Experientia 39 (1983), Birkhäuser Verlag, CH-4010 Basel/Switzerland

Genetic aspects of biodegradation by pseudomonads

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Microorganisms that are isolated on the basis of their ability to degrade natural or man-made organic substrates very often belong to the genus *Pseudomonas*. This may be because the pseudomonads have an extraordinary range of catabolic pathways, are easy to handle under laboratory conditions and generally grow on defined synthetic media. A single species such as *P. cepacia* utilizes more than 100 different substrates (out of 146 compounds tested) as the only C, N, or S source[22,98]. Many recalcitrant chemicals (e.g., aromatics, chlorinated aliphatic compounds, pesticides) can be degraded by different *Pseudomonas* species or related soil bacteria isolated from nature. It is therefore pertinent to discuss the genetic organization of biodegradation functions in those *Pseudomonas* species that have been examined in some detail by genetic techniques. In addition, numerous methods that are potentially applicable to the study of biodegradation have been developed for *Agrobacterium* and *Rhizobium* genetics and some of these will be mentioned.

Several reviews have been published describing the catabolic potential[22], the degradative plasmids[14,36] and chromosome maps[48] of *Pseudomonas* as well as the manipulation of soil bacteria by genetic engineering techniques[2,36,91].

Microbial communities and cometabolism of organic substrates undoubtedly play an important role in nature[97]. The geneticists, however, tend to shy away from such complex systems. They prefer pure cultures of distinct species and well-defined medium conditions allowing the selection of particular phenotypes. In the case of biodegradation pathways this means that a growth substrate should be utilized as the only C, N, S or P source. Occasionally, the disappearance of a colored substrate or the formation of a colored product may be helpful to monitor catabolic functions. Bacteria should grow on agar plates – some strains with interesting biodegradation properties refuse to do that – and allow at least one type of genetic transfer: conjugation, transduction, or transformation.

The genetic analysis of bacteria used to be confined to members of the same or closely related species. In recent years, however, the scope of genetic research has greatly widened due to 3 major developments:

a) Broad-host-range plasmids have been found that can replicate in virtually any gram-negative bacterium and enable the transfer of genetic information across the species barriers.

b) The properties of transposons and other insertion elements may be used to produce insertion mutations and deletions, and mutated genes carrying a transposon insert may subsequently be isolated by recombinant DNA techniques.

c) The gene cloning methods are universal in that the DNA from practically any organism can be inserted into cloning vectors. Several plasmid vectors that can be used in *Pseudomonas* species have been derived from broad-host-range plasmids.

1. Genome organization of Pseudomonas

The first *Pseudomonas* species to be studied extensively by genetic techniques was *P. aeruginosa*[48]. Some 50 auxotrophic and about the same number of catabolic markers have been located on the circular chromosome map of strain PAO and a representative selection of these is shown in figure 1A. Although the picture is far from complete, 2 conclusions can be drawn regarding the structural organization of catabolic pathways:

1. Enzymes that belong to the same pathway and may constitute a functional unit in the cell are often encoded by tightly clustered loci. Examples of such gene clusters are the Entner-Doudoroff pathway functions *zwf, edd* and *eda* (glucose-6-phosphate → pyruvate + triosephosphate), the *puuCDEF* genes (xanthine → glyoxylate), the *chuACDE* genes (choline → glycine), the *aguAB* loci (agmatine → putrescine) or the putative *arcABCD* operon (arginine + ADP → ornithine + ATP) (table 1). However, an entire catabolic pathway is rarely confined to a single gene cluster and thus the *puuA* (adenine deaminase) and *puuB* (guanine deaminase) loci are not linked to *puuCDEF*; genes involved in putrescine degradation are not near *aguAB*; and *oru* (ornithine utilization) genes are widely separated from *arcABCD* (fig. 1A).

2. Catabolic and auxotrophic markers are distributed over the entire chromosome; there is no indication of 'supra-operonic' clustering[107], even if more catabolic than auxotrophic mutations have been mapped in the 'late' chromosome region (60–90 min, fig. 1A).

Recently, Dean and Morgan[24] established a circular chromosome map of *P. putida* PPN (fig. 1B). The overall marker order is quite different from that of *P. aeruginosa* although both species belong to the group of fluorescent pseudomonads[98]. The most striking features appear to be the limited region to which auxotrophic markers are confined and a large cluster of genes with dissimilatory functions (e.g., encoding the utilization of benzoate, phenylacetate, mandelate, nicotinate) in an area with few auxotrophic markers

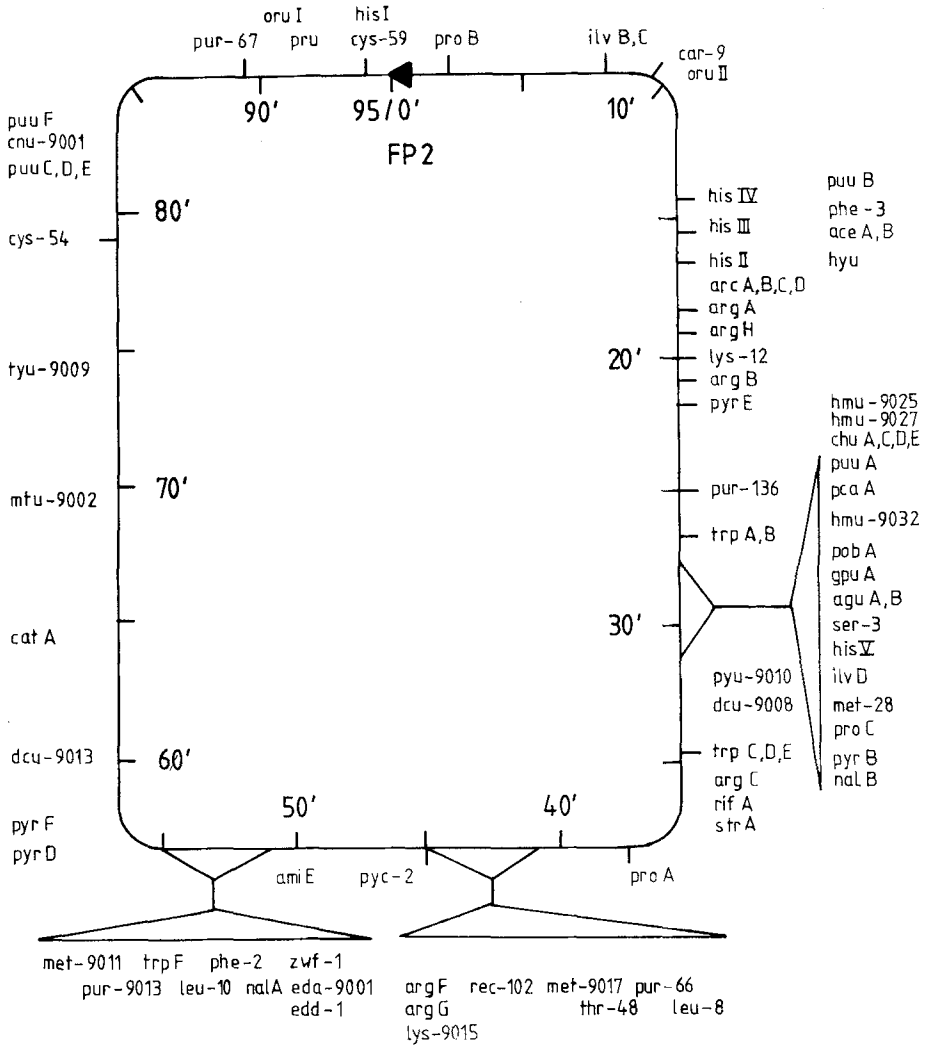

Figure 1A

Figure 1. *A* Chromosome map of *P. aeruginosa* PAO. It is based on the recent version of B.W. Holloway (in: Genetics and breeding of industrial microorganisms, C. Ball, ed., CRC Press, in the press) and contains unpublished data obtained in our laboratory but does not show all markers mapped in strain PAO. The zero min site corresponds to the major origin of chromosome transfer by FP2. A similar, albeit less extensive map is available for *P. aeruginosa* PAT[48]. The following genotype symbols have been used: *ace*, acetate utilization; *agu*, agmatine utilization; *ami*, amidase; *arc*, arginine catabolism (arginine → ornithine); *arg*, arginine requirement; *car*, carbamoylphosphate synthetase; *cat*, catechol utilization; *chu*, choline utilization; *cnu*, carnosine utilization; *cys*, cysteine requirement; *dcu*, dicarboxylic acid utilization; *eda*, Entner-Doudoroff aldolase (former designation: *hex*); *edd*, Entner-Doudoroff dehydratase; *gpu*, guanidino-propionate utilization; *his*, histidine requirement; *hmu*, histamine utilization; *hyu*, hydroxyproline utilization; *ilv*, isoleucine plus valine requirement; *leu*, leucine requirement; *lys*, lysine requirement; *met*, methionine requirement; *mtu*, mannitol utilization; *nal*, nalidixic acid resistance; *oru*, ornithine utilization; *pca*, protocatechuate utilization; *phe*, phenylalanine requirement; *pob*, *p*-hydroxybenzoate utilization; *pro*, proline requirement; *pru*, proline utilization; *pur*, purine requirement; *puu*, purine utilization; *pyc*, pyruvate carboxylase; *pyr*, pyrimidine requirement; *pyu*, pyrimidine utilization; *rec*, recombination ability; *rif*, rifampin resistance; *ser*, serine requirement; *str*, steptomycin resistance; *thr*, threonine requirement; *trp*, tryptophan requirement; *tyu*, tyrosine utilization; *zwf*, glucose-6-phosphate dehydrogenase.

(fig. 1B). This cluster of catabolic genes had previously been established by transduction[107] and it spans about 20% of the chromosome. Such clustering is amazing and leads to speculations about the evolution of catabolic functions in *Pseudomonas* (see below). The functions involved in glucose breakdown via the Entner-Doudoroff pathway (glucose → pyruvate + triosephosphate) have been mapped by transduction in a supra-operonic cluster separate from the first catabolic gene cluster mentioned[26], but it is not yet known whether these glucose utilization genes are located on the linkage map shown in figure 1B (A.F. Morgan, personal communication).

Extrachromosomal elements are abundant in *Pseudomonas* species. A class of plasmids termed 'degradative' plasmids[14] was first discovered in *Pseudomonas*[86] and is rapidly growing. Table 2 lists some examples of plasmid-encoded catabolic pathways in soil bacteria, mostly *P. putida*. The majority of the degradative plasmids are large (> 50 kb, often > 100 kb) and self-transmissible by conjugation. Some non-conjugative plasmids such as XYL or NIC can be transferred by the 'fertility factors' K and T, respectively[75,101]. TOL[5] and the 2,4-D (2,4-dichlorophenoxyacetate) plasmid pJP3[28] have a broad host range; they can be transferred to *E. coli* and other gram-negative bacteria.

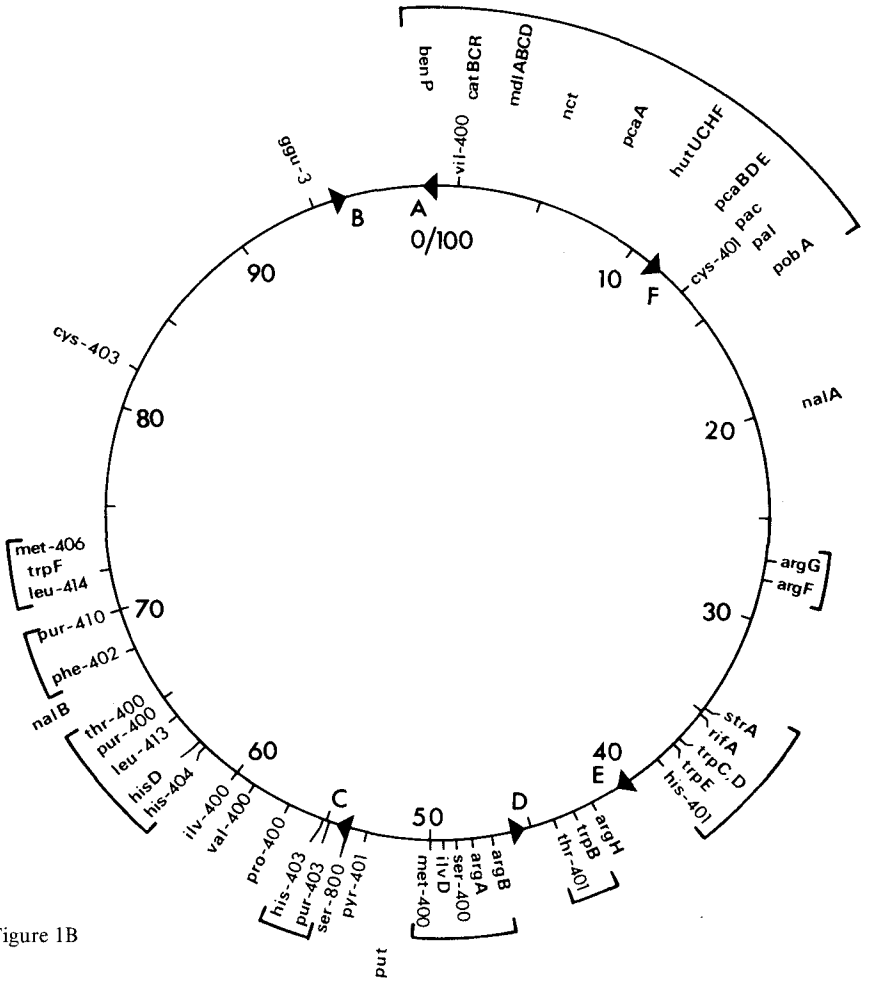

Figure 1B

B Chromosome map of *P. putida* PPN. Reproduced with kind permission of A.F. Morgan and B.W. Holloway and the Japan Scientific Societies Press[49]. It should be noted that strain PPN has also been called PRS1, Stanier's strain 90, and A3.12. The arrows designate the origins of Hfr donors formed by integration of R91-5::Tn*501*[24,49]. The genotype symbols are the same as those used for *P. aeruginosa* PAO; in addition, the following designations have been used: *benP*, benzoate transport; *ggu*, glucose and gluconate uptake; *hut*, histidine utilization; *mdl*, mandelate utilization; *nic*, nicotinate utilization; *pac*, phenylacetate utilization; *pal*, phenylalanine utilization; *put* (instead of *pru*), proline utilization; *vil*, valine + isoleucine + leucine requirement.

Many degradative plasmids studied code for the catabolism of aromatic compounds - both naturally occurring and xenobiotic ones; the catechol intermediates may be cleaved either at the *ortho-* or *meta*-position (table 2). Plasmids are involved in herbicide (e.g., 2,4-D) or insecticide (e.g., parathion) degradation, and pOAD2 enables *Flavobacterium* sp. KI72 to grow on the 6-aminohexanoate cyclic and linear dimers (byproducts of nylon production) as the only C and N sources (table 2). It appears that all degradative plasmids described thus far carry the genes of peripheral pathways; however, there is no apparent reason why central pathways involved in carbohydrate or amino acid utilization should not be encoded by large and stable plasmids.

Several compounds can be degraded either by a chromosomal or by a plasmid-encoded pathway in *P. putida*, depending on the particular isolate. Naphthalene degradation is specified by the plasmids NAH7 and pWW60-1 as well as other plasmids[11,113]. In strain PMD-1, however, the conversion of naphthalene to salicylate depends on chromosomal genes[115], and the subsequent salicylate degradation is under control of plasmid pMWD1[115]. The mineralization of phenylacetate and nicotinate appears to be specified by chromosomal genes in *P. putida* PPN (fig. 1B); in other strains the same compounds can be utilized only when the plasmids pWW17 and NIC-T, respectively, are present (table 2). An interesting situation arises when *P. putida* mt-2 carries the TOL plasmid pWW0; benzoate can be degraded either via the chromosomal *ortho*-pathway or via the plasmid-specified *meta*-pathway. Cells using the *ortho*-pathway grow faster than those using the *meta*-pathway. Benzoate medium thus selects for clones that have either lost the entire TOL plasmid or that have undergone a large deletion of the plasmid[4].

It is not uncommon for degradative pathways to be

Table 1. Some catabolic pathways encoded by chromosomal genes in *P. aeruginosa*

Growth substrate	Principal metabolic product(s)	Genetic loci mapped	Map location (min)	Reference
Acetamide	Acetate + NH_3	*amiE, R*	50	22
Agmatine	Putrescine (→ succinate)	*aguA, B*	28	[a]
Arginine	Ornithine, ATP	*arcA, B, C, D*	17	[b]
Benzoate	*cis, cis*-Muconate (→ succinate + acetyl-CoA)	*catA*	64	65
Choline	Glycine	*chuA* *chu-9012* (= *chuC*?) *chuD* *chuE*	24	65
Dicarboxylic acids (C6–C9)		*dcu-9008** *dcu-9013***	32 60	65
Glucose (carbohydrates)	Pyruvate (via Entner-Doudoroff pathway)	*zwf-1* *edd-1* *eda-9001*	53	[c]
Guanidinopropionate	β-Alanine + urea	*gpuA9001*	28	[d]
Histamine	Aspartate + formate	*hmu-9032*	28	[d]
		hmu-9027 *hmu-9025*	23	
Hydroxyproline	2-Ketoglutarate	*hyu* (4 loci, formerly called *hyp*)	16	63
p-Hydroxybenzoate	β-Carboxy-*cis, cis*-muconate	*pobA* *pcaA*	28	65
Proline	Glutamate	*pru-377* (= *pruAB*)	92	[e]
Purines	Glyoxylate + urea	*puuA*	27	64
		puuB	12	
		puuC, D, E, F	80	

* Pimelate, azelate utilization negative. ** Adipate, pimelate, suberate, azelate utilization negative. [a] D. Haas et al., in preparation. [b] C. Vander Wauven et al., in preparation. [c] R. A. Roehl, P. V. Phibbs, Jr, and T. W. Feary, Abstracts of the 82nd Annual Meeting of the American Society for Microbiology, K2, 1982. [d] H. Matsumoto, personal communication. [e] L. Soldati et al., in preparation.

encoded partly by a plasmid and partly by chromosomal genes in a given strain. The conversion of octane to octanaldehyde depends on OCT plasmid genes but the further oxidation reactions are specified by the *P. putida* or *P. aeruginosa* chromosome[31]. Similarly, camphor oxidation proceeds to isobutyrate under the control of the CAM plasmid; the genes for isobutyrate utilization are chromosomal in *P. putida*[86]. The situation is further complicated by plasmid-chromosome interactions. CAM[86], XYL-K[75], and NIC-T[101] mobilize the chromosome of *P. putida*. There is evidence that the *meta*-pathway genes specifying toluene degradation can transpose from the TOL plasmid into other plasmids[52] or into the *P. putida* chromosome[18]. These observations imply that some catabolic pathways may be able to spread in populations of gram-negative bacteria by transposition and transfer on promiscuous plasmids in much the same way as drug resistance transposons. Further support for this idea comes from hybridization studies. The NAH7 and SAL plasmids show extensive homology and are closely related, but SAL does not appear to have arisen from a simple deletion of NAH7 removing the information required for the conversion of naphthalene to salicylate[45]. The SAL, TOL and OCT plasmids also show some homology[45] but they do not all belong to the same incompatibility group: OCT is an IncP-2 plasmid whereas SAL, NAH and TOL belong to the IncP-9 group[53,108]. Both IncP-2 and IncP-9 groups also include R plasmids[53]. It seems reasonable to assume that both transposition and homologous recombination have contributed to the evolution of degradative and resistance plasmids[53].

Although *P. aeruginosa* possesses a wide range of catabolic pathways (table 1) and although some degradative plasmids have been successfully transferred to *P. aeruginosa* (e.g., CAM[86], TOL[76] and SAL[107]), natural isolates of this species have not been reported to harbor degradative plasmids[53]. Rather, R plasmids, FP plasmids ('sex factors') and plasmids conferring resistance to heavy metal ions are frequent in *P. aeruginosa*[48,53]. This may be a reflection of the habitat of this opportunistic pathogen, which prefers an aquatic environment (e.g., sediments in fresh water)[79] and survives quite poorly in soil, especially when the soil is dried[114]. By contrast, *P. putida* and other strains carrying degradative plasmids (table 2) are considered typical soil organisms[36]. Of course, there may be a human factor involved because researchers isolating *P. aeruginosa*, say, from hospital environments are primarily concerned with the presence of resistance plasmids.

Table 2. Some catabolic pathways encoded by plasmids in soil bacteria

Growth substrate	Principal metabolic product(s)	Plasmid	Original host	Reference
Toluene, *m*-xylene *p*-Xylene	Pyruvate + acetaldehyde Pyruvate + propionaldehyde (*meta*-cleavage)[b]	TOL (= pWW0)[a]	*P. putida*	36
Xylene, toluene	same as above (*meta*-cleavage)[b]	XYL-K	*Pseudomonas* Pxy	75
Naphthalene	Salicylate → pyruvate + acetaldehyde (*meta*-cleavage)[b]	NAH7[c]	*P. putida*	113
Naphthalene	Salicylate → succinate + acetyl-CoA (*ortho*-cleavage)[b]	pWW60-1[c]	*P. putida*	11
Salicylate	Pyruvate + acetaldehyde (*meta*-cleavage)[b]	SAL; pMWD1	*P. putida*	36; 115
p-Cresol	*p*-Hydroxybenzoate → succinate + acetyl-CoA (*ortho*-cleavage)[b]	pND50	*P. putida*	46
Camphor	Isobutyrate	CAM	*P. putida*	86
Nicotine, nicotinate	Maleamate + fumarate + succinate	NIC-T	*P. convexa*	101
n-Octane (C6–C10 alkane)	Alkane aldehyde	OCT	*P. putida*	31
Phenylacetate	Not known	pWW17	*P. putida*	81
3-Chlorobenzoate	Succinate + acetyl-CoA (*ortho*-cleavage)[b]	'pB13'; pAC25	*Pseudomonas* sp. B13; *P. putida*	20; 83
4-Chlorobiphenyl	4-Chlorobenzoate	pKF1	*Acinetobacter* sp. P6	38
2,4-Dichlorophenoxyacetate, 4-chloro-2-methylphenoxyacetate	Succinate + acetyl-CoA + glyoxylate (*ortho*-cleavage)[b]	pJP1; pJP3	*Alcaligenes paradoxus*	28; 34
2-Fluoroacetate	Glycolate	pUO1	*Moraxella* sp. B	55
2-Chloropropionate	Lactate	pUU204	*Pseudomonas* sp. E4	97
6-Aminohexanoate cyclic dimer	6-Aminohexanoate	pOAD2	*Flavobacterium* sp. KI72	77
Parathion	*p*-Nitrophenol + diethylthiophosphate	pCS1	*P. diminuta*	95

[a] The best studied of many ubiquitous toluene degradative plasmids is pWW0 isolated by P. A. Williams[83]. [b] Cleavage of catechol intermediate. [c] NAH7 and pWW60-1 are examples of several described naphthalene degradative plasmids.

2. *Genetic manipulation of Pseudomonas*

Conjugation

The classical narrow-host-range sex factors FP2 and FP5 have been used extensively for mapping chromosomal genes in *P. aeruginosa*[48,64,65]. FP2 mobilizes the chromosome from one major origin (shown in fig. 1A) and from some minor ones near *proB*[89]; FP5 has a similar major origin[64,65]. This situation precluded demonstration of genetic circularity until new plasmids having Cma (chromosome mobilizing ability) were found. A comparable situation existed in *P. putida* where XYL-K appears to have one principal origin of chromosome transfer[75].

The broad-host-range (IncP-1) Cma plasmid R68.45 has proved particularly useful in the genetic analysis of gram-negative bacteria. It was isolated in *P. aeruginosa*[41]. In this organism, chromosome mobilization by R68.45 occurs at about 10^{-4} per donor for any marker[41], from many sites and in both orientations[42]. These properties were important for establishing circularity of the PAO chromosome map[89]. R68.45-mediated conjugation was also used to construct circular linkage maps in *Rhizobium meliloti*[57], *R. leguminosarum*[6], *R. trifolii*[66] and *Agrobacterium tumefaciens*[51]. Furthermore, R68.45 has been shown to promote chromosome transfer in *E. coli*, *P. putida*, *P. glycinea*, *P. oxalaticus*, *P. fluorescens*, *Rhodopseudomonas sphaeroides*, *Klebsiella pneumoniae*, *Rhodopseudomonas capsulata*, *Rhizobium phaseoli*, *Paracoccus denitrificans*, *Azospirillum brasilense*, *Erwinia chrysanthemi*, *Erwinia carotovora*, *Methylophilus methylotrophus*, *Zymomonas mobilis* and *Caulobacter crescentus* (reviewed by Holloway[50]).

The ability of R68.45 to mobilize the chromosome or plasmids depends on the duplication of IS*21*, a 2.1-kb insertion element present in a single copy on the parental IncP-1 plasmid R68[109]. In R68.45, 2 copies of IS*21* are joined together as a direct repeat and in this configuration transposition of IS*21* (i.e., a single copy) to other replicons is increased by several orders of magnitude over the level of IS*21* transposition from R68 (Willetts et al.[109] and G. Riess, personal communication). Chromosome mobilization is thought to involve an unstable cointegrate of R68.45 with the chromosome[109]. The classical genetic analysis of chromosome mobilization by R68.45 suggests that IS*21* can transpose into the chromosome with low regional specificity and at high frequency[42].

R68.45 is unstable, especially after conjugal transfer to a new recipient[23,44]. In some cases, one copy of the IS*21* element is lost, presumably by homologous recombination (resulting in a plasmid indistinguishable from R68), but often functions adjacent to IS*21* are deleted as well[23,44]. In *P. aeruginosa,* spontaneous deletions preferentially remove one IS*21* copy, the kanamycin resistance gene, primase and some transfer functions[44]. Kanamycin selection thus eliminates this class of R68.45 deletion derivatives. There are a few reports of very poor chromosome mobilization by R68.45 in certain species of gram-negative bacteria. This may be due to inefficient transposition of IS*21* in these strains but there is also a possibility of deletions having occurred in R68.45. The structural integrity of the plasmid should always be verified by restriction enzyme analysis. (There is a unique *Hpa*I restriction site in IS*21*; hence IS*21* duplication is immediately apparent from a 2.1-kb *Hpa*I fragment[109].)

In *E. coli* R68.45 is able to mobilize non-transmissible plasmids by cointegrate formation[109]. This ability may be quite useful in the analysis of plasmids that do not have selectable phenotypes. The cointegrates can be expected to retain the antibiotic resistance markers of R68.45 (i.e., resistance to carbenicillin, tetracycline and kanamycin) and should also possess the broad host range of R68.45. This may permit the study of plasmids in a host (e.g., *E. coli*) in which they normally cannot replicate. Since the IS*21*-dependent cointegrates are resolved by the *recA*$^+$ protein in *E. coli* (G. Riess, personal communication) they are likely to be unstable in recombination-proficient hosts. An example of an R68.45-promoted cointegrate has been reported for a 270-kb nodulation plasmid in *Rhizobium trifolii*[94]. This cointegrate is transmissible to nodulation-negative, heat-cured *R. trifolii* strains at high frequency[94]. The Ti plasmid of *Agrobacterium tumefaciens* forms cointegrates with RP4 (which is very similar to R68) whereby direct repeats of IS*8* (very similar to IS*21*) occur at both junctions of the 2 replicons[25]. If R68.45 and RP4 fail to mobilize a plasmid, the use of RP1::Tn*501* may prove more successful[73].

R68.45 has an additional useful property: it can pick up chromosomal genes to form R′ plasmids. Insertion of chromosomal DNA into R68.45 has been demonstrated in *P. aeruginosa*[47], *P. putida*[72], *Klebsiella pneumoniae*[30], *Methylophilus methylotrophus*[71] and in *Rhizobium* species[58]. R′s can be selected in 2 ways: An R68.45 donor is mated with a recombination-deficient recipient of the same species or with a recipient belonging to a different species which has little overall chromosome homology with the donor species but nevertheless allows expression of donor genes. Thus, the recombinants formed in a PAO(R68.45)×PPN cross are due to R′s carrying PAO chromosomal DNA[72]. Many R′plasmids, as first isolated, are highly unstable – even in recombination-deficient hosts – but often continued purification under selective pressure will produce stable derivatives. The maximum size of a chromosomal insert is not known but can certainly exceed 100 kb.

The use of R68.45 in biodegradation studies is still very limited but evidence has been obtained for R68.45-mediated gene transfer in a 2-member microbial community growing on 2-chlorobutanoate[97]: *P. putida* PP3 dehalogenates 2-chlorobutanoate to 2-hydroxybutanoate, a product which is not utilized by strain PP3 but serves as a growth substrate for HB2001, an unidentified dehalogenase-negative bacterium. A community of *P. putida* PP3 (R68.45) and HB2001 in a chemostat formed recombinants able to grow on both 2-chlorobutanoate and 2-hydroxybutanoate[97].

Hfr donor strains provide a very efficient means to analyze chromosomal markers. Such strains have been constructed in *P. putida* by integration of plasmid R91-5::Tn*501* into various chromosomal sites (fig. 1B). R91-5, a *P. aeruginosa* plasmid, is derepressed for conjugal transfer and cannot replicate autonomously in *P. putida.* When loaded with transposon Tn*501*, the plasmid integrates spontaneously into the chromosome at a frequency of 10^{-6}, yielding Hfr donors[24]. In *P. aeruginosa,* temperature-sensitive replication mutants of RP1[43] or R68[49] have been forced into the chromosome by selection for the R plasmid resistance markers at a non-permissive temperature (43 °C). Naturally occurring Hfr donors have not been found in *Pseudomonas* but it now appears feasible to construct them by one of the methods mentioned above.

Transposon mutagenesis

In many cases, transposon mutagenesis has clear-cut advantages over the traditional chemical mutagenesis using ethyl methane sulfonate or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. First, the mutation created by transposon insertion is tagged by a selectable antibiotic resistance[88]. Second, most (but not all) transposon insertions into operons result in polar mutations[21]. Third, transposons often form spontaneous deletions in the vicinity of their insertion site and in the case of the tetracycline-resistance transposon Tn*10* it is possible to find these deletions easily by enrichment for tetracycline-sensitive cells[9]. And fourth, the mutated gene region can be cloned by digesting total DNA with a restriction enzyme that does not cut the integrated transposon (e.g., *Eco*RI or *Sst*I in the case of Tn*5*) followed by in vitro insertion of the fragment into a vector plasmid, with selection for the transposon-encoded resistance[16,35]. The cloned mutated gene can then serve as a probe to screen gene libraries for the corresponding wildtype allele. This procedure is most useful when a cloned gene cannot be recognized because its function is unknown or not assayable.

The transposons Tn*1*, Tn*501* and Tn*7* have been used to mark FP plasmids in *P. aeruginosa*[33]. Transposition was accomplished in the following way: Cells harboring a transfer-deficient (Tra^-) transposon donor plasmid plus an FP plasmid (by definition Tra^+ and Cma^+) were mated with rec^+ recipients and selection was made for the resistance conferred by the transposon[33]. (Mobilization of the Tra^- plasmid by FP could be distinguished from transposition of the transposon to FP by checking transconjugants for non-inheritance of other markers of the Tra^- plasmid.) The transposition frequencies observed ranged from 10^{-5} to 10^{-8}. An analysis of Tn*501* and Tn*7* insertions into the narrow-host-range plasmid R91-5 revealed moderate regional specificity with some hotspots, but clearly many different insertions could be observed[70]. Insertions of Tn*1* and Tn*501* into the PAO chromosome have been obtained[60,103] but these are very rare ($< 10^{-8}$ when a temperature-sensitive RP1 plasmid is used as the donor; unpublished results of M. Rella and D. Haas) and probably non-random. Tn*1* and Tn*501* are thus unsuitable for random transposon mutagenesis of chromosomal genes. Tn*7* has one hotspot of integration in the PAO chromosome[13] and is equally unsuitable. However, the chromosomal insertions of Tn*1*, Tn*501* and Tn*7* may be used to mutagenize conjugative plasmids that are able to replicate in strain PAO. Alternatively, if a plasmid (such as TOL) can be maintained in *E. coli*, chromosomally inserted transposons can be picked up by the plasmid in the same way[35].

The transposon Tn*5* (encoding kanamycin-resistance) displays a low specificity of insertion and transposes readily into the chromosome of *E. coli*[96]. Therefore, a number of systems have been tried in order to use Tn*5* in *Pseudomonas*. The '*suicide*' plasmids (RP4::Mu::Tn*5*), which work well in *Agrobacterium*[105] and *Rhizobium*[7], have not been found suitable for *P. aeruginosa* and other *Pseudomonas* species (Sato et al.[92] and our unpublished results). Three systems have been applied successfully to different *Pseudomonas* species and at least one of these methods should work in any *Pseudomonas* strain of interest. a) An RP4-ColE1 hybrid plasmid whose replication depends on the ColE1 moiety was loaded with Tn*7*. The plasmid is maintained stably in *E. coli* but shows no or abortive replication in phytopathogenic *Pseudomonas* species[92] and in *P. putida*[32], because ColE1 plasmids cannot be maintained in *Pseudomonas*. A related RK2-ColE1 hybrid plasmid (pRKTV14) containing Tn*5* has been used to mutagenize a *P. putida* strain which degrades toluene via *cis*-toluene-dihydrodiol (rather than via benzoate)[32]. Evidence was obtained that the toluene degradation *(tod)* genes are organized in an operon, presumably located in the chromosome. Tn*5* could also be used to isolate amino acid auxotrophs in this strain[32]. b) The ColI plasmid replicates in *E. coli* but not in *Pseudomonas*. Derivatives of ColI which are derepressed for conjugal transfer were charged with Tn*5* or Tn*10*; they were found to be convenient suicide plasmids for *P. putida* and *Pseudomonas* sp. B13 (F.C.H. Franklin and G. Boulnois, personal communication). Both chromosomal and plasmid mutations were obtained. In *P. aeruginosa*, the ColI plasmids did not work as transposon donors (M. Rella, personal communication).

c) A temperature-sensitive RP1 plasmid was deleted for IS*21*, kanamycin resistance and primase; into this vector (pME305)[44] was introduced a Tn*5* derivative (Tn*5-751*) carrying an additional resistance marker (trimethoprim resistance) on a 3.3 kb *Bam*HI fragment[85]. When an *E. coli* pME305::Tn*5-751* donor and a *P. aeruginosa* recipient were crossed and selection was made for kanamycin and trimethoprim resistance together at 43 °C (the non-permissive temperature), the frequency of Tn*5-751* transposition into the PAO genome was ca. 10^{-6} per donor; the abortive transfer of the vector was about 10^{-2} per donor. Amino acid auxotrophs were recovered at 0.5–1%[85]. Because of the considerable instability of the pME305 vector in *P. aeruginosa* even at 30 °C, transposition mutagenesis may also be carried out at this temperature. Double selection for kanamycin plus trimethoprim resistance is necessary because *P. aeruginosa* is intrinsically resistant to kanamycin and it is thus difficult to select for the wildtype Tn*5*[85].

Transduction and transformation

Generalized transducing phages are available for *P. aeruginosa* and *P. putida* PpG and PPN; transduction has also been used in *P. fluorescens* and *P. acidovorans*[48]. Transformation of *P. aeruginosa* and *P. putida* with plasmid or phage DNA is possible in cells made 'competent' by $CaCl_2$ treatment[36,48,67]. Although the transformation efficiency in *Pseudomonas* is somewhat lower than that in *E. coli*, recombinant plasmids can usually be introduced into *P. aeruginosa* and *P. putida* without problems. Transformation of these strains by linear chromosomal DNA is inefficient. By contrast, *P. stutzeri* and the related species *P. mendocina*, *P. alcaligenes* and *P. pseudoalcaligenes* can undergo natural transformation without chemical treatment using linear double-stranded chromosomal DNA as the substrate[12].

Cloning

The IncP-4/IncQ plasmids RSF1010, R300B and R1162, which are very similar or identical, have a broad-host-range in gram-negative bacteria, are maintained at about 20 copies per chromosome equivalent, mobilizable by conjugative plasmids (e.g., RPl, R64*drd*11) and suitable replicons for vector construction[1,2,3,36]. 5 vector plasmids and 1 cosmid (pMMB34)

based on IncQ replicons are listed in table 3. Versatile, stable vectors are, e.g., pKT231 for *P. putida* and pKT240 for *P. aeruginosa* (where carbenicillin selection works well but kanamycin or streptomycin are inadequate for selection).

The IncP-1 conjugative plasmid RK2 (very closely related to the RP4 and R68 plasmids already mentioned) also possesses a broad-host-range but has a low copy number (2–8 copies per chromosome equivalent)[2]. The mobilizable vector pRK290 (table 3) is stable in *E. coli* and *Rhizobium* but its stability in different *Pseudomonas* species has not been tested[27]. Cosmid derivatives of pRK290 are available (table 3) and allow cloning of DNA fragments in the size range of 15–31 kb[37,56]. These cosmids offer a very efficient system for R prime construction in vitro[37,56]. The primary host for cloning with IncQ or IncP vectors will usually be *E. coli*; the recombinant plasmids are then mobilized (or perhaps transformed) into the *Pseudomonas* strain of interest[2].

The *P. aeruginosa* plasmid pVS1[99] can be transferred by mobilization to a number of *Pseudomonas* species, to *Agrobacterium tumefaciens* and *Rhizobium leguminosarum*, but not to *E. coli*. A single 3-kb region of the 30-kb plasmid is essential for maintenance, stability and host range (Y. Itoh, unpublished results). By contrast, the broad-host-range plasmids mentioned above carry essential determinants in more than one

Table 3. Some cloning vectors for *Pseudomonas*[a]

Vector	Replicon(s)	Size (kb)	Cloning sites	Selected marker[b]	Insertional inactivation	Mobilization by	Reference
pKT210	RSF1010 (IncQ = IncP-4)	11.8	*Sst*I	Cm	Sm	RP1 or R64*drd*11	1, 2
			*Hin*dIII	Cm, Sm	–		
			*Eco*RI, *Hpa*I	Cm	–		
pKT231	RSF1010	13.0	*Sst*I	Km	Sm	as for pKT210	1, 2
			*Hin*dIII, *Cla*I, *Xho*I, *Xma*I	Sm	Km		
			*Eco*RI, *Hpa*I	Sm	–		
pKT240	RSF1010	12.5	*Hin*dIII, *Cla*I, *Xho*I, *Xma*I	Cb	Km	as for pKT210	M. Bagdasarian, personal communication
			*Bam*HI	Cb, Km	–		
			*Sst*I	Cb, Km	Sm (low level)		
			*Eco*RI, *Hpa*I	Cb, Km	–		
pTB107	R300B (IncQ)	10	*Hin*dIII, *Cla*I, *Xho*I, *Xma*I	Sm (Su)	Km	as for pKT210	3, P. Barth, personal communication
			*Sst*I	Km (Su)	Sm		
			*Eco*RI, *Hpa*I	Km (Su)	–		
pGSS8	R300B	9.5	*Sal*I, *Bam*HI	Sm	Tc		3
			*Sst*I	Tc	Sm		
			*Eco*RI, *Hpa*I	Tc	–		
pRO1614[c]	Cryptic PAO plasmid, pBR322	6.2	*Bam*HI, *Hin*dIII	Cb	Tc		78
pME290[d]	pVS1 (IncP, unclassified)	6.8	*Hin*dIII, *Xho*I, *Xma*I	Cb	Km	Mob$^-$	Y. Itoh, personal communication
			*Pst*I	Km	Cb		
pRK290[e]	RK2 (IncP-1)	20	*Eco*RI, *Bgl*II	Tc	–	pRK2013 (a ColE1 replicon with RK2 *tra* genes)	27
pVK101	pRK290	21.3	*Sal*I	Km	Tc	pRK2013	56
			*Hin*dIII, *Xho*I	Tc	Km		
			*Eco*RI	Km, Tc	–		
pVK102 (cosmid)	pRK290	23	*Sal*I	Km	Tc	pRK2013	56
			*Hin*dIII, *Xho*I	Tc	Km		
pLAFR1 (cosmid)	pRK290	21.6	*Eco*RI	Tc	–	pRK2013	37
pMMB34[f] (cosmid)	RSF1010	13.8	*Bam*HI	Km	–	as for pKT210	M. Bagdasarian and J. Frey, personal communication

[a] References 1, 2, 3, 36 and 91 provide a more extensive compilation of *Pseudomonas* vectors. Several RSF1010-pBR322 hybrids have been constructed which are unstable in *Pseudomonas* and can be maintained only by constant selection[39,112]. Plasmid pGSS8 (which contains the tetracycline resistance region of pBR322) and other hybrids containing only part of pBR322 are stable in at least *E. coli*, *P. aeruginosa*, *Methylophilus methylotrophus* and *Alcaligenes eutrophus*[3]. [b] Abbreviations used for resistance markers: Cm, chloramphenicol; Sm, streptomycin; Km, kanamycin; Cb, carbenicillin; Tc, tetracycline; Su, sulfonamide. [c] pRO1614 has been introduced into *P. aeruginosa*, *P. putida* and *P. fluorescens* but its stability in the two latter strains has not been reported. [d] pME290 is stable in *P. aeruginosa*; it cannot replicate in *E. coli*. [e] The stability of pRK290 in *Pseudomonas* has not been reported. [f] pMMB34 is more stable than pKT247 (Ref. 1) and does not give polycosmid artifacts (J. Frey and M. Bagdasarian, personal communication).

region and cannot be reduced to stable replicons as small as 3 kb. A series of vector plasmids of convenient size (6–8 kb) has been derived from the pVS1 replicon by Y. Itoh and J. Watson. In some vectors the original mercury (Hg^{II}) resistance of pVS1 (specified by Tn*501*) has been retained without the transposition functions, whereas in other vectors (e.g., pME290; see table 3) carbenicillin resistance has been introduced as a selectable marker. The pVS1-derived vectors are present in circa 8 copies per chromosome equivalent (Y. Itoh, personal communication) and because of their natural containment may offer an alternative to the IncQ- and IncP-systems.

P. aeruginosa and *P. putida* are appropriate hosts for in vitro recombinant DNA work[2]. In both species restriction-deficient mutants[2,50] are available facilitating the introduction of foreign DNA; transformation can be accomplished[36,67], and recombination-deficient *recA*-like strains have been isolated that allow the maintenance of partial diploids[15,17]. Recently, the *recA*-like mutation *rec-102* has been mapped in *P. aeruginosa*; it is now possible to construct recombination-deficient mutants with desired genotypes in this bacterium[37a]. Moreover, *P. aeruginosa* PAO can be rendered phenotypically restriction-deficient by growing it at 43 °C without aeration[87]. Although the 43 °C-grown strain PAO still appears to restrict (albeit with reduced efficiency) the DNA coming from *E. coli*, the '43 °C-effect' is useful in 2-stage cloning experiments in which *E. coli* is used for primary cloning and hybrid plasmids are then mobilized into strain PAO. Because of its extensive genetic characterization, *P. aeruginosa* PAO may be the host of choice for many cloning experiments in *Pseudomonas*. An inconvenient aspect of strain PAO is that it harbors cryptic plasmids[80] which usually remain truly cryptic (i.e., they are not extracted by the plasmid extraction procedures commonly used) but occasionally pop out and the chances of this happening are predicted by Murphy's Law[8]. It should be emphasized, however, that the cryptic plasmids have never been observed to interfere with cloning experiments.

Very little is known about the expression of cloned DNA from gram-negative bacteria in heterologous host systems. In early experiments, the transfer of TOL::Tn*401* or TOL-RP4 hybrid to *E. coli* could be demonstrated but this host was unable to grow on *m*-toluate as the only carbon and energy source[5,52]. *P. aeruginosa* transconjugants carrying TOL can grow on *m*-toluate, albeit more slowly than *P. putida* (TOL^+), suggesting that the *m*-toluate degradative functions are expressed in *P. aeruginosa* but only poorly so in *E. coli*[52,76]. Cloning experiments and enzyme assays have shown that TOL enzyme levels in *E. coli* are about 1–2% of those in *P. putida*, calculated per TOL gene copy[35]. However, when a high copy number vector (pKT210) was used for TOL gene cloning, the enzyme levels in *E. coli* were sufficiently high to permit growth on *m*-toluate. Still, the growth rate was 10 times lower than that of a comparable *P. putida* strain[35]. High TOL gene expression (catechol-2,3-dioxygenase) in *E. coli* is observed when transcription is under control of a vector promoter. These experiments suggest that *P. putida* promoters function less efficiently in *E. coli* than in their natural host[35].

There may be additional problems associated with the expression of foreign genes: if positive regulatory elements are needed for optimal gene expression and if their expression in *E. coli* is poor, then the structural genes cannot be fully expressed. Moreover, the uptake of exotic substrates may be rate-limiting and some intermediate products quite toxic in *E. coli*. For instance, the NAH7 genes cloned into high copy number vectors gave enzyme levels in *E. coli* of about 10% of those measured in *P. putida*. In spite of this, *E. coli* cells containing the NAH7 genes required 14 days of incubation to form small colonies in the presence of naphthalene[93].

We have cloned arginine catabolic genes (the putative *arcABCD* operon) from *P. aeruginosa* into broad-host-range plasmids (R68.45 and pKT240) and transferred the recombinant plasmids into *E. coli* (A. Mercenier and D. Haas, unpublished data). Although at least 2 *arc* enzymes, arginine deiminase and catabolic ornithine carbamoyltransferase, are expressed in *E. coli* at about 2% of the non-induced level observed in *P. aeruginosa*, the *E. coli* transconjugants are unable to grow on arginine.

The cloned *amiE* gene of strain PAO enables *E. coli* to utilize acetamide as an N source[29]. By contrast, *P. aeruginosa* grows very well on acetamide as the only nutrient. Another example is the expression of cosmid-cloned D-galactose dehydrogenase from *P. fluorescens*[10]. The specific activity of this enzyme in *E. coli* was below detection but when the structural gene for the enzyme was cloned into an expression vector using the *E. coli lac* promoter, the specific activity could be increased at least 10,000-fold[10].

The reverse situation, i.e., the expression of *E. coli* genes in *Pseudomonas*, is less well documented. The *E. coli argE* and *argH* biosynthetic genes carried by an f′ plasmid are expressed well in *P. fluorescens* strain 6.2[68]. The *trp* operon of *E. coli* cloned into RSF1010 was expressed in *P. aeruginosa* and *P. putida*[91]. Although tryptophan synthetase levels were very high in *P. aeruginosa* immediately after RSF1010-*trp* plasmid transfer, they dropped after subculturing of the transconjugant, apparently because of plasmid mutations[91].

Much remains to be learned about gene expression in heterologous systems involving different pseudomonads. As quantitative data are scarce it is difficult to make predictions. It may be a good idea to remember

that *'Pseudomonas'* means 'false unity' - physiologically very different bacteria have been placed into one huge taxonomic family.

Reversed genetics

Although efficient methods for conjugation, transposon mutagenesis, transformation, and gene cloning have been worked out for several *Pseudomonas* species and although, in principle, these methods may be applicable to any new *Pseudomonas* species, their adaptation may well be an arduous task. In certain cases, the strategy of reversed genetics[106] has distinct advantages. When the gene-function relationships are to be studied and if the relevant genetic information is carried by a relatively small DNA segment (its maximum size is determined by the amount that can be cloned into an appropriate vector in *E. coli*), the methodology of reversed genetics can be applied to a wide range of gram-negative bacteria. A straightforward method has been elaborated to study the T-DNA of the *Agrobacterium* Ti plasmid[104] and is outlined here (see fig. 2).

a) A genome fragment from a bacterium of interest (e.g., *Agrobacterium)* is cloned into pBR322, the host being *E. coli.* b) Site-directed mutagenesis[102] of the insert is conducted in *E. coli.* c) Efficient mobilization of the mutated recombinant plasmid into *Agrobacterium* is achieved by using 2 helper plasmids (pGJ28, a ColD replicon providing mobilization functions for pBR322, and R64*drd*II, an I_α-type plasmid supplying transfer functions). Selection for a pBR322-encoded antibiotic resistance in *Agrobacterium* will result in the integration, by recombination, of the recombinant plasmid into the homologous part of the genome, because the ColE1 replicon pBR322 and the helper plasmids cannot replicate autonomously in *Agrobacterium.* d) A second recombination event leads to looping out and e) subsequent loss of pBR322 plus the target part of the genome. The procedure should be applicable to *Pseudomonas*[104], since pBR322 cannot replicate in this organism. Similar but somewhat more laborious experimental strategies have been used to investigate *nif* (nitrogen fixation) genes and nodulation genes from *R. meliloti*[59,90].

3. Construction of strains with novel catabolic abilities

The method of mutation and selection has been used frequently to endow microorganisms with novel catabolic abilities[22,74]. A well-documented case is the utilization of aliphatic amides by *P. aeruginosa,* stud-

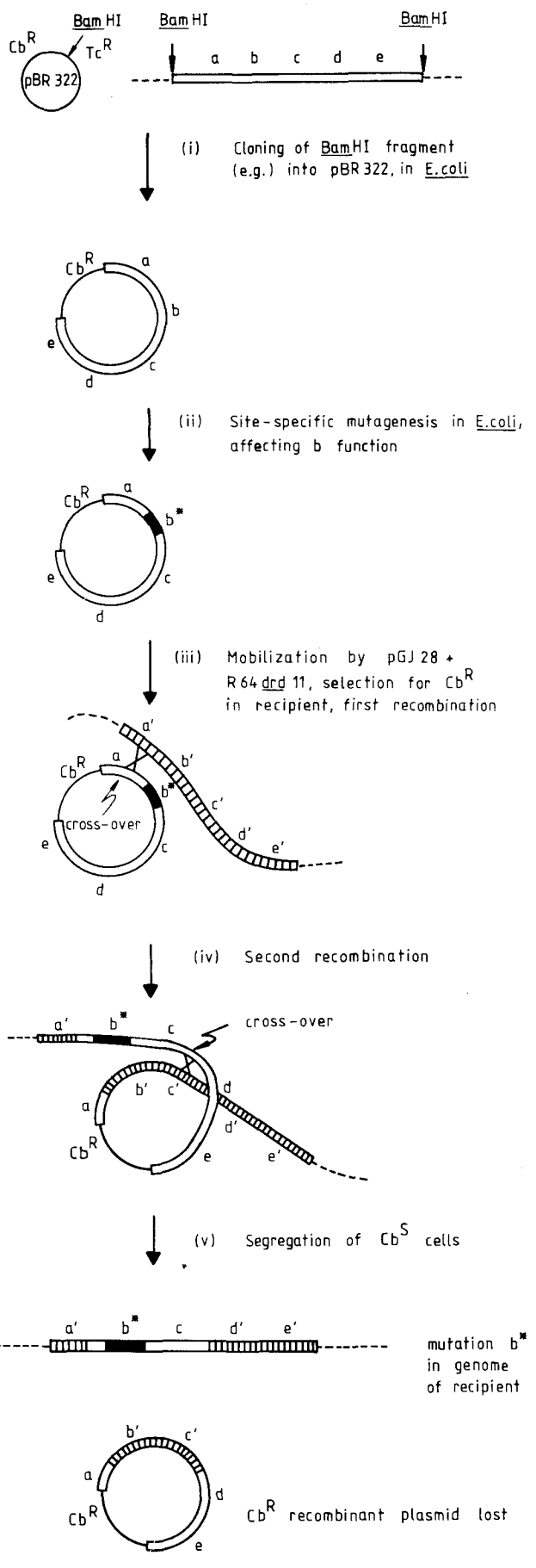

Figure 2. Introduction of specific mutations into the genome of a non-*E. coli* strain, by a strategy of reversed genetics. Adapted from Van Haute et al.[104]. The abcde fragment shown is part of the genome of interest. For further details, see text. Cb^R: resistance to carbenicillin.

ied by P. Clarke and co-workers[22]. By sequential mutational changes of substrate and inducer specificities, *P. aeruginosa* mutants were obtained which can utilize phenylacetamide (e.g.) as the growth substrate; in the wildtype strain this compound is neither a substrate for amidase nor an inducer for this enzyme and, therefore, the wildtype cannot grow on phenylacetamide (for review, see Clarke[22]). Clearly, the scope of the mutation-selection approach is limited to changes of enzymes, regulatory systems and, perhaps, silent genes that already exist in a microorganism. If novel pathways or new combinations of pathways are to be created then it is often practical to supply the new function on a plasmid, which either occurs naturally or may be constructed in vivo or in vitro. The potential of this approach is illustrated by the following examples.

1. *P. putida* strain PP1-2 can grow on phenol as the carbon and energy source but tolerates only relatively low phenol concentrations[111]. Phenol is converted to catechol, which is degraded via the (chromosomal) *ortho*-pathway. A mutant (PP1-3) defective in the first enzyme of this pathway, catechol-1,2-oxygenase, cannot grow on phenol or benzoate. When a variant of the TOL plasmid (M1-1) is introduced into strain PP1-3, the transconjugants acquire the ability to metabolize *m*-toluate, benzoate, and phenol because catechols can now be cleaved by the plasmid-encoded *meta*-pathway enzymes. Interestingly, the PP1-3 (M1-1) transconjugants tolerate higher phenol concentrations (up to 10 mM) than does the natural isolate PP1-2 and phenol can now act as an inducer of the *meta*-pathway[111].

2. Floc-forming bacteria behave like immobilized cells and may offer advantages in biological continuous systems, e.g., in waste-water treatment. The flocforming *Pseudomonas* species No. 12 cannot degrade and grow on aromatic compounds such as toluene and xylene but can do so when the TOL plasmid has been introduced by conjugation[100].

3. *Pseudomonas* species B13 degrades 3-chlorobenzoate via the plasmid pB13-encoded[20,84] *ortho*-cleavage pathway[83] (fig. 3). 4-Chlorobenzoate cannot be utilized because of the narrow substrate specificity of the B13 toluate oxidase and this limitation has not been overcome by mutation and selection[83]. When the non-specific toluate (benzoate) oxidase (the *xylD+ xylL* products) encoded by the TOL plasmid is present in strain B13, 4-chlorobenzoate can be degraded to 4-chlorocatechol, which is then converted to central metabolites via the pB13 *ortho*-cleavage pathway (fig. 3). However, a *Pseudomonas* B13 (TOL^+) transconjugant can only grow on chlorobenzoates when part of the TOL *meta*-cleavage pathway is blocked by a mutation in *xylE* (specifying catechol-2,3-dioxygenase) because otherwise the *meta* pathway would produce toxic ('suicidal') products from the chlorocatechols (fig. 3)[83]. Spontaneous mutations in *xylE* are found when 4-chlorobenzoate utilization is selected in strain B13 (TOL^+) and they involve complex structural changes in the TOL plasmid[54].

4. There are *Alcaligenes* and *Pseudomonas* strains which utilize phenol and methylsalicylates, respectively. Introduction of the pB13 plasmid into these strains enables them to utilize chloroaromatics (chlorophenol and chlorosalicylates) as their only C sources[84]. These

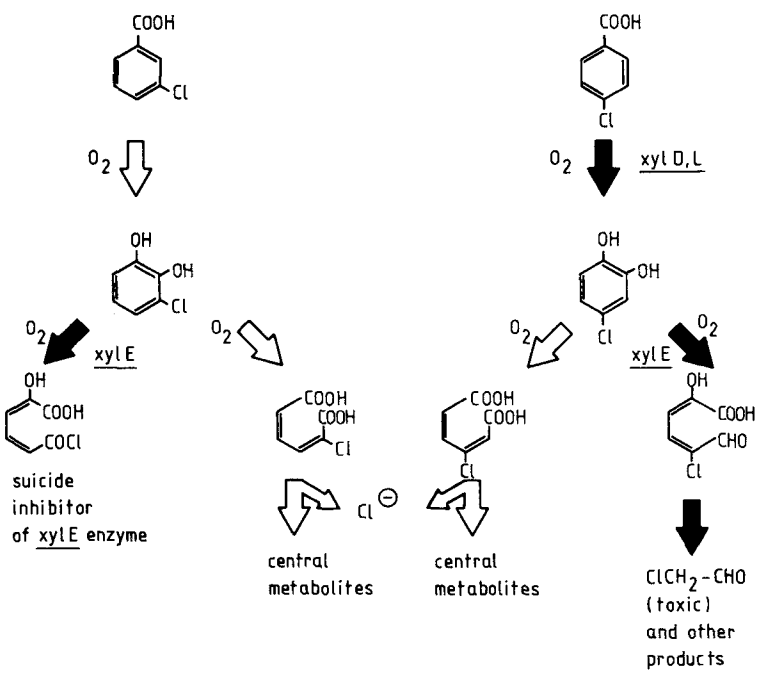

Figure 3. Degradation of 3-chlorobenzoate and 4-chlorobenzoate by *Pseudomonas* sp. B13 (TOL^+). Adapted from Reineke et al.[83]; further particulars are described in the text. ⇒, reactions determined by pB13; →, reactions determined by TOL.

observations have now enabled Lehrbach et al. (manuscript in preparation) to construct chloroaromatics-degrading *Pseudomonas* strains by gene cloning techniques. The genes for toluate oxidase *(xylD* and *xylL)* from the TOL plasmid and the gene for salicylate hydroxylase *(nahG)* from the NAH7 plasmid were cloned. When introduced into *Pseudomonas* sp. B13, these broad-substrate-specificity enzymes permitted the strain to utilize 4-chlorobenzoate and chlorosalicylates, respectively. This directed evolution of new pathways requires extensive knowledge of degradation pathways and their genetic and regulatory organization but avoids a lengthy analysis of complex genetic changes that may take place during adaptation experiments involving transfers, deletions, insertions and rearrangements of large degradative plasmids.

4. Prospects and problems

The manipulation of degradative pathways by genetic techniques is still in its infancy. The 4 examples given in the preceding section all involve the degradation of aromatic compounds. The main reason for this is the fact that a great deal is known about the structure-function relationships of the TOL and NAH7 plasmids whereas there is much less information available on the genetic background of most catabolic pathways, especially those catalyzing the mineralization of xenobiotic compounds. Shotgun cloning approaches are unlikely to produce strains with useful novel degradative properties. As pointed out before, the chromosomal genes specifying the enzymes of an entire catabolic pathway in *Pseudomonas* tend to be grouped in several and separate clusters, and the same holds true for the genetic organization of degradative plasmids (studied in detail in the case of TOL[35], NAH7[113], and OCT[31]).

Moreover, several regulatory elements may be needed to ensure induction of the whole pathway. Substrate uptake can be expected to require specific permeases, which may also be under induction control. Because of this natural complexity, genetic engineering techniques will be most useful in those cases in which the recruitment of a *single* function (i.e., one enzyme or one group of co-regulated enzymes) suffices to widen the range of growth substrates for a particular strain or to improve the rate of a degradative process.

In waste-water systems the salinity may be a factor limiting biodegradation. It has been suggested that osmotolerance of bacterial strains could be improved by cloning the genes of proline biosynthesis because bacteria overproducing proline are more tolerant to osmotic stress[61]. Some of the strains degrading xenobiotic compounds (e.g., dichloromethane or sulfanilic acid, T. Leisinger, personal communication) grow very slowly by comparison with standard *P. putida* strains. Genetic methods might help to construct hybrid strains able to utilize exotic compounds and yet possessing a reasonable growth rate.

Very little is known about the stability of recombinant DNA molecules in *Pseudomonas.* In studies on cloned PAO DNA in recombination-deficient and -proficient PAO strains we have observed that some recombinant plasmids are quite unstable even under selective pressure although the cloning vector used (pKT240, an RSF1010 replicon; table 3) is very stable in *P. aeruginosa.* However, after a period of adaptation stable deletion derivatives of recombinant plasmids could be obtained (our unpublished experiments). Plasmid instability was also observed in *P. putida* when insertions were made into non-essential regions of plasmid R1162[69]. In *E. coli* the plasmids carrying these insertions were stable. R1162::Tn*5* plasmids could recover their stability in *P. putida* by internal deletion of Tn*5* sequences[69].

Recombinant plasmids which are stable in small-scale cultures under laboratory conditions may nevertheless present problems when large-scale cultures and/or continuous systems have be used. This point is illustrated by the single-cell protein production using *Methylophilus methylotrophus.* An IncQ recombinant plasmid carrying the glutamate dehydrogenase *(gdh^+)* gene of *E. coli* enables the methylotroph to assimilate ammonia with improved efficiency and thus the recombinant organism converts more methanol, the C source, into cellular material[110]. After 1 week of continuous culture in a 5-l fermenter more than 90% of the cells had lost 3 unselected plasmid markers by deletions of the recombinant plasmid whereas the selected gdh^+ gene was retained[82].

It is impossible to anticipate the stability of engineered strains in open systems where it may be difficult or even prohibitive to maintain some kind of selective pressure. The fate of several microbial species of potential use in genetic engineering has been studied after their introduction into open systems such as sewage and soil[62]. It was found that most species persisted only when the environment had been sterilized before inoculation[62]. Unfortunately, no *Pseudomonas* strains were included in this study. Would a *Pseudomonas* culture able to degrade a particular xenobiotic compound persist in ordinary soil? An engineered *P. cepacia* strain utilizing the herbicide 2,4,5-trichlorophenoxyacetate (2,4,5-T) as the sole C source was able to degrade 2,4,5-T in soil and under optimal growth conditions gave 1% survival after 2 weeks of incubation in this environment[19]. However, the soil samples used were incubated in the laboratory and field studies have not yet been carried out.

The problem of instability of recombinant plasmids might be overcome by the use of transposable elements as vectors for foreign DNA. The transposon Tn*7*, e.g., has several internal restriction sites which

can be used for cloning[40]. Because insertion of foreign DNA at the *Hin*dIII sites abolishes Tn*7* transposition, a helper plasmid supplying transposition functions is needed to allow the insertion of the recombinant Tn*7* into the host chromosome[40]. After elimination of the helper plasmid, a Tn*7-trpE* recombinant transposon was found stable in *E. coli, M. methylotrophus* and *P. aeruginosa*[40].

5. Conclusion

The detoxification and mineralization of recalcitrant compounds in industrial waste with the aid of microorganisms is a central topic of biodegradation studies. There is no doubt that genetic methods can be used to improve the degradative abilities of microbes but it should be realized that the successful application of genetics to practical problems of biodegradation is a long shot. Whether or not engineered strains can be used to control environmental pollution remains to be seen. At any rate, a rigorous control of toxic products at their source and a cautious use of recalcitrant compounds are the most effective methods to avoid further pollution of the environment.

Acknowledgments. I am very grateful to many colleagues for suggestions, communication of unpublished data and discussion: M. Bagdasarian, P. Barth, G. Boulnois, A.M. Chakrabarty, A.M. Cook, F.C.H. Franklin, J. Frey, R. Früh, R.W. Hedges, B.W. Holloway, Y. Itoh, Ph. Lehrbach, T. Leisinger, H. Matsumoto, A. Mercenier, A.F. Morgan, M. Rella, G. Riess, L. Soldati, K.N. Timmis, M. Van Montagu, and J. Watson Work in this laboratory was supported by the Schweizerische Nationalfonds (project 3.041-0.81).

1 Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F.C.H., Bagdasarian, M.M., Frey, J., and Timmis, K.N., Specific-purpose plasmid cloning vectors, II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene *16* (1981) 237–247.

2 Bagdasarian, M., and Timmis, K.N., Host: vector systems for gene cloning in *Pseudomonas*, in: Current topics in microbiology and immunology, vol. 96, pp. 47–67. Eds P.H. Hofschneider and W. Goebel. Springer, Berlin 1982.

3 Barth, P.T., Tobin, L., and Sharpe, G.S., Development of broad host-range plasmid vectors, in: Molecular biology, pathogenicity and ecology of bacterial plasmids, pp. 439–448. Eds S.B. Levy, R.C. Clowes and E.L. Koenig. Plenum Press, New York 1981.

4 Bayley, S.A., Duggleby, C.J., Worsey, M.J., Williams, P.A., Hardy, K.G., and Broda, P., Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. Molec. gen. Genet. *154* (1977) 203–204.

5 Benson, S., and Shapiro, J., TOL is a broad-host-range plasmid. J. Bact. *135* (1978) 278–280.

6 Beringer, J.E., Hoggan, S.A., and Johnston, A.W.B., Linkage mapping in *Rhizobium leguminosarum* by means of R plasmid-mediated recombination. J. gen. Microbiol. *104* (1978) 201–207.

7 Beringer, J.E., Beynon, J.L., Buchanan-Wollaston, A.V., and Johnston, A.W.B., Transfer of the drug-resistance transposon Tn*5* to *Rhizobium*. Nature *276* (1978) 633–634.

8 Bloch, A., Murphy's law and other reasons why things go wrong. Price/Stern/Sloan Publishers Inc., Los Angeles 1981.

9 Bochner, B.R., Huang, H.-C., Schieven, G.L., and Ames, B.N., Positive selection for loss of tetracycline resistance. J. Bact. *143* (1980) 926–933.

10 Buckel, P., and Zehelein, E., Expression of *Pseudomonas fluorescens* D-galactose dehydrogenase in *E. coli*. Gene *16* (1981) 149–159.

11 Cane, P.A., and Williams, P.A., The plasmid-coded metabolism of naphthalene and 2-methylnaphthalene in *Pseudomonas* strains: phenotypic changes correlated with structural modification of the plasmid pWW60-1. J. gen. Microbiol. *128* (1982) 2281–2290.

12 Carlson, C.A., Pierson, L.S., Rosen, J.J., and Ingraham, J.L., *Pseudomonas stutzeri* and related species undergo natural transformation. J. Bact. *153* (1983) 93–99.

13 Caruso, M., and Shapiro, J.A., Interactions of Tn*7* and temperate phage F116L of *Pseudomonas aeruginosa*. Molec. gen. Genet. *188* (1982) 292–298.

14 Chakrabarty, A.M., Plasmids in *Pseudomonas*. A. Rev. Genet. *10* (1976) 7–30.

15 Chakrabarty, A.M., and Gunsalus, I.C., Chromosomal mobilization from a *rec*A mutant of *Pseudomonas putida*. Molec. gen. Genet. *176* (1979) 151–154.

16 Chakravorty, A.K., Zurkowski, W., Shine, J., and Rolfe, B.G., Symbiotic nitrogen fixation: molecular cloning of *Rhizobium* genes involved in exopolysaccharide synthesis and effective nodulation. J. molec. appl. Genet. *1* (1982) 585–596.

17 Chandler, P.M., and Krishnapillai, V., Isolation and properties of recombination-deficient mutants of *Pseudomonas aeruginosa*. Mutation Res. *23* (1974) 15–23.

18 Chatterjee, D.K., and Chakrabarty, A.M., Genetic rearrangements in plasmids specifying total degradation of chlorinated benzoic acids. Molec. gen. Genet. *188* (1982) 279–285.

19 Chatterjee, D.K., Kilbane, J.J., and Chakrabarty, A.M., Biodegradation of 2,4,5-trichlorophenoxyacetic acid in soil by a pure culture of *Pseudomonas cepacia*. Appl. environ. Microbiol. *44* (1982) 514–516.

20 Chatterjee, D.K., and Chakrabarty, A.M., Genetic homology between independently isolated chlorobenzoate-degradative plasmids. J. Bact. *153* (1983) 532–534.

21 Ciampi, M.S., Schmid, M.B., and Roth, J.R., Transposon Tn*10* provides a promoter for transcription of adjacent sequences. Proc. natl Acad. Sci. USA *79* (1982) 5016–5020.

22 Clarke, P.H., The metabolic versatility of pseudomonads. Antonie van Leeuwenhoek *48* (1982) 105–130.

23 Currier, T.C., and Morgan, M.K., Direct DNA repeat in plasmid R68.45 is associated with deletion formation and concomitant loss of chromosome mobilization ability. J. Bact. *150* (1982) 251–259.

24 Dean, H.F., and Morgan, A.F., Integration of R91-5::Tn*501* into the *Pseudomonas putida* PPN chromosome and genetic circularity of the chromosomal map. J. Bact. *153* (1983) 485–497.

25 Depicker, A., De Block, M., Inze, D., Van Montagu, M., and Schell, J., IS-like element IS*8* in RP4 plasmid and its involvement in cointegration. Gene *10* (1980) 329–338.

26 de Torrontegui, G., Diaz, R., Wheelis, M.L., and Cánovas, J.L., Supra-operonic clustering of genes specifying glucose dissimilation in *Pseudomonas putida*. Molec. Gen. Genet. *144* (1976) 307–311.

27 Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R., Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. natl Acad. Sci. USA *77* (1980) 7347–7351.

28 Don, R.H., and Pemberton, J.M., Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bact. *145* (1981) 681–686.

29 Drew, R.E., and Clarke, P.H., The construction in vitro of derivatives of bacteriophage lambda carrying the amidase genes of *Pseudomonas aeruginosa*. Molec. gen. Genet. *177* (1980) 311–320.

30 Espin, G., Alvarez-Morales, A., and Merrick, M., Complementation analysis of *gln*A-linked mutations which affect nitrogen fixation in *Klebsiella pneumoniae*. Molec. gen. Genet. *184* (1981) 213–217.

31 Fennewald, M., Benson, S., Oppici, M., and Shapiro, J., Insertion element analysis and mapping of the *Pseudomonas* plasmid *alk* regulon. J. Bact. *139* (1979) 940–952.

32 Finette, B.A., and Gibson, D.T., Transpositional mutations and genetic organization of the toluene *(tod)* operon of *Pseudomonas putida.* Abstracts of the annual meeting of the American Society for Microbiology, 1983, H127.

33 Finger, J., and Krishnapillai, V., Host range, entry exclusion, and incompatibility of *Pseudomonas aeruginosa* FP plasmids. Plasmid *3* (1980) 332-342.

34 Fisher, P.R., Appleton, J., and Pemberton, J.M., Isolation and characterization of the pesticide-degrading plasmid pJP1 from *Alcaligenes paradoxus.* J. Bact. *135* (1978) 798-804.

35 Franklin, F.C.H., Bagdasarian, M., Bagdasarian, M.M., and Timmis, K.N., Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring *meta* cleavage pathway. Proc. natl Acad. Sci. USA *78* (1981) 7458-7462.

36 Franklin, F.C.H., Bagdasarian, M., and Timmis, K.N., Manipulation of degradative genes of soil bacteria, in: Microbial degradation of xenobiotics and recalcitrant compounds, pp.109-130. Eds Th. Leisinger, A.M. Cook, R. Hütter and J. Nüesch. Academic Press, London 1981.

37 Friedman, A.M., Long, S.R., Brown, S.E., Buikema, W.J., and Ausubel, F.M., Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene *18* (1982) 289-296.

37a Früh, R., Watson, J.M., and Haas, D., Construction of recombination-deficient strains of *Pseudomonas aeruginosa.* Molec. gen. Genet. *191* (1983) 334-337.

38 Furukawa, K., and Chakrabarty, A.M., Involvement of plasmids in total degradation of chorinated biphenyls. Appl. environ. Microbiol. *44* (1982) 619-626.

39 Gautier, F., and Bonewald, R., The use of plasmid R1162 and derivatives for gene cloning in the methanol-utilizing *Pseudomonas* AM1. Molec. gen. Genet. *178* (1980) 375-380.

40 Grinter, N.J., A broad-host-range cloning vector transposable to various replicons. Gene *21* (1983) 133-143.

41 Haas, D., and Holloway, B.W., R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa.* Molec. gen. Genet. *144* (1976) 243-251.

42 Haas, D., and Holloway, B.W., Chromosome mobilization by the R plasmid R68.45: a tool in *Pseudomonas* genetics. Molec. gen. Genet. *158* (1978) 229-237.

43 Haas, D., Watson, J., Krieg, R., and Leisinger, Th., Isolation of an Hfr donor of *Pseudomonas aeruginosa* PAO by insertion of the plasmid RP1 into the tryptophan synthase gene. Molec. gen. Genet. *182* (1981) 240-244.

44 Haas, D., and Riess, G., Spontaneous deletions of the chromosome-mobilizing plasmid R68.45 in *Pseudomonas aeruginosa* PAO. Plasmid *9* (1983) 42-52.

45 Heinaru, A.L., Duggleby, C.J., and Broda, P., Molecular relationships of degradative plasmids determined by in situ hybridisation of their endonuclease-generated fragments. Molec. gen. Genet. *160* (1978) 347-351.

46 Hewetson, L., Dunn, H.M., and Dunn, N.W., Evidence for a transmissible catabolic plasmid in *Pseudomonas putida* encoding the degradation of *p*-cresol via the protocatechuate ortho cleavage pathway. Genet. Res., Camb. *32* (1978) 249-255.

47 Holloway, B.W., Isolation and characterization of an R′ plasmid in *Pseudomonas aeruginosa.* J. Bact. *133* (1978) 1078-1082.

48 Holloway, B.W., Krishnapillai, V., and Morgan, A.F., Chromosomal genetics of *Pseudomonas.* Microbiol. Rev. *43* (1979) 73-102.

49 Holloway, B.W., Crowther, C., Dean, H., Hagedorn, J., Holmes, N., and Morgan, A.F., Integration of plasmids into the *Pseudomonas* chromosome, in: Drug resistance in bacteria, pp.231-242. Ed. S. Mitsuhashi. Japan Scientific Societies Press, Tokyo 1982.

50 Holloway, B.W., *Pseudomonas* genetics and its application to other bacteria, in: Genetics of industrial microorganisms, pp.41-45. Eds Y. Ikeda and T. Beppu. Kodansha Ltd, Tokyo 1983.

51 Hooykaas, P.J.J., Peerbolte, R., Regensburg-Tuink, A.J.G., de Vries, P., and Schilperoort, R.A., A chromosomal linkage map of *Agrobacterium tumefaciens* and a comparison with the maps of *Rhizobium* spp. Molec. gen. Genet. *188* (1982) 12-17.

52 Jacoby, G.A., Rogers, J.E., Jacob, A.E., and Hedges, R.W., Transposition of *Pseudomonas* toluene-degrading genes and expression in *Escherichia coli.* Nature *274* (1978) 179-180.

53 Jacoby, G.A., Plasmids of *Pseudomonas aeruginosa,* in: *Pseudomonas aeruginosa.* Clinical manifestations of infection and current therapy, pp.271-309. Ed. R.G. Doggett. Academic Press, New York 1979.

54 Jeenes, D.J., Reineke, W., Knackmuss, H.-J., and Williams, P.A., TOL plasmid pWW0 in constructed halobenzoate-degrading *Pseudomonas* strains: enzyme regulation and DNA structure. J. Bact. *150* (1982) 180-187.

55 Kawasaki, H., Yahara, H., and Tonomura, K., Isolation and characterization of plasmid pUO1 mediating dehalogenation of haloacetate and mercury resistance in *Moraxella* sp. B. Agric. biol. Chem. *45* (1981) 1477-1481.

56 Knauf, V.C., and Nester, E.W., Wide host range cloning vectors: a cosmid clone bank of an Agrobacterium Ti plasmid. Plasmid *8* (1982) 45-54.

57 Kondorosi, A., Kiss, G.B., Forrai, T., Vincze, E., and Banfalvi, Z., Circular linkage map of *Rhizobium meliloti* chromosome. Nature *268* (1977) 525-527.

58 Kondorosi, A., Vincze, E., Johnston, A.W.B., and Beringer, J.E., A comparison of three *Rhizobium* linkage maps. Molec. gen. Genet. *178* (1980) 403-408.

59 Kondorosi, A., Kondorosi, E., Pankhurst, C.E., Broughton, W.J., and Banfalvi, Z., Mobilization of a *Rhizobium meliloti* megaplasmid carrying nodulation and nitrogen fixation genes into other *Rhizobia* and *Agrobacterium.* Molec. gen. Genet. *188* (1982) 433-439.

60 Krishnapillai, V., Royle, P., and Lehrer, J., Insertions of the transposon Tn*1* into the *Pseudomonas aeruginosa* chromosome. Genetics *97* (1981) 495-511.

61 Le Rudulier, D., and Valentine, R.C., Genetic engineering in agriculture: osmoregulation. TIBS *7* (1982) 431-433.

62 Liang, L.N., Sinclair, J.L., Mallory, L.M., and Alexander, M., Fate in model ecosystems of microbial species of potential use in genetic engineering. Appl. environ. Microbiol. *44* (1982) 708-714.

63 Manoharan, H.T., and Jayaraman, K., Mapping of the loci involved in the catabolic oxidation of L-hydroxyproline in *Pseudomonas aeruginosa* PAO. Molec. gen. Genet. *172* (1979) 99-105.

64 Matsumoto, H., Ohta, S. Kobayashi, R., and Terawaki, Y., Chromosomal location of genes participating in the degradation of purines in *Pseudomonas aeruginosa.* Molec. gen. Genet. *167* (1978) 165-176.

65 Matsumoto, H., Nakazawa, T., Ohta, S., and Terawaki, Y., Chromosomal locations of *cat*A, *pob*A, *pca*A, *dcu* and *chu* genes in *Pseudomonas aeruginosa.* Genet. Res., Camb. *38* (1981) 251-266.

66 Megias, M., Caviedes, M.A., Palomares, A.J., and Perez-Silva, J., Use of plasmid R68.45 for constructing a circular linkage map of the *Rhizobium trifolii* chromosome. J. Bact. *149* (1982) 59-64.

67 Mercer, A.A., and Loutit, J.S., Transformation and transfection of *Pseudomonas aeruginosa*: effects of metal ions. J. Bact. *140* (1979) 37-42.

68 Mergeay, M., Boyen, A., Legrain, C., and Glansdorff, N., Expression of *Escherichia coli* K-12 arginine genes in *Pseudomonas fluorescens.* J. Bact. *136* (1978) 1187-1188.

69 Meyer, R., Laux, R., Boch, G., Hinds, M., Bayly, R., and Shapiro, J.A., Broad-host-range IncP-4 plasmid R1162: effects of deletions and insertions on plasmid maintenance and host range. J. Bact. *152* (1982) 140-150.

70 Moore, R.J., and Krishnapillai, V., Tn*7* and Tn*501* insertions into *Pseudomonas aeruginosa* plasmid R91-5: mapping of two transfer regions. J. Bact. *149* (1982) 276-283.

71 Moore, A.T., Nayudu, M., and Holloway, B.W., Genetic mapping in *Methylophilus methylotrophus* AS1. J. gen. Microbiol. *129* (1983) 785-799.

72 Morgan, A.F., Isolation and characterization of *Pseudomonas aeruginosa* R′ plasmids constructed by interspecific mating. J. Bact. *149* (1982) 654-661.

73 Morrison, N.A., Hau, C.Y., Trinick, M.J., Shine, J., and Rolfe, B.G., Heat curing of a sym plasmid in a fast-growing *Rhizobium* sp. that is able to nodulate legumes and the nonlegume *Parasponia* sp. J. Bact. *153* (1983) 527-531.

74 Mortlock, R.P., Metabolic acquisitions through laboratory selection. A. Rev. Microbiol. *36* (1982) 259-284.

75 Mylroie, J.R., Friello, D.A., Siemens, T.V., and Chakrabarty, A.M., Mapping of *Pseudomonas putida* chromosomal genes with a recombinant sex-factor plasmid. Molec. gen. Genet. *157* (1977) 231-237.

76 Nakazawa, T., TOL plasmid in *Pseudomonas aeruginosa* PAO: thermosensitivity of self-maintenance and inhibition of host cell growth. J. Bact. *133* (1978) 527–535.

77 Negoro, S., and Okada, H., Physical map of nylon oligomer degradative plasmid pOAD2 harbored in *Flavobacterium* sp. KI72 Agric. biol. Chem. *46* (1982) 745–750.

78 Olsen, R.H., DeBusscher, G., and McCombie, W.R., Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. J. Bact. *150* (1982) 60–69.

79 Pellett, S., Bigley, D.V., and Grimes, D.J., Distribution of *Pseudomonas aeruginosa* in a riverine ecosystem. Appl. environ. Microbiol. *45* (1983) 328–332.

80 Pemberton, J.M., and Clark, A.J., Detection and characterization of plasmids in *Pseudomonas aeruginosa* strain PAO. J. Bact. *114* (1973) 424–433.

81 Pickup, R.W., Lewis, R.J., and Williams, P.A., *Pseudomonas* sp. MT14, a soil isolate which contains two large catabolic plasmids, one a TOL plasmid and one coding for phenylacetate catabolism and mercury resistance. J. gen. Microbiol. *129* (1983) 153–158.

82 Powell, K.A., and Byrom, D., Culture stability in strains used for single cell protein production, in: Genetics of industrial microorganisms, pp.345–350. Eds Y. Ikeda and T. Beppu. Kodansha Ltd., Tokyo 1983.

83 Reineke, W., Jeenes, D.J., Williams, P.A., and Knackmuss, H.-J., TOL plasmid pWW0 in constructed halobenzoate-degrading *Pseudomonas* strains: prevention of *meta* pathway. J. Bact. *150* (1982) 195–201.

84 Reineke, W., Wessels, S.W., Rubio, M.A., Latorre, J., Schwien, U., Schmidt, E., Schlömann, M., and Knackmuss, H.-J., Degradation of monochlorinated aromatics following transfer of genes encoding chlorocatechol catabolism. FEMS Microbiol. Lett. *14* (1982) 291–294.

85 Rella, M., and Haas, D., Transposon mutagenesis using a Tn*5* derivative in *Pseudomonas aeruginosa.* Abstracts of the ASM/DGHM spring meeting, 1983, p.37.

86 Rheinwald, J.G., Chakrabarty, A.M., and Gunsalus, I.C., A transmissible plasmid controlling camphor oxidation in *Pseudomonas putida.* Proc. natl Acad. Sci. USA *70* (1973) 885–889.

87 Rolfe, B., and Holloway, B.W., Alterations in host specificity of bacterial deoxyribonucleic acid after an increase in growth temperature of *Pseudomonas aeruginosa.* J. Bact. *92* (1966) 43–48.

88 Roth, J.R., New genetic techniques using transposable drug resistance elements, in: Microbiology 1981, pp.402–404. Ed. D. Schlessinger. ASM, Washington D.C. 1981.

89 Royle, P.L., Matsumoto, H., and Holloway, B.W., Genetic circularity of the *Pseudomonas aeruginosa* PAO chromosome. J. Bact. *145* (1981) 145–155.

90 Ruvkun, G.B., and Ausubel, F.M., A general method for site-directed mutagenesis in prokaryotes. Nature *289* (1981) 85–88.

91 Sakaguchi, K., Vectors for gene cloning in *Pseudomonas* and their applications, in: Current topics in microbiology and immunology, vol.96, pp.31–45. Eds P.H. Hofschneider and W. Goebel. Springer, Berlin 1982.

92 Sato, M., Staskawicz, B.J., Panopoulos, N.J., Peters, S., and Honma, M., A host-dependent hybrid plasmid suitable as a suicidal carrier for transposable elements. Plasmid *6* (1981) 325–331.

93 Schell, M.A., Cloning and expression in *Escherichia coli* of the naphthalene degradation genes from plasmid NAH7. J. Bact. *153* (1983) 822–829.

94 Scott, D.B., and Ronson, C.W., Identification and mobilization by cointegrate formation of a nodulation plasmid in *Rhizobium trifolii.* J. Bact. *151* (1982) 36–43.

95 Serdar, C.M., Gibson, D.T., Munnecke, D.M., and Lancaster, J.H., Plasmid involvement in parathion hydrolysis by *Pseudomonas diminuta.* Appl. environ. Microbiol. *44* (1982) 246–249.

96 Shaw, K.J., and Berg, C.M., *Escherichia coli* K-12 auxotrophs induced by insertion of the transposable element Tn*5*. Genetics *92* (1979) 741–747.

97 Slater, J.H., and Bull, A.T., Environmental microbiology: biodegradation. Phil. Trans. R. Soc. Lond. B *297* (1982) 575–597.

98 Stanier, R.Y., Palleroni, N.J., and Doudoroff, M., The aerobic pseudomonads: a taxonomic study. J. gen. Microbiol. *43* (1966) 159–271.

99 Stanisich, V.A., Bennett, P.M., and Richmond, M.H., Characterization of a translocation unit encoding resistance to mercuric ions that occurs on a nonconjugative plasmid in *Pseudomonas aeruginosa.* J. Bact. *129* (1977) 1227–1233.

100 Tago, Y., Oishi, K., Yano, K., and Aida, K., Transfer of TOL plasmid to a floc-forming bacterium, *Pseudomonas* sp. No. 12, in: Abstracts of the 4th international symposium on genetics of industrial microorganisms, P-I-9, Kyoto, Japan 1982.

101 Thacker, R., and Gunsalus, I.C., Dissociation of the NIC plasmid aggregate in *Pseudomonas putida.* J. Bact. *137* (1979) 697–699.

102 Timmis, K.N., Gene manipulation *in vitro*, in: Genetics as a tool in microbiology, pp.49–109. Eds S.W. Glover and D.A. Hopwood. Cambridge University Press, Cambridge 1981.

103 Tsuda, M., Harayama, S., and Iino, T., Use of transposons for the genetic analysis in *Pseudomonas aeruginosa,* in: Abstracts of the 4th international symposium on genetics of industrial microorganisms, O-IX-3, Kyoto, Japan 1982.

104 Van Haute, E., Joos, H., Maes, M., Warren, G., Van Montagu, M., and Schell, J., Intergeneric transfer and exchange recombination of restriction fragments cloned in pBR322: a novel strategy for the reversed genetics of the Ti plasmids of *Agrobacterium tumefaciens.* EMBO J. *2* (1983) 411–417.

105 Van Vliet, F., Silva, B., Van Montagu, M., and Schell, J., Transfer of RP4::Mu plasmids to *Agrobacterium tumefaciens.* Plasmid *1* (1978) 446–455.

106 Weissmann, C., Reversed genetics. TIBS *3* (1978) N109–N111.

107 Wheelis, M.L., The genetics of dissimilatory pathways in *Pseudomonas.* A. Rev. Microbiol. *29* (1975) 505–524.

108 White, G.P., and Dunn, N.W., Compatibility and sex specific phage plating characteristics of the TOL and NAH catabolic plasmids. Genet. Res., Camb. *32* (1978) 207–213.

109 Willetts, N.S., Crowther, C., and Holloway, B.W., The insertion sequence IS*21* of R68.45 and the molecular basis for mobilization of the bacterial chromosome. Plasmid *6* (1981) 30–52.

110 Windass, J.D., Worsey, M.J., Pioli, E.M., Pioli, D., Barth, P.T., Atherton, K.T., Dart, E.C., Byrom, D., Powell, K., and Senior, P.J., Improved conversion of methanol to single-cell protein by *Methylophilus methylotrophus.* Nature *287* (1980) 396–401.

111 Wong, C.L., and Dunn, N.W., Combined chromosomal and plasmid encoded control for the degradation of phenol in *Pseudomonas putida.* Genet. Res., Camb. *27* (1976) 405–412.

112 Wood, D.O., Hollinger, M.F., and Tindol, M.B., Versatile cloning vector for *Pseudomonas aeruginosa.* J. Bact. *145* (1981) 1448–1451.

113 Yen, K.-M., and Gunsalus, I.C., Plasmid gene organization: naphthalene/salicylate oxidation. Proc. natl Acad. Sci. USA *79* (1982) 874–878.

114 Zechman, J.M., and Casida, L.E., Jr., Death of *Pseudomonas aeruginosa* in soil. Can. J. Microbiol. *28* (1982) 788–794.

115 Zuniga, M.C., Durham, D.R., and Welch, R.A., Plasmid- and chromosome-mediated dissimilation of naphthalene and salicylate in *Pseudomonas putida* PMD-1. J. Bact. *147* (1981) 836–843.

0014-4754/83/111199-15$1.50+0.20/0