Strategy for Cloning Large Gene Assemblages as Illustrated Using the Phenylacetate and Polyhydroxyalkanoate Gene Clusters

Belén García,¹ Elías R. Olivera,¹ Ángel Sandoval,¹ Elsa Arias-Barrau,¹ Sagrario Arias,¹
Germán Naharro,² and José M. Luengo^{1*}

*Departamento de Bioquímica y Biología Molecular¹ and Departamento de Patología Animal (Sanidad Animal),²
Facultad de Veterinaria, Universidad de León, 24007 León, Spain*

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We report an easy procedure for isolating chromosome-clustered genes. By following this methodology, the entire set of genes belonging to the phenylacetic acid (PhAc; 18-kb) pathway as well as those required for the synthesis and mobilization of different polyhydroxyalkanoates (PHAs; 6.4 kb) in *Pseudomonas putida* U were recovered directly from the bacterial chromosome and cloned into a plasmid for the first time. The transformation of different bacteria with these genetic constructions conferred on them the ability to either degrade PhAc or synthesize bioplastics (PHAs).

The cloning and functional analyses of large gene clusters usually entail significant difficulties. Cloning procedures are often limited by the absence of the required restriction sites on the DNA fragment to be cloned, and the expression of the encoded proteins is always affected by mutations introduced in the sequence when large DNA regions are to be amplified (2, 3, 9, 17, 22, 30). These problems are further exacerbated when little information about the sequence, organization, and regulation of the genes to be cloned is available. Thus, in order to facilitate the cloning of all of the genes and regulatory elements included in large genetic assemblages, we have developed a different strategy. Using a simple method based on a single recombination event (27), we have been able to (i) recover the selected genes from the genome, obtaining a faithful copy of the desired genetic construction; (ii) clone them into a plasmid; and (iii) transfer their complete biosynthetic or catabolic routes to other microbes, enhancing the metabolic capabilities of these microbes. Our approach, which is of undoubted scientific interest, is also very useful from a practical point of view, since it facilitates the cloning of linked sets of genes that might be required to reproduce basic processes of biochemical or genetic interest as well as others of biotechnological or industrial importance.

Accordingly, we describe here the methodological bases of the procedure and illustrate its practical use by describing the isolation and expression of the entire phenylacetic acid (PhAc) and polyhydroxyalkanoate (PHA) pathways, which are of great scientific interest (6, 13, 20) and have important biotechnological applications (11, 12).

Cloning of the PhAc catabolic pathway from *Pseudomonas putida* U. The aerobic degradation of PhAc in *P. putida* U (Colección Española de Cultivos Tipo 4848) (21) is carried out by 13 catabolic enzymes (PaaABCEFGHIJLMN) organized into five functional units, namely, (i) a transport system integrated by a permease (PaaL) and a porine (PaaM), which

catalyzes the uptake of PhAc; (ii) an acyl coenzyme A (acyl-CoA)-activating enzyme (PaaF), which converts PhAc into phenylacetyl-CoA (PhAc-CoA); (iii) a ring-hydroxylation complex (PaaGHIJK) that catalyzes the hydroxylation of PhAc-CoA to 2-hydroxy-phenylacetyl-CoA (2-OH-PhAc-CoA); (iv) a ring-opening enzyme (PaaN), which transforms 2-OH-PhAc-CoA into an aliphatic intermediate; and (v) a β -oxidation system integrated by two enoyl-CoA hydratases (PaaA and PaaB), a 3-OH-acyl-CoA dehydrogenase (PaaC), and a ketothiolase (PaaE), which, finally, converts this compound into general catabolites (14, 15, 20, 28). Additionally, two regulatory proteins (PaaX and PaaY) are involved in the control of the flux of intermediates through the pathway (5, 20). All of these enzymes are encoded by 15 genes organized into five operons, three of them (*paaABCEF*, *paaGHIJK*, and *paaLMN*) carrying the catabolic genes and two regulatory ones (*paaX* and *paaY*), which are divergently translated (20).

This catabolic route is not only present in some strains belonging to the genus *Pseudomonas* (14) but also widely distributed among many phylogenetically unrelated microbes (11). It was previously shown (20) that this catabolic pathway is the central route of a more complex degradative unit (named catabolon), which is integrated by the different catabolic routes involved in the degradation of all of the aromatic compounds that generate PhAc or phenylacetyl-CoA as catabolic intermediates (phenylacetyl-CoA catabolon). Furthermore, it has also been reported that some of the enzymes belonging to the central pathway of the phenylacetyl-CoA catabolon may have important biotechnological applications (1, 10, 15, 19), thus expanding interest in the study of this route. For this reason, we attempted the cloning of the entire Paa catabolic pathway into a plasmid since this construction would facilitate additional genetic, biochemical, regulatory, structural, and biotechnological studies.

We approached the construction of a recombinant strain carrying the entire Paa pathway by following different strategies. First, we attempted to obtain several fragments, either by direct digestion with restriction enzymes or by PCR (6, 15, 16, 20, 25, 26); unfortunately, we failed to obtain a construction containing the entire functional route. In order to reproduce

* Corresponding author. Mailing address: Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de León, Campus de Vegazana s/n, 24007 León, Spain. Phone: 34-987291228. Fax: 34-987291226. E-mail: dbbjlr@unileon.es.

FIG. 1. Schematic representation of the procedure followed for cloning the *paa* cluster with plasmid *pK18::mob*.

this pathway exactly, we changed our research strategy. Flanking the DNA sequence involved in the catabolism of PhAc (*paaXYABCEFGHIJKLMN*) are three genes nonessential for the catabolism of PhAc that code for a transcriptional activator (5' region; *orfA*) and a two-component regulatory system (3' region; *orfS* and *orfR*). The *orfA* gene (located outside the DNA fragment required for the aerobic catabolism of PhAc) was amplified by PCR and cloned into the integrative plasmid *pK18::mob* (27) (Fig. 1), and this construction was used to transform *P. putida* U by triparental mating (7). The recombinant bacteria, which showed kanamycin resistance (*Km^r*), were analyzed to determine the correct insertion of *pK18::mob* in the chromosome (at the *orfA* site; see Fig. 1). One of these colonies was cultured in Luria-Bertani (LB) medium, and its DNA was extracted, purified, and digested with the restriction enzyme *Bam*HI, which cuts at the multicloning (polylinker) site of *pK18::mob* and 5 kb downstream from *paaN* (3' end). The *Bam*HI-digested DNA fragments, which were greater than

23 kb in size, were extracted from the agarose, repurified, re-circularized using T4 DNA ligase, and used to transform *Escherichia coli* DH10B (14). Several colonies of the recombinant bacteria showing *Km^r* were analyzed. All of them contained a large-molecular-size plasmid (*pKpaa*; about 27 kb) carrying the *pK18::mob* sequence and an insert of 23 kb that corresponded to the entire pathway (including the regulatory elements) involved in the aerobic catabolism of PhAc in *P. putida* U. The complete procedure we followed is illustrated in Fig. 1.

A second approach, one which is more convenient when no restriction sites outside the clustered genes are known, involves a variant of the procedure described above consisting of the use of two different plasmids (in our case, *pK18::mob* and *pJQ200KS*) (23, 29). The recombinant bacteria containing *pK18::mob* inserted after the *orfA* gene (see above) were transformed with a genetic construction that contained the *paaN* gene (3' end of the DNA region containing the *paa* cluster) in plasmid *pJQ200KS* (gentamicin resistant [*Gm^r*]).

FIG. 2. Bacterial growth (absorbance at 540 nm) under different culture conditions. (a) *E. coli* W14 and *E. coli* W14(pKpaa) cultured in chemically defined medium containing 5 mM PhAc (○ and ●, respectively) or 5 mM 2-phenylethylamine (◇ and ◆, respectively) as the sole carbon source; (b) *A. hydrophila* (□) and *A. hydrophila*(pKpaa) (■) cultured in chemically defined medium containing 5 mM PhAc; (c and d) *P. aeruginosa* (△) and *P. aeruginosa*(pKpaa) (▲) (c) and *P. stutzeri* (▽) and *P. stutzeri*(pKpaa) (▼) (d) cultured under the same conditions described for panel b. When *E. coli* W14 or *E. coli* W14(pKpaa) was employed, 1 µg of cobalamine/ml was added to the medium (4). *A. hydrophila* ATCC 7966 (ampicillin resistant), *P. aeruginosa* PG201 (kanamycin resistant), and *P. stutzeri* DK301 (rifampin resistant) were obtained from different collections. *E. coli* W14 was derived from *E. coli* W (ATCC 11105) after being subjected to a deletion of 33 kb (4).

The double recombinant strains (containing both plasmids, pK18::mob and pJQ200KS, inserted at the 5' and 3' ends of the *paa* cluster) were selected in LB medium containing kanamycin and gentamicin. DNA from several colonies was independently extracted, purified, and digested with the restriction enzyme BamHI (which cuts at both polylinkers but not at the *paa* region), and fragments with sizes greater than 23 kb were ligated and used to transform *E. coli* DH10B, as indicated above. The bacteria were plated on LB medium containing kanamycin, and the different colonies were analyzed. Those containing a high-molecular-weight plasmid were selected and further analyzed. With this procedure, we were also able to clone the entire PhAc catabolic pathway into the pK18::mob plasmid. This second approach has an advantage over the first in that it is possible to select the required fragment by using one of the two resistance phenotypes (Km^r or Gm^r) as selection markers; additionally, it is not necessary to know of a specific restriction site outside the region to be cloned since it is possible to use the restriction sites belonging to the polylinkers of both plasmids.

Functional analysis of the cloned pathway. Restriction and sequence analyses of the pKpaa plasmid obtained from recombinant *E. coli* DH10B(pKpaa) revealed that it contains a faithful copy of the pKpaa plasmid present in *P. putida* U. Furthermore, analysis of the cell extracts of a recombinant strain of bacteria cultured in LB medium supplemented with PhAc revealed the existence of phenylacetyl-CoA ligase activity (a Paa marker enzyme), which was not found in *E. coli* DH10B trans-

formed with the plasmid pK18::mob and grown under similar conditions. Unfortunately, owing to the inability of the *E. coli* DH10B strain to grow in chemically defined medium, we were unable to confirm whether this pathway enables this bacterium to grow in defined media containing PhAc as the sole carbon source. To clarify this point, we used *E. coli* W14. This bacterium is a mutant obtained by subjecting *E. coli* W to a deletion of 33 kb. The deleted DNA fragment contains all of the genes required for the catabolism of PhAc as well as others necessary for the assimilation of 2-phenylethylamine (4). Thus, *E. coli* W14 is unable to grow in chemically defined medium containing PhAc as the sole carbon source. Transformation of this mutant with plasmid pKpaa conferred upon the recombinant strain *E. coli* W14(pKpaa) the ability to efficiently degrade PhAc (Fig. 2), whereas it was still unable to grow in minimal medium containing 2-phenylethylamine as the sole carbon source. Moreover, analysis of the phenylacetyl-CoA ligase activity in *E. coli* W14(pKpaa) cell extracts (measured colorimetrically or by high-pressure liquid chromatography [14, 15]) revealed that such activity was present, whereas it was not detected in *E. coli* W14 carrying plasmid pK18::mob. Furthermore, when *E. coli* W14(pKpaa) was cultured in a similar medium containing PhAc plus glucose (a *paa* repressor) at 10 mM or in which PhAc had been replaced by 4-OH-PhAc (a close structural compound that does not induce the PhAc pathway in *P. putida* U) at 5 mM, neither phenylacetyl-CoA ligase nor the PhAc transport system (two target enzymatic systems of the Paa pathway [5, 20, 28]) was detected. This

FIG. 3. Schematic representation of the procedure followed for cloning the *pha* clusters with plasmids pK18::mob and pJQ200KS.

finding suggested that the regulatory elements that control the expression of the pathway in *P. putida* U had also been included in the recombinant plasmid.

Expression of the PhAc catabolic pathway in other microbes. To confirm the efficacy of plasmid pK*paa*, we used it to transform other bacteria (*Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Pseudomonas stutzeri*). Taking into account that this plasmid is replicative only in *E. coli*, the transformation of *A. hydrophila* and *P. stutzeri* would require a random recombinant event for *Km^r* to be maintained. In such cases, if some of the genes included in the *paa* cluster bore a certain homology with others present in the recipient strain, the cluster could be truncated and hence the function of the entire pathway would be lost. To avoid this result, all of the bacteria were first transformed with plasmid pK18::mob; the recombinant bacteria, which were selected in LB medium supple-

mented with kanamycin, were later transformed by triparental mating (7) with the pK*paa* plasmid and were then cultured in a chemically defined medium containing PhAc as the sole carbon source. The recombination event in these bacteria should occur mainly between the two copies of pK18::mob, one integrated in the chromosome of the recipient strain and the other contained in pK*paa*. In the case of *P. aeruginosa*, the bacterium has a chromosomal gene that confers *Km^r*; this gene was used as a target for the recombinant event. Recombinant bacteria with a functional set of *paa* genes were selected in chemically defined medium containing PhAc as the sole carbon source. In all cases, we were able to confer upon the recipient bacteria the ability to catabolize PhAc (Fig. 2).

These data indicate that (i) the expression of the genes cloned in plasmid pK*paa* drove a functional catabolic pathway that efficiently catalyzed the assimilation of PhAc in different

FIG. 4. Scanning (a, b, e, and f) and transmission (c, d, g, and h) electron micrographs of the *P. putida* U Δ pha mutant (a to d) and of the same strain transformed with plasmid pKpha (e to h) when the strain was cultured in a minimal medium containing either 10 mM PhAc and 10 mM octanoic acid (a, c, e, and g) or 10 mM PhAc and 10 mM 8-phenyloctanoate (b, d, f, and h) as carbon sources. Bars, 1 μ m.

microbes, enhancing their catabolic potential; and (ii) the *P. putida* U *paa* promoters, as well as their regulatory elements, worked efficiently in these bacteria. Thus, this genetic construction could be useful for studying the role of the *paa* regulatory genes in different recombinant bacteria as well as for establishing the molecular basis of this regulation. However, although all of the recombinant species carrying the

pKpaa plasmid efficiently catabolized PhAc when they were reseeded three or four times in chemically defined medium containing PhAc as the sole carbon source, the single recombinant colonies obtained after the initial transformation with the pKpaa plasmid showed a very low rate of growth in that medium (several [7 to 10] days were required for growth to begin). This growth rate suggests that the bacteria need to

accommodate these genes into their genetic or metabolic machinery before they can efficiently catabolize PhAc.

Cloning of the metabolic pathway involved in the biosynthesis and degradation of PHAs in *P. putida* U. The second example that illustrates the efficacy of our method for cloning clustered genes is that of the PHA pathway. PHAs are naturally occurring polyesters synthesized by different bacteria when cultured under a broad variety of nutritional and environmental conditions (6, 12, 13, 18, 24, 31). PHAs are accumulated intracellularly as reserve materials and begin to be mobilized when the carbon or the energy source in the culture broth is exhausted (6, 8, 12, 13, 33). These polymers are of considerable industrial interest owing to the fact that their physicochemical properties resemble those of traditional plastics (32). However, PHAs offer certain advantages over plastics of fossil origin since they are biodegradable and biocompatible, making them very interesting from a biotechnological point of view (12). The genes encoding the PHA metabolic pathway in *P. putida* U are included in a cluster (*pha*) integrated by two different operons. The first (*phaC1ZC2D*) contains four genes encoding two polymerases (PhaC1 and PhaC2), a depolymerase (PhaZ), and a different protein (PhaD), one whose function is unknown but that seems to be required for PHA formation (12, 31, 32, 33). The second operon (*phaFI*) is located downstream from *phaC1ZC2D* and is divergently translated. These genes encode two proteins (PhaF and PhaI) that seem to be involved in the formation and stabilization of PHA inclusions as well as in the regulation of *phaC1ZC2D* gene expression (12, 33). The considerable biotechnological interest in PHAs prompted us to clone the genes encoding the entire pathway into a plasmid, since their expression in different selected microbes could circumvent the biosynthetic, regulatory, or physiological limitations observed in the original strain, thereby contributing to the synthesis of new bioplastics with different monomer compositions, physicochemical properties, characteristics, and biotechnological applications.

The genes encoding the entire PHA pathway (*pha* clusters) were cloned into plasmid pK18:*mob* (pK*pha*) by following the strategy illustrated in Fig. 3. Sequence analysis of this plasmid confirmed that it contained a complete and faithfully reproduced copy of the chromosomal clusters involved in the synthesis, storage, and catabolism of PHAs. Furthermore, the transformation of a *P. putida* U Δ *pha* mutant (a strain in which the complete *pha* gene clusters had been deleted) (Fig. 4a to d) with the pK*pha* plasmid revealed that the genetic information cloned in this plasmid restored the ability to accumulate and catabolize aromatic and aliphatic PHAs (Fig. 4e to h). These findings are very important, not only because they include the first description of the cloning of the entire Pha pathway into a plasmid, but also because of their important biotechnological repercussions. Thus, by use of this construction or one derived from it, the Pha pathway could be transferred to many other organisms, including bacteria, yeasts, or higher plants, thus helping to increase the production yield of a given polymer or to obtain different plastics with different physicochemical properties and characteristics and, hence, broadening the potential biotechnological applications.

In sum, the results reported here describe a very simple strategy that can be efficiently used for cloning many other chromosomal clustered genes that, like the Paa or Pha path-

ways, are of academic, medical, environmental, or biotechnological interest. Thus, the usefulness of this procedure for recovering chromosomal gene-carrying routes, organized in a single operon, in several consecutive operons, or in different operons located close to one another, is evident.

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All experiments reported in this paper comply with present Spanish legislation.

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