolites of the upper pathway may be formed as dead-end products (Furukawa et al., 1979; Bedard and Haberl, 1990; Seeger et al., 1997). Microorganisms capable of mineralizing a subset of PCB congeners have been obtained by judicious combination of pathway segments comprising the biphenyl upper pathway, a benzoate/toluate or 2-chlorobenzoate dioxygenase system and a chlorocatechol pathway (Mokross et al., 1990; Havel and Reineke, 1991; Hickey et al., 1992; McCullar et al., 1994; Hrywna et al., 1999), usually by in vivo conjugative mating of appropriate strains.

Transformation of Biarylethers Studies by Cerniglia et al. (1979) and Klecka and Gibson (Klecka and Gibson, 1979; Klecka and Gibson, 1981), using naphthalene or biphenyl-degrading strains of *Pseudomonas* or *Sphingomonas* species, indicated that dibenzo-*p*-dioxin, dibenzofuran and chlorinated derivatives were transformed into dead-end products. Analysis of bacterial degradation of dibenzofuran (Engesser et al., 1989; Fortnagel et al., 1990), carbazole (Sato et al., 1997), dibenzo-*p*-dioxin (Wittich et al., 1992), and diphenyl ether (Schmidt et al., 1992; Dehmel et al., 1995) revealed a novel mode of dioxygenation reaction for the aromatic nucleus, termed "angular dioxygenation," in which the carbon bonded to the heteroatoms and the adjacent carbon in the aromatic ring are oxidized. Angular dioxygenation of dibenzofuran, carbazole, dibenzo-*p*-dioxin, and diphenyl ether produces chemically unstable hemiacetal-like intermediates, which are spontaneously converted to 2,2′,3-trihydroxybiphenyl, 2′-amino-2,3-dihydroxybiphenyl, 2,2′,3-trihydroxydiphenyl ether, and phenol plus catechol, respectively. Angular dioxygenation thus results in the cleavage of the diphenyl ether three-ring structure. The 2′-substituted 2,3-dihydroxybiphenyls that are formed are degraded further by *meta*-cleavage and hydrolysis, resulting in the formation of salicylate (from dibenzofuran; Fortnagel et al., 1990; Strubel et al., 1991), catechol (from dibenzo-*p*-dioxin; Wittich et al., 1992), or anthranilate (from carbazole; Sato et al., 1997), respectively. So far, only a few bacterial strains capable of catalyzing angular dioxygenation have been described, predominantly strains of *Pseudomonas*, *Sphingomonas* and *Terrabacter* species. The first angular dioxygenase described originated from *Sphingomonas wittichii* strain RW1 (Armengaud et al., 1998). Like dioxygenase α-subunits of angular dioxygenases from other strains of *Sphingomonas*, *Terrabacter* and *Rhodococcus* species, the α-subunit of the RW1-derived dioxygenase is closely related to the toluene/biphenyl and naphthalene families of Rieske nonheme iron oxygenases (Shepherd and

Lloyd-Jones, 1998; Kasuga et al., 2001; Iida et al., 2002).

These angular dioxygenases do not comprise a monophylogenetic group, but are derived from at least four lineages. None of these genes has been reported thus far in *Pseudomonas* species, even though some angular dioxygenase genes were reported to be located on mobile elements (Iida et al., 2002). In contrast, carbazole dioxygenases of the phthalate family are frequently observed in *Pseudomonas* species and the gene products have been observed to transform dibenzofuran and dibenzo-*p*-dioxin (Sato et al., 1997; Habe et al., 2002), as well as chlorinated derivatives (Habe et al., 2001; Habe et al., 2002).

Anaerobic Degradation of Haloaromatic Compounds The capability for denitrification under anaerobic conditions is widespread among *Pseudomonas* species. Additionally, various dehalogenating enzymes do not require oxygen and function under anaerobic conditions. The observation of *Pseudomonas* species degrading chloroaromatics under anaerobic conditions would not be surprising, and the establishment of the *Pseudomonas* sp. strain CBS3 4-chlorobenzoate dehalogenase genes in *Thauera aromatica* T1, which is capable of degrading toluene and 4-hydroxybenzoate under denitrifying conditions, resulted in a derivative mineralizing 4-chlorobenzoate anaerobically (Coschigano et al., 1994).

Under denitrifying conditions, 2-fluoro- and 3-fluoro- as well as 3-chloro- and 4-chlorobenzoate can be degraded readily (Häggblom et al., 1996; Vargas et al., 2000). Analyses have shown, however, that the ability to degrade such halobenzoates under anoxic conditions is widespread among the Proteobacteria, and various strains of *Pseudomonas* species, most closely related to *P. stutzeri* (Song et al., 2000; Vargas et al., 2000), have been isolated that are capable of degrading 2-fluoro-, and 4-fluoro- as well as 3-chlorobenzoate. Evidently, *P. stutzeri* is important for halobenzoate degradation under natural conditions. However, the mechanisms of halobenzoate degradation under denitrifying conditions have not been elucidated.

Importance of *Pseudomonas* for Chloroaromatic Degradation In Situ The frequent isolation of strains of *Pseudomonas* species capable of degrading chloroaromatics seems to indicate that they are of some importance for degradation under natural environmental conditions. However, various catabolic pathways for chloroaromatics are plasmid encoded, and gene transfer is known to occur under environmental conditions. Different plasmids and mobile elements, specifically those involved in the degradation of chlorobenzoate, 2,4-dichlorophenoxyacetic acid (2,4-D) or chloroaniline, have been examined for their transfer under natural conditions, and transconjugants receiving the plasmids have been characterized. Generally, the type of species recovered might be inherent to the environment that provided the selection pressure. Selection pressures might include to the ability to survive in biofilms or to scavenge nutrients. In a detailed study on two surface horizons from an agricultural soil inoculated with a *P. putida* harboring one of two distinct catabolic plasmids, pJP4 (Don and Pemberton, 1981) or pEMT1 (Top et al., 1995), changes were observed in the overall community because of proliferation of the transconjugants (Dejonghe et al., 2000), and a detailed phylogenetic analysis of these transconjugants showed that the plasmids were preferentially acquired and expressed in soil by representatives of *Ralstonia* and *Burkholderia*. Similarly, no *Pseudomonas* species could be isolated after bioaugmentation of a sandy loam soil with pJP4 donor strains (Newby et al., 2000).

Other studies have examined collections of strains obtained from environmental samples (McGowan et al., 1998). Degradation of 2,4-D among easily cultivated organisms is more widespread among strains of *Ralstonia* and *Burkholderia* species than among *Pseudomonas* species. However, such studies are dependent upon the environment analyzed. Subsurface aquifers constitute environments that are physically, chemically and biologically different from surface soils with reduced concentrations and availabilities of oxygen, carbon and nutrients, as well as lower bacterial densities (Ghiorse and Wilson, 1988). The continuous exposure of such aquifers to chlorinated phenoxyacetic acid herbicides has been shown to result in changes in the community composition with a resulting increased abundance of *Pseudomonas* species (De Lipthay et al., 2003). Though only one of 50 *Pseudomonas* species mineralized 2,4-D, an important role of *Pseudomonas* on 2,4-D metabolism in situ was suggested. Rhizosphere bacteria, such as fluorescent *Pseudomonas* species, are ecologically adapted to colonize and compete in the rhizosphere environment and it can be speculated that *Pseudomonas* constitute a significant fraction of rhizosphere-associated 2,4-D degraders.

Although *Pseudomonas* sp. strain B13 was isolated as a host of the *clc* element, in inoculation experiments into natural ecosystems, the *clc* element, like *tfd* genes, ended up in *Ralstonia* or related Betaproteobacteria, like *Comamonas* (Zhou and Tiedje, 1995; Ravatn et al., 1998; Springael et al., 2002). This was assumed to

indicate that the *clc* genes are most efficiently expressed in strains belonging to those genera rather than in fluorescent pseudomonads. However, additional factors govern the acquisition and spread of a 3-chlorobenzoate-degrading phenotype in nature. 3-Chlorobenzoate is activated by a chromosomally encoded benzoate dioxygenase and dehydrogenase by organisms harboring chlorocatechol genes. Thus, properties of the benzoate dioxygenase system of possible recipient strains can be regarded as selectivity factors (Bott and Kaplan, 2002). *Pseudomonas* species were prevalent among 3-chlorobenzoate-degrading isolates harboring a chlorocatechol *ortho*-cleavage pathway (Peel and Wyndham, 1999; Krooneman et al., 2000).

Diverse pathways capable of achieving mineralization add another layer of complexity to the degradation of chlorobenzoates. Degradation of chlorobenzoates can occur via chlorocatechol (*clc* pathway), hydrolytic dehalogenation of 4-chlorobenzoate to give 4-hydroxybenzoate (*fcb* pathway; Klages and Lingens, 1980), dioxygenation of 3-chlorobenzoate to give 5-chloroprotocatechuate (Nakatsu and Wyndham, 1993), and probably via a fourth pathway with gentisate as intermediate (gp-pathway; Krooneman et al., 1996; Krooneman et al., 1998). The archetype of the gentisate pathway for chlorobenzoate degradation was enriched under oxygen-limiting conditions (Krooneman et al., 1996). Obviously, bacteria using this pathway possessed relatively low growth rates on 3-chlorobenzoate and benzoate, along with relatively high substrate and oxygen affinities for these compounds (Krooneman et al., 2000). This is in contrast to bacteria harboring the *clc* pathway, which seem to be characterized by high maximum specific growth rates on aromatic substrates and relatively high apparent half saturation constants. Thus, presumably, bacteria degrading chlorobenzoate via gentisate or protocatechuate might be better adapted to reduced growth rate conditions (i.e., low oxygen and low substrate concentration). Evidently, the spread of the different pathways throughout different taxa has been observed, and *Pseudomonas* species were observed to harbor *clc* genes, whereas the gp-pathway was dominant in *Bordetella* and *Alcaligenes* species (Krooneman et al., 2000). Evidently, such clustering is due to the general genetic and metabolic equipment of the organisms. However, gentisate dioxygenase genes were observed in different *P. aeruginosa* isolates (Hickey et al., 2001) and in the genome of *P. aeruginosa* PAO1 (Stover et al., 2000) but not in the genome of *P. putida* KT2440 (Nelson et al., 2002).

Whereas a degradative pathway of chlorobenzoate via gentisate has not been analyzed in detail, the pathway via protocatechuate has been elucidated and the crucial chlorobenzoate dioxygenase was analyzed also at the genetic level. The host range of *cbaAB* genes for 3-chlorobenzoate 4,5-dioxygenase has been analyzed both in defined mating experiments as well as by analysis of transconjugants (Fulthorpe and Wyndham, 1991; Nakatsu et al., 1995) formed under environmental conditions. Transconjugants were predominantly Betaproteobacteria, but also some *Pseudomonas* transconjugants were observed. However, those transconjugants predominantly formed 3-chlorocatechol from 3-chlorobenzoate, indicating an active benzoate 1,2-dioxygenase was interfering with 3-chlorobenzoate 4,5-dioxygenase. Moreover, 5-chloroprotocatechuate accumulated, indicating that protocatechuate 3,4-dioxygenase cannot adequately deal with this metabolite. In contrast, mineralization of 3-chlorobenzoate was observed in organisms harboring a protocatechuate 4,5-dioxygenase, an enzyme that can dehalogenate 5-chloroprotocatechuate (Kersten et al., 1982). Thus, the host range of the *cbaAB* genes can be correlated with the distribution of the protocatechuate *meta*-ring-fission pathway (Nakatsu et al., 1995). Hosts of the *fcb* genotype were, in contrast, mainly *Pseudomonas* (Peel and Wyndham, 1999), which correlates with the ability of *Pseudomonas* to mineralize 4-hydroxybenzoate.

Biotransformation

Biotransformation refers to the biocatalytic conversion of a preformed substrate into a product by a single or a few enzymatic steps. Although free or immobilized enzymes may be applied as such, cofactor-dependent reactions are performed preferentially in living cells to allow inexpensive cofactor regeneration. Several cofactor-independent enzymes are applied in industry (lipases, nitrilases, isomerases, etc.) and bulk processes are based on the stable action of such enzymes. In contrast, cofactor-dependent whole cell reactions (oxidations, reductions, etc.) still have not been applied to tap their inherent potential. A key element in terms of the economics of these processes is the susceptibility of the living cell to the substrate and other components of the reaction solution and the resulting chemical produced. With the molecular tools available, it is possible to transfer genetic information from a variety of organisms into a suitable host.

Several species of *Pseudomonas* have been shown to be good host strains for the expression of heterologous genes providing considerable potential for biotechnological exploitation. Furthermore, strains of *Pseudomonas* species are

often tolerant of, or resistant to, noxious agents present in soils, including antibiotics, disinfectants, detergents, heavy metals, and organic solvents. Several species of *Pseudomonas*, in particular *P. putida*, have been shown to be good host strains for the expression of heterologous genes (Mermod et al., 1986; Ramos et al., 1987; Cases and de Lorenzo, 1998). As a result, these bacteria are viewed as potential cell factories in a diverse range of biotechnological applications, including bioremediation of contaminated sites (Dejonghe et al., 2000), quality improvement of fossil fuels, for example by desulfurization (Galan et al., 2000), biocatalysis for the production of fine chemicals (Zeyer et al., 1985; Schmid et al., 2001), the production of bioplastics (Olivera et al., 2001), and as agents of plant growth promotion and plant pest control (Walsh et al., 2001).

Moreover, a limited number of catabolic enzymes that typically mediate chemically difficult-to-achieve reactions as a consequence of their regio- and stereoselectivity, and thus have considerable potential as biocatalysts for the production of fine chemicals, have been characterized. Some of the previously unknown catabolic enzymes revealed by genome analysis may equally have promise for industrial biotransformations, such as those for the production of epoxides, substituted catechols, enantiopure alcohols and amides and heterocyclic compounds (Zeyer et al., 1985; Wubbolts and Timmis, 1990; Faber and Franssen, 1993; Schmid et al., 2001). Additionally, identification of the genes involved in the synthesis of poly-hydroxy-(phenyl)alkanoic acids, which are currently being studied as biodegradable aromatic polymers, and their precursors, will aid the development of novel bioplastics (Gorenflo et al., 2001; Olivera et al., 2001).

Biotransformations by Solvent-tolerant Pseudomonas Owing to their versatility and particularly to their chiral and positional selectivities, enzyme-mediated processes have become increasingly important for the production of fine chemicals. Many such processes, however, require cofactor regeneration, which is the major reason for the use of whole-cells biocatalysis. A number of high-value biocatalytic conversions involve apolar substrates and products, such as aliphatic, aromatic and heterocyclic compounds (Schmid et al., 2001). Such substances are generally water-insoluble and toxic to whole cells. By using multiple-phase media, it is often possible to integrate conversion and downstream processing within a single reactor system. Therefore, solvent-tolerance of the microorganisms carrying out the conversions is essential. Unlike *E. coli* or many other microorganisms, *P. putida* strains, such as DOT-T1 (Ramos et al., 1994) or S12 (De Bont, 1998), tolerate high concentrations of solvents with low logP values, which, coupled to their metabolic versatility, make these bacteria particularly suited for the production of fine chemicals in nonconventional media. The most critical mechanism for solvent-tolerance in these bacteria is the existence of ATP-driven specific solvent-efflux pumps that extrude the solvent and hinder its accumulation in the cell membranes. Solvent-tolerant strains of *P. putida* harbor specific mechanisms that deal with environmental stresses. They have special solvent efflux pumps, and their outer membrane has specific characteristics, including an unusual fatty acid composition. Presumably, other mechanisms (stress proteins, specific compounds overproduced, etc.) involved in tolerance remain to be elucidated. The combination of these adaptive responses not only allows the organisms to withstand solvents but also places them in a far better position to cope with chemicals that are toxic to other organisms. Many of these toxic chemicals are the desired products of industry, including aldehydes, alcohols, hydroxylated aromatics and epoxides.

Summary

This review has attempted to present an overview of *Pseudomonas* in light of much of the genotypic analyses that were initiated more than 30 years ago and that have served to provide the groundwork for proposing a taxonomic framework based upon estimations of phylogenetic relationships. Certainly, these data comprise the majority of new taxonomic information obtained since the last two compilations of Pseudomonadaceae and *Pseudomonas* in the last two editions of *The Prokaryotes* and play an important role in revealing the heterogeneity of *Pseudomonas* sensu lato, and in improving and stabilizing the taxonomy of *Pseudomonas* sensu stricto and other pseudomonads. The recognition of the "natural" relationships of these bacteria, in turn, should lead to better appreciation and understanding of the overall biology of *Pseudomonas* spp. As new species of *Pseudomonas* are isolated and described, the correlation of metabolic and genetic characteristics with the phylogeny and taxonomy of these organisms should shed more light on the ecological and biotechnological potential of this complex bacterial genus.

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