Bi-functionality of the PhaF protein of Pseudomonas putida in the polyhydroxyalkanoate production process

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Bi-functionality of the PhaF protein of Pseudomonas putida in the polyhydroxyalkanoate production process

A dissertation submitted to the
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presented by
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2005
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Summary

Polyhydroxyalkanoates (PHAs) are bacterial polyesters accumulated as discrete granules and used as a storage material for carbon and reducing equivalents. In *Pseudomonas putida* GPo1, PHAs are synthesized when the carbon source is present in excess and another nutrient such as nitrogen is limiting growth. The recovery of PHA from bacterial cells yields a material with properties similar to common plastics. These properties depend among others on the carbon source used for synthesis. Due to their bacterial origin, PHAs are degraded by a wide range of microorganisms. Typical degradation rates for a piece of polyhydroxybutyrate range from a few months in anaerobic sewage to several years in seawater.

The *pha* gene cluster is responsible for the accumulation of polyhydroxyalkanoates in *Pseudomonas putida* GPo1. It includes genes coding for the two polymerases PhaC1 and PhaC2, for the PhaZ depolymerase, as well as for the PhaD regulatory protein and the PhaF and PhaI phasins. All these proteins, with the exception of PhaD, are found on the PHA granule surface. Furthermore, evidence of the existence of promoters upstream of the *phaC1*, *phaF* and *phaI* genes was found.

The discovery of the PhaF and PhaI phasins being recent, not much is know about these proteins. The granule-bound PhaF phasin consists of two domains, one of them being homologous to histone H1-like proteins and thus potentially able to bind DNA. To investigate this possibility, experiments were carried out using PHA granules harboring PhaF on their surface and free phasins released from PHA granules. These assays were carried out using DIG and radioactively labeled DNA. While no DNA binding could be observed with free PhaF phasins, it was shown that native PHA granules, harboring PhaF phasins, bind any DNA fragment in the presence of magnesium, regardless of its size, sequence or origin, thereby supporting a model in which PhaF is able to bind DNA. Calculations showed that the ratio of base pairs per PhaF phasin molecule varies from 0.5 to 53.1, presumably depending on the amount of active PhaF on the PHA granule surface.

To better understand the role of the PhaF and PhaI phasins, knockouts were generated. In the *phaF* knockout, no changes regarding the PHA granule size or number could be identified, whereas in the *phaI* knockout, a decreased PHA accumulation was observed. This phenotype resembles that obtained with the *phaD* knockout, which is missing PhaI on the
surface of the PHA granules. Additionally, in the phaI knockout, the PhaF phasin was not visible on the PHA granule surface.

Before studying the influence of the PhaF and PhaI phasins and that of the PhaD regulatory proteins on the promoters of the pha gene cluster, the existence of the Pc2 promoter upstream of the PhaC2 polymerase gene was established. Furthermore, the Pc1 promoter located upstream of the PhaC1 polymerase gene was better characterized. A shortened version of the Pc1 promoter used in previous experiments was created by removing a fragment of DNA where no significant secondary structure and no recognition site could be identified. Comparing the activities of the original and the shortened version showed a four fold lower activity for the shorter promoter.

Study of the pha promoters activity in P. putida GPo1 and the phaD, phaF and phaI knockouts in various media using β-galactosidase reporters showed that PhaF requires the presence of PhaD, PhaF and PhaI to be active at a high level. Furthermore, PhaI is a strong repressor for Pc1, Pc2 and Pi and an activator for Pf, while PhaD is an activator for both phasins.
Résumé

Les polyhydroxyalkanoates (PHAs) sont des polyesters bactériens accumulés sous forme de granules et utilisés comme moyen de stockage pour le carbone et les équivalents de réduction. Dans Pseudomonas putida GPo1, les PHAs sont synthétisés lorsque la source de carbone est présente en excès et qu’un autre élément nutritif tel que l’azote limite la croissance. La récupération du PHA à partir des cellules bactériennes produit une matière dont les propriétés sont similaires aux plastiques communs. Ces propriétés dépendent entre autre de la source de carbone utilisée au cours de la synthèse. En raison de leurs origines bactériennes, les PHAs sont dégradés par une vaste gamme de microorganismes. Les taux de dégradation habituels pour un morceau de polyhydroxybutyrate varie de quelques mois dans un milieu anaérobie à plusieurs années dans l’eau de mer.

Le groupe de gènes pha est responsable de l’accumulation de polyhydroxyalkanoates dans Pseudomonas putida GPo1. Il comprend les gènes codant les deux polyméras PhaC1 et PhaC2, la dépolymerase PhaZ, ainsi que la protéine régulatrice PhaD et les phasines PhaF et PhaI. Toutes ces protéines, à l’exception de PhaD, sont présentes à la surface des granules de PHA. De plus, des preuves de l’existence de promoteurs en amont des gènes phaC1, phaF et phaI ont été trouvées.

La découverte des phasines PhaF et PhaI étant récente, peu de choses sont connues à leur sujet. La phasine PhaF, qui est attachée aux granules, consiste en deux domaines, l’un d’eux étant homologue aux protéines de la famille de l’histone H1 et donc potentiellement capable de se lier à l’ADN. Afin d’examiner cette possibilité, des expériences ont été faites avec des granules présentant des phasines PhaF à leur surface ainsi qu’avec des phasines libres provenant de celles-ci. Ces expériences ont été faites en utilisant de l’ADN marqué à la digoxygénine ou radioactivement. Alors qu’aucun attachement à de l’ADN n’a pu être observé avec des phasines PhaF libres, il a été démontré que des granules de PHA présentant PhaF lient n’importe quel fragment d’ADN en présence de magnésium, indépendamment de sa taille, de sa séquence ou de son origine, soutenant ainsi un modèle selon lequel PhaF est capable de lier l’ADN. Des calculs ont montré que le nombre de paires de bases par molécule de phasine PhaF varie de 0.5 à 53.1, probablement en fonction de la quantité de PhaF actives à la surface des granules de PHA.
Afin de mieux comprendre le rôle des phasines PhaF et PhaI, des mutants ont été générés. Dans le mutant dépourvu de \textit{phaF}, aucun changement concernant la taille ou la quantité des granules n’a été identifié, alors que dans le mutant dépourvu de \textit{phaI}, une diminution de l’accumulation de PHA a été observée. Ce phénotype ressemble à celui obtenu avec le mutant dépourvu de \textit{phaD}, à qui il manque PhaI à la surface des granules. De plus, dans le mutant dépourvu de \textit{phaI}, la phasine PhaF n’était pas visible à la surface des granules de PHA.

Avant d’étudier l’influence des phasines PhaF et PhaI et celle de la protéine régulatrice PhaD sur les promoteurs du groupe de gènes \textit{pha}, l’existence d’un promoteur en amont du gène de la polymerase PhaC2 à été établie. De plus, le promoteur Pc1 situé en amont du gène de la polymerase PhaC1 a été mieux caractérisé. Une version courte du promoteur Pc1 utilisé dans des expériences antérieures a été créée en ôtant un fragment d’ADN dans lequel aucune structure secondaire significative et aucun site de reconnaissance n’ont pu être identifiés. La comparaison des activités résultant de l’expression de la version originale et raccourcie ont montré une activité quatre fois inférieure pour le promoteur raccourci.

L’étude de l’activité des promoteurs du groupe \textit{pha} dans \textit{P. putida} GPo1 et les mutants dépourvus de \textit{phaD}, \textit{phaF} et \textit{phaI} dans différents milieux à l’aide de rapporteurs utilisant la β-galactosidase a montré que PhaF nécessite la présence de PhaD, PhaF et PhaI pour être actif à un niveau élevé. De plus, PhaI est un fort répresseur pour Pc1, Pc2 et Pi et un activateur pour Pf, alors que PhaD est un activateur pour les deux phasines.
Chapter 1

Introduction
The annual world production of synthetic polymers amounts to about 140 million tons. A vast majority of this volume is composed of chemically stable polymers that are not easily degraded (Shimao, 2001). These are mostly synthetic polymers produced essentially by chemical addition or condensation reactions in which a large number of monomers are joined sequentially. The presence of stabilizers in widely used plastics such as nylon, polyethylene (PE), polyethylene terephthalate (PET) or polyvinyl chloride (PVC) is partially responsible for their poor biodegradability. Consequently, interest in the degradation process of several polymers and in the use of environmental-friendly alternatives has increased.

1.1. Biopolymers

Polymers generated from renewable natural sources are known as biopolymers. They are often biodegradable and can be synthesized in vivo by organisms (i.e. polyhydroxyalkanoates, alginate, carrageenan, polyisoprene), in vitro using isolated enzymes (i.e. acrylamide), or chemically from biological starting materials such as sugars, starch or natural fats and oils (i.e. polylactic acid).

Polylactic acid is a bio-based alternative to chemical plastics used in clinical devices because of its high mechanical strength, because it can be resorbed by animal and human bodies, and because it is non-toxic before and during biodegradation. Here, non-enzymatic hydrolysis is thought to be responsible for the degradation of PLA. In the environment, only a few microorganisms showing PLA-degradation activity have been identified even though proteins such as proteinase K, pronase or bromelain can degrade PLA in vitro (Shimao, 2001). In aerobic or anaerobic environments, degradation of PLA is a two-step process. In the first phase, low molecular weight chains are formed by non-enzymatic hydrolysis of the high molecular weight polyester. Below a molecular weight of 40'000, the polymer is degraded to carbon dioxide, water and humus by microorganisms (Drumright et al., 2000). Polylactic acid is obtained by polymerization of lactic acid monomers resulting from the fermentation of corn dextrose. During the polymerization process, D-, L- and meso-lactides are formed by condensation of two lactic acid molecules (Drumright et al., 2000). The architecture and molecular mass of the PLA polymer determine properties such as melting point, rate of crystallization or crystallinity. Incorporation of D- or meso-lactide in PLA made from pure L-lactide results in a decrease of those three parameters, but has little effect on the glass
transition temperature. At a content of L-lactide lower than 85%, PLA is amorphous (Drumright *et al.*, 2000).

Polyhydroxyalkanoates (PHAs) are another class of polymer synthesized by many gram-positive and gram-negative bacteria. PHAs are accumulated as discrete granules and are used as a storage material for carbon and reducing equivalents. The amount of polymer accumulated by bacteria can reach levels as high as 90% of their cell dry weight (*Madison et al.*, 1999). PHAs are synthesized when the carbon source is present in excess and another nutrient such as nitrogen, sulphur, phosphate, iron, magnesium, potassium or oxygen is limiting growth (*Schlegel et al.*, 1961). Polymerization of soluble carbon monomers into an insoluble polymer allows the bacteria to store valuable compounds without altering their osmotic state (*Madison et al.*, 1999). The recovery of PHA from bacterial cells yields a material with properties similar to common plastics. Due to their bacterial origin, PHAs are degraded by a wide range of microorganisms that secrete PHA hydrolases and PHA depolymerases (*Jendrossek et al.*, 1996). However, typical degradation rates for a piece of polyhydroxybutyrate range from a few months in anaerobic sewage to several years in seawater (*Jendrossek et al.*, 1996; *Mergaert et al.*, 1993; *Mergaert et al.*, 1995).

The various available PHAs have several potential applications (table 1). Their use as bulk biodegradable bioplastics to replace petrochemical polymers is unlikely due to their cost, but they could serve in diverse areas where the distinctive properties of each polymer are more important. Several patents have been granted concerning the application of specific PHAs. These patents include use for packaging and coating such as bottles or cosmetics containers (*Baptist*, 1963; *Baptist et al.*, 1963; *Webb*, 1990), diaper backsheet (*Martini et al.*, 1989a; 1989b), nonwoven fabrics (*Steel et al.*, 1986), hot-melt adhesives (*Kaufman et al.*, 1992), pressure-sensitive adhesive formulations (*Rutherford et al.*, 1997), replacement of petrochemical polymers in toner and developer compositions (*Fuller et al.*, 1991), ion-conducting polymers (*Reusch et al.*, 1993; 1996) or latex for paper-coating applications (*Marchessault et al.*, 1995). PHAs could even be used to produce dairy cream substitutes (*Yalpani*, 1993a) or flavor delivery agents in foods (*Yalpani*, 1993b). Other potential industrial applications of PHAs include their use as biodegradable carrier for long-term dosage of fertilizers, herbicides and other chemical compounds. Such large-scale applications would clearly require low cost PHA production.

Another interesting field for the application of PHA is the medical area. Potential products in this field include surgical devices such as pins, staples or swabs, blood vessel and
bone replacements or biodegradable carriers for long-term dosage of drugs and medicines. Furthermore, PHAs or PHA-derivatives could be used as a matrix to give a specific structure to synthetically grown tissues.

PHAs can not only be used as polymers, but also as a source of enantiomerically pure monomers. PHAs can easily be hydrolyzed chemically (de Roo et al., 2002) and the resulting β-hydroxy acids can be transformed into a wide range of commercially attractive molecules such as 2-alkenoic acids, β-hydroxyalkanols, β-acyllactones, β-amino acids and β-hydroxyacid esters (Williams et al., 1996; Witholt et al., 1992; 1994).

<table>
<thead>
<tr>
<th>Industry</th>
<th>Packaging and coating</th>
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<td>Diaper backsheet</td>
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<td>Nonwoven fabrics</td>
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<td>Hot-melt or pressure-sensitive adhesives</td>
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<td>Petrochemical polymer replacement in toners and developers</td>
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<td>Delivery of fertilizers, herbicides or insecticides</td>
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<th>Medicine</th>
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<td>Tissue matrices</td>
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| Chemistry | Chiral monomer source |

Table 1: Possible use of PHAs in the industrial, medical and chemical fields.

1.2. Polyhydroxyalkanoates

Polyhydroxyalkanoates are linear polyesters composed of hydroxy fatty acid monomers in which the carboxy group and the hydroxy group of two different monomers form an ester bond (Madison et al., 1999). Except for a few special cases where no chiral center is present, the hydroxy-substituted carbon is always of the $R$ configuration. The side chain found at the chiral center can vary from methyl to tridecyl and does not have to be a saturated alkyl chain. Reports have shown that unsaturated (Fritzsche et al., 1990; Lageveen et al., 1988), halogenated (Abe et al., 1990; Doi et al., 1990; Kim et al., 1992), aromatic
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(Curley et al., 1996; Kim et al., 1991; Song et al., 1996) and branched monomers (Choi et al., 1994; Hazer et al., 1994) can be incorporated in PHAs (Madison et al., 1999). Furthermore, variation of the position of the hydroxy group is also possible and accumulation of polymers containing 4- (Kunioka et al., 1989; Valentin et al., 1992; 1996), 5- (Valentin et al., 1996) and 6- (Madison et al., 1999) hydroxy acids have been reported (Eggink et al., 1995) (figure 1).

Figure 1: Versatility of the chemical structure of accumulated polyhydroxyalkanoates.

(A) variation of the position of the hydroxy group. ① 3-hydroxyalkanoate, ② 4-hydroxyalkanoate, ③ 5-hydroxyalkanoate, ④ 6-hydroxyalkanoate.

(B) example of the diversity of the accumulated side chains. ① saturated side chain, ② unsaturated side chain, ③ halogenated side chain, ④ aromatic side chain, ⑤ branched side chain.

PHAs are classified in two groups, according to their side-chain length. The first group, called short chain length PHAs (scl-PHAs), contains polymers having side-chains of
one or two carbon units. Polymers with longer side-chains form the medium chain length PHAs (mcl-PHAs). A vast majority of the microorganisms accumulating PHAs synthesize either scl-PHAs containing 3-hydroxybutyrate or mcl-PHAs containing 3-hydroxyoctanoate and 3-hydroxydecanoate monomers (Anderson et al., 1990; Lee, 1996; Steinbüchel, 1991; Steinbüchel et al., 1991).

In the late 1920s, the molecular weights of aliphatic polyesters obtained in a classical condensation polymerization of diacids and diols never exceeded 20'000 to 30'000 Da. Industries had at that time a limited interest for these aliphatic polyesters due to their low molecular weights, and preferred working with aromatic polyesters such as polyethylene terephthalate (PET) (Marchessault, 1996). Nowadays however, PHAs with molecular masses varying from 50'000 to 1'000'000 Da can be produced depending on the carbon source and the bacterial strain used (Madison et al., 1999). These high molecular weight chiral aliphatic polyesters were hard to produce chemically, and their recent availability from biological sources has raised a new interest in their study and utilization.

1.2.1. scl-PHAs

scl-PHAs contain 3-hydroxybutyrate, 3-hydroxyvalerate or a mix of both monomers, and are named poly-3-hydroxybutyrate (P(3HB)), poly-3-hydroxyvalerate (P(3HV)) and poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P(3HB-3HV)) respectively. 4-hydroxybutyrate can also be incorporated, yielding poly-4-hydroxybutyrate (P(4HB)) (Madison et al., 1999).

Inside the bacterium, P(3HB) is in a fluid, amorphous state. However, extraction with organic solvents yields a highly crystalline material, which is stiff but brittle (Doi, 1995). It is this brittleness of the recovered P(3HB) that is responsible for its lack of stress resistance. Furthermore, P(3HB) has a high melting temperature, around 170°C, which is near the temperature at which the polymer degrades thermally, thus limiting the potential use of the homopolymer (Madison et al., 1999).

The P(3HB-3HV) copolymer obtained by incorporating 3-hydroxyvalerate monomers into P(3HB) is less stiff and brittle and can be used in different applications. This copolymer has excellent water and gas barrier properties and can be processed at a lower temperature than P(3HB) while retaining most of its mechanical properties.
Due to its early discovery in 1926 by Lemoigne (Lemoigne, 1926), P(3HB) is the most thoroughly characterized biopolymer. Biochemical investigations of the enzymes involved in its accumulation have focused on only two of the natural producers, Zoogloea ramigera and Ralstonia eutropha (Madison et al., 1999). In Ralstonia eutropha, the biosynthetic pathway of P(3HB) includes three enzymatic reactions: the condensation of two acetyl-CoA molecules into acetoacetyl-CoA, the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA and the polymerisation of the (R)-3-hydroxybutyryl-CoA into poly(3-hydroxybutyrate) (figure 2). These steps are carried out by three enzymes: a β-ketoacyl-CoA thiolase (encoded by phbA), an NADPH-dependent acetoacyl-CoA dehydrogenase (encoded by phbB) and a P(3HB) polymerase (encoded by phbC) respectively.

![Figure 2: Ralstonia eutropha scl-PHA biosynthetic pathway.](image)

1.2.2. mcl-PHAs

mcl-PHAs were discovered in the late 1970s, when a material forming mushroom-like structures in freeze-fracture electron micrographs of Pseudomonas oleovorans was found. This structures were clearly different from the spikes isolated formed by poly-3-hydroxybutyrate (de Smet et al., 1983). Unlike scl-PHAs, mcl-PHAs have low levels of
crystallinity and are more elastic (Gross et al., 1989; Preusting et al., 1990). These properties depend on the side-chain length of the incorporated monomer. Furthermore, unlike PHB, PHAs are composed of different monomer units, giving different properties to the polymer (Lageveen et al., 1988; Williams et al., 1996). All PHAs known up to now are made of monomers containing the $R$-configuration at their chiral center (Brandl et al., 1990). The mechanical and physical properties of PHAs such as stiffness, brittleness, melting point, glass transition temperature or resistance to organic solvents can change considerably with the monomer composition (Bluhm et al., 1986; Gross et al., 1989; Preusting et al., 1990). The two most important factors influencing the monomer composition of PHAs are the bacterial strain that produces the polymer and the carbon source available to the bacterium. Indeed, the species and strain used determines the substrate range of the PHA polymerase as well as the peripheral metabolism responsible for supplying substrate for the polymerization from the provided carbon source (Steinbüchel, 1996). Presently, more than 90 different hydroxyalkanoic acids have been detected as constituents of biosynthetic poly(3-hydroxyalkanoate) (Steinbüchel, 1996).

The biosynthesis of PHA can be divided in three different stages: the uptake of the carbon source, the conversion of this source to a PHA precursor and finally the synthesis of the polymer (Steinbüchel, 1996). mcl-PHAs are synthesized via either the $\beta$-oxidation or the fatty acid synthesis pathways. In the first case, fatty acids or other aliphatic carbon sources are taken up, the fatty acid degradation pathway successively removes one acetyl-CoA unit to the substrate, and the resulting PHA composition depends on the carbon source (Brandl et al., 1988; Huisman et al., 1989; Lageveen et al., 1988). In the second case, no relationship between the carbohydrates used as carbon sources and the resulting PHA composition exists (Haywood et al., 1990; Huijberts et al., 1992; Timm et al., 1990).

1.2.2.1. Biosynthesis via the $\beta$-oxidation pathway

The transport of fatty acids into the cell relies on at least two proteins encoded by the $fadD$ and $fadL$ genes. The $fadD$ gene product, acyl-CoA synthase, appears to be required for uptake of both medium and long chain fatty acids, while the $fadL$ gene product, FLP, is only essential for the uptake of long chain fatty acids. In the absence of FLP, medium chain fatty acids diffuse across the cell membrane to the acyl-CoA synthase, where they are coupled with coenzyme A and released into the cell. The fatty acids are degraded in the $\beta$-oxidation cycle
which requires enzymes encoded by the *fadA, fadB, fadE, fadF, fadG* and *fadH* genes (figure 3). The fatty acid degradation pathway is regulated by the protein encoded by the *fadR* gene (Nunn, 1986). Fatty acids activated to coenzyme A thioesters are successively converted to 2-trans-enoyl-CoA, S-3-hydroxyacyl-CoA and 3-ketoacyl-CoA. Finally, acetyl-CoA is released and a fatty acid shorter by one C2 unit is formed. Possible precursors of PHA that could be channeled from the β-oxidation pathway to the PHA polymerase are 3-ketoacyl-CoA, S-3-hydroxyacyl-CoA and enoyl-CoA (van der Leij et al., 1995).

In *E. coli*, the reduction of 3-ketoacyl-CoA to R-3-hydroxyacyl-CoA has been shown using the 3-ketoacyl reductase encoded by the *fabG* genes of *E. coli* (Taguchi et al., 1999) and *P. aeruginosa* (Ren et al., 2000). In the *E. coli* JMU194 *fadA* strain (deficient in the 3-ketoacyl-CoA thiolase) equipped with a PHA synthase, the *P. aeruginosa* FabG increases the amount of PHA accumulated using hexadecanoate as carbon source from 14% to 21% of the cell dry weight without changing the monomer composition (Ren et al., 2000). In the similar strain *E. coli* WA101 however, FabG shifts the monomer composition from 15% 3-hydroxyhexanoate, 36% 3-hydroxyoctanoate and 49% 3-hydroxydecanoate to 7% 3-hydroxyoctanoate and 93% 3-hydroxydecanoate without changing the amount of accumulated PHA when using decanoate as carbon source (Park et al., 2002). Furthermore, the RhlG 3-ketoacyl reductase from *P. aeruginosa*, which plays a role in rhamnolipid synthesis, also appears to be involved in PHA synthesis, probably by converting 3-ketoacyl esters to 3-hydroxyacyl esters (Campos-García et al., 1998). Coexpression of *rhlG* and the PHA synthase *phaC2* of *Pseudomonas* sp. 61-3 in a *fadA*-negative *E. coli* strain increases the amount of accumulated PHA from 21% to 33% of the cell dry weight without significantly changing the monomer composition (Park et al., 2002).

Pramanik et al. showed that the *E. coli* multienzyme complex encoded by *fadB* is involved in five different enzymatic activities, including the epimerisation of S-3-hydroxyacyl-CoA to R-3-hydroxyacyl-CoA (Pramanik et al., 1979), making S-3-hydroxyacyl-CoA a possible precursor for PHA biosynthesis. Indeed, 3-ketoacyl-CoA was reduced to R-3-hydroxyacyl-CoA by a 3-ketoacyl-CoA reductase activity, S-3-hydroxyacyl-CoA was epimerized to R-3-hydroxyacyl-CoA by a 3-hydroxyacyl-CoA epimerase activity, and enoyl-CoA was transformed to R-3-hydroxyacyl-CoA by enoyl-CoA hydratase II activity.

The conversion of enoyl-CoA to R-3-hydroxyacyl-CoA has been shown in *E. coli* using *(R)-specific enoyl-CoA hydratases from Aeromonas caviae* and *P. aeruginosa* (Fukui et al., 1998; Tsuge et al., 1999). In *A. caviae*, an enoyl-CoA hydratase, encoded by *phaJ* and
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showing \((R)\)-specific hydratase activity towards 2-\textit{trans}-enoyl-CoA with four to six carbon atoms, was identified (Fukui \textit{et al.}, 1998). In \textit{P. aeruginosa}, however, two enoyl-CoA hydratases, encoded by \textit{phaJ1} and \textit{phaJ2}, were found. These two enzymes exhibit high \((R)\)-specific enoyl-CoA hydratase activities and different substrate specificities. The hydratase encoded by \textit{phaJ1} favors short-chain length enoyl-CoA while the one encoded by \textit{phaJ2} prefers medium-chain length enoyl-CoA (Tsuge \textit{et al.}, 1999).

\[ \text{R-SCoA} \rightarrow \text{HSCoA, ATP} \]

\[ \text{AMP+PPI, } \text{H}_2 \text{O} \]

\[ \text{Fatty acids} \]

\[ \text{FadD} \]

\[ \text{Acetyl-CoA} \rightarrow \text{Acyl-CoA} \]

\[ \text{CoA} \rightarrow \text{HSCoA} \]

\[ \text{FadA} \]

\[ \text{FadE} \]

\[ \text{FAD} \rightarrow \text{FADH}_2 \]

\[ \text{R-SCoA} \rightarrow \text{R-2-trans-enoyl-CoA} \]

\[ \text{PhaJ} \]

\[ \text{PhaC} \]

\[ \text{Poly(3-Hydroxyalkanoate)} \]

\[ \text{FabG} \]

\[ \text{FadB} \]

\[ \text{FadA} \]

\[ \text{FadE} \]

\[ \text{FadD} \]

\[ \text{FadE} \]

\[ \text{FadA} \]

\[ \text{PhaC} \]

\[ \text{HSCoA} \]

\[ \text{FadB} \]

\[ \text{PhaJ} \]

\[ \text{R-3-hydroxyacyl-CoA} \]

\[ \text{S-3-hydroxyacyl-CoA} \]

\[ \text{R-3-hydroxyacyl-CoA} \]

\[ \text{3-ketoacyl-CoA} \]

\[ \text{Acetyl-CoA} \rightarrow \text{Acyl-CoA} \]

\[ \text{CoA} \rightarrow \text{HSCoA} \]

\[ \text{FadA} \]

\[ \text{FadE} \]

\[ \text{FAD} \rightarrow \text{FADH}_2 \]

\[ \text{R-SCoA} \rightarrow \text{R-2-trans-enoyl-CoA} \]

\[ \text{PhaJ} \]

\[ \text{PhaC} \]

\[ \text{Poly(3-Hydroxyalkanoate)} \]

\[ \text{FadD} \]

\[ \text{FadE} \]

\[ \text{FadA} \]

\[ \text{PhaC} \]

\[ \text{Poly(3-Hydroxyalkanoate)} \]

\[ \text{FabG} \]

\[ \text{FadB} \]

\[ \text{PhaJ} \]

\[ \text{R-3-hydroxyacyl-CoA} \]

\[ \text{S-3-hydroxyacyl-CoA} \]

\[ \text{R-3-hydroxyacyl-CoA} \]

\[ \text{3-ketoacyl-CoA} \]

\[ \text{Acetyl-CoA} \rightarrow \text{Acyl-CoA} \]

\[ \text{CoA} \rightarrow \text{HSCoA} \]

\[ \text{FadA} \]

\[ \text{FadE} \]

\[ \text{FAD} \rightarrow \text{FADH}_2 \]

\[ \text{R-SCoA} \rightarrow \text{R-2-trans-enoyl-CoA} \]

\[ \text{PhaJ} \]

\[ \text{PhaC} \]

\[ \text{Poly(3-Hydroxyalkanoate)} \]

\[ \text{FadD} \]

\[ \text{FadE} \]

\[ \text{FadA} \]

\[ \text{PhaC} \]

\[ \text{Poly(3-Hydroxyalkanoate)} \]

\[ \text{FabG} \]

\[ \text{FadB} \]

\[ \text{PhaJ} \]

\[ \text{R-3-hydroxyacyl-CoA} \]

\[ \text{S-3-hydroxyacyl-CoA} \]

\[ \text{R-3-hydroxyacyl-CoA} \]

\[ \text{3-ketoacyl-CoA} \]

\[ \text{Acetyl-CoA} \rightarrow \text{Acyl-CoA} \]

\[ \text{CoA} \rightarrow \text{HSCoA} \]

\[ \text{FadA} \]

\[ \text{FadE} \]

\[ \text{FAD} \rightarrow \text{FADH}_2 \]

\[ \text{R-SCoA} \rightarrow \text{R-2-trans-enoyl-CoA} \]

\[ \text{PhaJ} \]

\[ \text{PhaC} \]

\[ \text{Poly(3-Hydroxyalkanoate)} \]

\[ \text{FadD} \]

\[ \text{FadE} \]

\[ \text{FadA} \]

\[ \text{PhaC} \]

\[ \text{Poly(3-Hydroxyalkanoate)} \]

\[ \text{FabG} \]

\[ \text{FadB} \]

\[ \text{PhaJ} \]

\[ \text{R-3-hydroxyacyl-CoA} \]

\[ \text{S-3-hydroxyacyl-CoA} \]

\[ \text{R-3-hydroxyacyl-CoA} \]

\[ \text{3-ketoacyl-CoA} \]

\[ \text{Acetyl-CoA} \rightarrow \text{Acyl-CoA} \]

\[ \text{CoA} \rightarrow \text{HSCoA} \]

\[ \text{FadA} \]

\[ \text{FadE} \]

\[ \text{FAD} \rightarrow \text{FADH}_2 \]

\[ \text{R-SCoA} \rightarrow \text{R-2-trans-enoyl-CoA} \]

\[ \text{PhaJ} \]

\[ \text{PhaC} \]

\[ \text{Poly(3-Hydroxyalkanoate)} \]
1.2.2.2. Biosynthesis via the fatty acid synthesis pathway

The fatty acid synthesis route is a source of monomers for the accumulation of PHA from structurally unrelated substrates such as glucose or gluconate (Haywood et al., 1990; Huijberts et al., 1994; Huijberts et al., 1992; Timm et al., 1990).

![Diagram of fatty acid synthesis pathway]

**Figure 4: Pathway for the biosynthesis of mcl-PHA from carbohydrates in fluorescent pseudomonads.**

1.3-ketoacyl-ACP synthase, 2. 3-ketoacyl-ACP reductase, 3. 3-hydroxyacyl-ACP dehydratase, 4. enoyl-ACP reductase, 5. acyl-ACP thiolase, 6. R-3-hydroxyacyl-ACP-CoA transacylase, 7. P(3HA) polymerase.
In this pathway, the carbon source is first converted to acetyl-CoA and then carboxylated to malonyl-CoA. A malonyl transacylase links the malonyl-CoA to an acyl carrier protein (ACP), releasing coenzyme A. Malonyl-ACP is further transformed to 3-ketoacyl-ACP, R-3-hydroxyacyl-ACP, enoyl-ACP and finally acyl-ACP. The latter compound is then coupled to a malonyl-ACP to yield a new 3-ketoacyl-ACP molecule. In this pathway, R-3-hydroxyacyl-ACP is a putative precursor for the synthesis of PHA.

When grown on sugars, fluorescent pseudomonads of rRNA homology group I accumulate a polymer consisting mainly of C\textsubscript{10} and C\textsubscript{8} monomer units, which are apparently derived from intermediates of the fatty acid biosynthesis (figure 4) (Huijberts et al., 1994; Huijberts et al., 1992; Timm et al., 1990). In several \textit{Pseudomonas} strains, it has indeed been shown that the transacylase \textit{PhaG} catalyzes the reversible transfer of the acyl group from (\textit{R})-3-hydroxyacyl-ACP to (\textit{R})-3-hydroxyacyl-CoA. This activity was first described for \textit{P. putida} KT2440 with \textit{in vitro} synthesized 3-hydroxydecanoyl-CoA and the acyl carrier protein (Rehm et al., 1998). Subsequently, the involvement of \textit{phaG} in the biosynthesis of polyhydroxyalkanoic acids has been shown in several \textit{Pseudomonas} strains including \textit{P. aeruginosa} (Hoffmann et al., 2000b), \textit{P. oleovorans} (Hoffmann et al., 2000a), \textit{Pseudomonas} sp.61-3 (Matsumoto et al., 2001) and \textit{P. fragi} (Fiedler et al., 2000).

1.3. Phasins

Phasins are defined as proteins that bind to the surface of the polyhydroxyalkanoate granules, and have been shown to play various roles in PHA synthesis and degradation. Synthases and depolymerases have been studied for more than a decade because of their key roles in the accumulation or degradation of PHAs. Other phasins, especially the so-called granule-associated proteins (GAPs), have only recently gained attention, because they were found much later, and appear to have a role in the regulation of the PHA accumulation process (Maehara et al., 2002; Prieto et al., 1999), have an influence on the granule size and number (Klinke et al., 2000; Maehara et al., 1999), or have an influence on the molecular weight of the synthesized PHA (Maehara et al., 1999). A better understanding of their involvement in the PHA accumulation process could be beneficial for large-scale production of PHA or implementation of PHA-producing plant systems.
1.3.1. Phasins associated with scl-PHAs

Three major classes of proteins are found bound on the PHB granule surface: the synthases, the depolymerases and the granule-associated proteins. Synthases bound to the granule surface have been identified in *R. eutropha* (Haywood et al., 1989) and *Chromatium vinosum* (Liebergessell et al., 1994). Depolymerases have been found on the surface of PHB granules in *R. eutropha* (Hippe et al., 1967) or *Rhodospirillum rubrum* (Griebel et al., 1971; Griebel et al., 1968). Many other proteins that are neither synthases nor depolymerases have been found associated to the surface of PHB granules.

PhaP is a 24 kDa protein that binds to P(3HB) granules and determines the size of the PHA granules in *R. eutropha*. Immunochemical analyses have shown that PhaP is always bound to the granule and that no free PhaP is present in the cytoplasm of the wild-type strain. Furthermore, it has been shown that the PhaP concentration is inversely related to the granule size, the lack of PhaP leading to the presence of only one large granule, while its overexpression results in many small granules (Wieczorek et al., 1995). According to York et al., the synthesis of PhaP in *R. eutropha* is triggered by either net PHB synthesis or intracellular PHB content higher than 50% of the cell dry-weight. Similarly, degradation of PhaP occurs when PHB is being utilized and a low amount of PHB is present in the cells (York et al., 2001). Similar proteins have been identified in several strains, for instance *Aeromonas caviae* PhaP (Fukui et al., 2001), *Chromatium vinosum* PhaP (Liebergessell et al., 1996) or *Paracoccus denitrificans* PhaP*$_Pd$* (Maehara et al., 1999), as well as the *Rhodococcus ruber* GA14 (Pieper-Fürst et al., 1994), the *Methylobacterium rhodesenium* GA11 and GA20 (Föllner et al., 1995), or the *Acinetobacter* GA13 (Schembri et al., 1995b).

In the *Rhodococcus ruber* GA14, two carboxy-terminal hydrophobic stretches have been identified that are essential for binding to the P(3HB) granules (Pieper-Fürst et al., 1995).

In *Paracoccus denitrificans*, the 16 kDa phasin PhaP*$_Pd$* gene is located downstream of the PhaC*$_Pd$* polymerase and is followed by a gene encoding the regulatory protein PhaR*$_Pd$* (Maehara et al., 2001; Maehara et al., 2002; Maehara et al., 1999). Coexpression of the PhaP*$_Pd$* protein and PHA biosynthesis genes in *E. coli* results in a higher number of PHB granules as well as a higher PHB content, but at the same time also in a decreased granule size and a lower molecular weight of PHB.
1.3.2. Phasins associated with mcl-PHAs

In *P. putida* GPo1, five proteins encoded by the *pha* gene cluster are found on the PHA granule surface: the two PHA polymerases PhaC1 and PhaC2, the PHA depolymerase PhaZ and the two granule-associated proteins PhaF and PhaI.

Even though the role of PhaI is not yet clear, its amino acid sequence shows a clear homology with the N-terminal domain of PhaF. As PhaI is bound to the granule, this domain is thought to be responsible for binding of PhaF to the PHA granule. Sequence analysis has revealed similarities between the C-terminal domain of PhaF and histone-H1-like proteins. In fact, this domain contains nine copies of the AAKP motif characteristic of this family of proteins (Prieto *et al.*, 1999), and may therefore bind DNA.

The absence of PhaF does not affect PHA content and granule formation under nitrogen-limited batch fermentations, but in nitrogen-limited continuous cultures, a threefold decrease in the PHA content is observed. Since in continuous cultures, cells are constantly dividing, it could be that PhaF plays a role in formation of new granules in recently divided cells (Prieto *et al.*, 1999).

1.4. Regulatory elements

PHA-producing microorganisms have evolved different pathways for PHA formation depending on their ecological niche. Studies have shown the importance of both the cellular physiology of the cell and the central metabolism in the PHA accumulation process. Regulation of the PHA accumulation process is quite complex and occurs at three different levels; first at the physiological level, by cofactor inhibition and metabolite availability, second at the genetic level, through alternative σ-factors, two-component regulatory systems and autoinducing molecules, and third at the protein level, through granule size and molecular weight control by PHA polymerase and other phasins (Madison *et al.*, 1999).

1.4.1. Regulation of scl-PHA accumulation

It has been known for three decades that P(3HB) synthesis is regulated at the enzymatic level (Senior *et al.*, 1973). Furthermore, it has been established that the
intracellular concentration of acetyl-CoA and free coenzyme A play a central role in the regulation of polymer synthesis (Haywood et al., 1988) and that high concentrations of NAD(P)H as well as high ratios of NAD(P)H/NAD(P) stimulate P(3HB) accumulation (Lee et al., 1995). Recently, it has also been shown that the monomer composition of scl-PHAs accumulated by *R. eutropha* is controlled by the PHB synthase, whereas the biosynthesis rate is controlled by both the 3-ketothiolase and the acetoacetyl-CoA reductase. Increasing the ratio between the PHB synthase activity and the 3-ketothiolase and the acetoacetyl-CoA reductase activities results in more numerous granules. Decreasing this ratio yields larger but fewer granules (Jung et al., 2000).

At the transcription level a promoter inducible by phosphate starvation (possibly under control of the *pho* regulon) has been identified upstream of the *phaB* gene in *Acinetobacter* sp. (Schembri et al., 1995a). Other possible promoters have been identified or postulated based on sequence analysis in various microorganisms (Huisman et al., 1991; Liebergesell et al., 1992; Matsusaki et al., 1998; McCool et al., 1999; Schembri et al., 1995a; Schubert et al., 1991; Timm et al., 1992), but until now not much is known about specific regulatory proteins involved in *phb* and *pha* gene expression.

The two genes *ntrB* and *ntrC*, which are part of the nitrogen regulation system of various bacteria, have recently been implicated in the control of P(3HB) biosynthesis by ammonia availability in *Azospirillum brasilense* SP7 (Sun et al., 2000).

In *Pseudomonas* sp. 61-3, the transcriptional regulator PhbR<sub>Ps</sub> was found. It has a high sequence identity to the AraC/XylS family of transcriptional activators (Matsusaki et al., 1998). This strain is able to accumulate at the same time a P(3HB) homopolymer and a random copolymer containing monomeric units ranging from four to twelve carbon atoms. The PhbR<sub>Ps</sub> protein is however only involved in the regulation of the genes required for the biosynthesis of the P(3HB) homopolymer.

A specific *pha* regulatory protein, PhaR<sub>Pd</sub>, has been identified immediately downstream of the *phaZCP* locus of *Paracoccus denitrificans*, and its involvement in the expression and regulation of granule associated proteins has been shown in *E. coli* (Maehara et al., 1999). The purified 22 kDa PhaR<sub>Pd</sub> protein, which probably forms a tetramer in its native state, was shown to specifically bind to the intergenic region of *phaC-phaP*. Furthermore, *in vitro* assays have shown that addition of purified PhaR<sub>Pd</sub> was able to repress expression of PhaP<sub>Pd</sub> in a cell-free protein synthesis system using *E. coli* S30 extract (Maehara et al., 2001). Using DNaseI footprinting, it was shown that PhaR<sub>Pd</sub> binds
specifically to two regions located upstream of \textit{phaP} and \textit{phaR}. This suggests that \textit{PhaR}_{\text{Pd}} plays a role not only in the regulation of \textit{phaP}, but also in its own regulation. Furthermore, it was established that \textit{PhaR}_{\text{Pd}} is able to bind to P(3HB) granules if those granules are not covered by \textit{PhaP}_{\text{Pd}} phasins, and that this interaction breaks up already existing \textit{PhaR}_{\text{Pd}}-DNA complexes (Maehara \textit{et al.}, 2002). Based on these results, Maehara \textit{et al.} propose a model in which \textit{PhaR}_{\text{Pd}} is synthesized at basal levels under non-PHA accumulating condition, and binds DNA upstream of \textit{phaP} and \textit{phaR}, preventing their expression (figure 5). Once PHA accumulation is initiated, oligomeric and polymeric forms of 3HB bind to \textit{PhaR}_{\text{Pd}}, releasing it from the DNA and thus initiating the expression of \textit{phaP}. When PHA synthesis is stopped or under PHA degrading conditions mature granules are present, which are covered by \textit{PhaP}_{\text{Pd}}. Released and newly produced \textit{PhaR}_{\text{Pd}} does not bind to the granule and therefore binds again to DNA, repressing the \textit{PhaP}_{\text{Pd}} expression (Maehara \textit{et al.}, 2002).

\textbf{Figure 5:} Hypothetical model of \textit{PhaR}-mediated \textit{phaP} expression in \textit{P. denitrificans} (Maehara \textit{et al.}, 2002) (reprinted with the permission of the American Society for Microbiology). \textit{Z}, \textit{C}, \textit{P} and \textit{R} indicate the names of \textit{pha} genes in \textit{P. denitrificans}. (A) Gene organization of the \textit{pha} locus and produced proteins. (B) Repression of the expression of \textit{phaP} and \textit{phaR} under non-PHA accumulation conditions. \textit{PhaR} that is produced at a basal level binds to both IRCP and IRPR, and therefore \textit{phaP} expression is repressed. (C) Derepression of expression of \textit{phaP} and \textit{phaR} under PHA accumulation conditions. Once PHA accumulation is initiated, \textit{PhaR} is released from DNA by the binding of \textit{PhaR} to both oligomeric and polymeric forms of 3HB, and then the \textit{phaP} expression is initiated at the onset of dissociation of \textit{PhaR} from the upstream element for \textit{phaP}. (D) Repression of the expression of \textit{phaP} and \textit{phaR} when PHA synthesis is stopped or under PHA degradation conditions. Since PHA granules accumulated are already covered with proteins (the predominant protein is \textit{PhaP}), \textit{PhaR} that is newly produced at a basal level or that is released from PHA granules by degradation of PHA binds to both IRCP and IRPR, and therefore \textit{phaP} expression is repressed by the binding of \textit{PhaR} to the upstream element of \textit{phaP}. 

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1.4.2. Regulation of mcl-PHA accumulation

In *P. aeruginosa* PAO1, RpoN, the $\sigma^{54}$ subunit of the RNA polymerase involved in growth phase dependent activation of certain non-housekeeping promoters, strictly controls the PHA accumulation pathway from gluconate, while the accumulation of PHA from fatty acids does not depend on RpoN (Timm *et al.*, 1992).

In *P. putida* KT2442, a two-component system showing homology to the LemA/GacA sensor kinase/response regulator couple may be involved in the regulation of PHA synthesis (Madison *et al.*, 1999). This protein couple is known for sensing environmental conditions and relaying the resulting signals to control the expression of various genes. Considering the role of PHA as carbon and reducing equivalent storage in specific environments, the involvement in the PHA accumulation process of a similar protein system controlling a set of genes based on growth conditions is not unlikely (Madison *et al.*, 1999).

In *P. putida* GPo1, the PhaF phasin plays a role in the regulation of the expression of the PhaC1 polymerase, the PhaI phasin and PhaF itself (Prieto *et al.*, 1999). When *P. putida* GPo1 is cultivated in medium containing citrate or glucose, PhaF represses the transcription of the *phaC1* and the *phaIF* mRNAs. Under these conditions, *phaF* is expressed from its own promoter. In the presence of octanoic acid, however, the expression of *phaC1*, *phaF* and *phaI* is induced and PHA granules are formed. Even though the C-terminal domain of PhaF shows similarities to histone H1-like proteins, which are known to bind DNA, a direct interaction of PhaF with promoter regions located upstream of the *phaC1* and *phaI* genes has not yet been shown. Based on the observation that the *phaF* transcript is always expressed, Prieto *et al.* propose a model where, in the presence of glucose or citrate, PhaF is free in the cytoplasm because PHA granules are not formed from these substrates. Therefore PhaF can bind to DNA and repress the expression of *phaC1* and *phaIF*. In the presence of octanoic acid, PhaF is bound to the PHA granule and expression of *phaC1* and *phaIF* is possible (figure 6) (Prieto *et al.*, 1999).
Figure 6: Hypothetical model for the regulation of pha genes in *P. putida* GPo1 (Prieto et al., 1999) (reprinted with the permission of the American Society for Microbiology). *C1, Z, C2, D, F* and *I* represent the names of *pha* genes. White arrows indicate the directions of transcription of the genes. The *phaC1, phaF* and *phaIF* transcripts are marked as thick black arrows. Discontinuous arrows denote mRNAs that have not been detected. The hatched circles bound to the granules denote PhaC2 and PhaZ proteins. (A) Repression of the transcription of the *phaC1* and the *phaIF* mRNAs when *P. putida* is cultured in medium containing citrate or glucose as the carbon source. Under these growth conditions *phaF* is transcribed. (B) Induction of the expression of *phaC1, phaI*, and *phaF* genes in the presence of octanoic acid and association with PHA granules.
Another protein involved in the PHA accumulation process in *P. putida* GPo1 is PhaD. PhaD is the only known protein expressed by the *pha* cluster that is not found on the PHA granule surface. Sequence analysis has not shown any similarities with PHA polymerases, PHA depolymerases, or other phasins (Klinke *et al.*, 2000). Knocking out the *phaD* gene results in a PHA production that is less than 20% of that in the wild-type. Furthermore, the size of the PHA granules decreases while their number increases. Interestingly, in a *phaD* negative mutant, PhaI is not found on the granule surface (Klinke *et al.*, 2000). Whether PhaD interacts at the protein level, enabling the binding of PhaI to the granule, or at the DNA level, activating the expression of the *phaI* gene is still unclear. Furthermore, it remains to be determined if PhaD interacts directly with *phaI* or its gene product, or if other proteins are involved. Another question raised by the experiment of Klinke et al. is whether the phenotype observed in the *phaD* negative mutant (PHA content and PHA granule size and amount) is due to the missing PhaD protein, or to the absence of PhaI on the PHA granule surface.

### 1.5. Aim and scope of this thesis

In spite of the enormous research effort carried out on PHAs, only little is known about the regulation and expression of the genes responsible for the accumulation of medium chain length polyhydroxyalkanoates. Previous work by Prieto *et al.* (Prieto *et al.*) has shown that the PhaF protein is involved in the regulation of the PHA accumulation process in *P. putida* GPo1. Furthermore, sequence similarity searches have highlighted two domains in this protein: the C-terminal domain resembles histone H1-like proteins, while the N-terminal domain is similar to another granule-associated protein, PhaI.

The aim of this work is first to investigate the potential DNA binding ability of PhaF derived from its C-terminal domain, and second to investigate the activity of the promoters present in the *pha* gene cluster.

The first two chapters describe the interaction of the PhaF protein with DNA.

Chapter 2 presents various attempts to demonstrate DNA binding by PhaF proteins when not attached to a PHA granule using different DNA labelling techniques as well as different PhaF purification methods.
In chapter 3, the binding of different DNA fragments by native PHA granules harboring PhaF on their surface was demonstrated using PHA granules isolated from *P. putida* GPo1 and its *phaF*-negative mutant *P. putida* GPG-Tc6.

The next chapter describes the expression profiles observed in both wild-type strains and mutants. In chapter 4, the effects of carbon source and medium on the activity levels of promoters from the *pha* gene cluster were investigated using LacZ fusion reporter genes. Furthermore, the influence of *phaD*, *phaF* and *phaI* on those activities was evaluated using the same reporters in knockout strains.

Chapter 5 discusses the work presented in the previous chapters and proposes a model for the role of the PhaF phasin in *Pseudomonas putida* GPo1.

The last chapter describes the tools developed to investigate the activity of promoters of the *pha* gene cluster as well as interesting observations made during the present work that require further investigation.
1.6. References


Chapter 1


Chapter 1


Chapter 1


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ketoacyl-ACP reductase and polyhydroxyalkanoate synthase genes induces PHA
length 3-hydroxyalkanoic acids from gluconate by Pseudomonas aeruginosa and other

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Chapter 2

DNA binding by free PhaF phasins

Summary

The capacity of the PhaF protein to bind DNA was investigated using both DIG and radioactively labeled DNA gel shift assays. Band shifts were observed in control reaction using the Oct2A transcription factor. However, crude extract from *Pseudomonas putida* GPo1 or proteins released from its PHA granules showed no band shifts with DNA fragments containing promoters of the *pha* cluster or with DIG labeled control DNA.
2.1. Introduction

In *P. putida* GPo1, the proteins involved in the accumulation of PHA as distinct hydrophobic granules are encoded by a gene cluster containing two polymerase genes, a depolymerase gene and three regulatory genes. The two PHA polymerase genes *phaC1* and *phaC2* are separated by the *phaZ* depolymerase gene and followed by the *phaD* regulatory gene, all transcribed in the same direction. The two phasins PhaF and PhaI are encoded downstream of *phaD* by genes transcribed in the opposite direction. The existence of at least two promoters regulated by PhaF in the *pha* gene cluster has been established (Prieto *et al.*, 1999). The first, Pc1, is located upstream of the *phaC1* polymerase gene, while the second, Pi, is located upstream of the *phaI* phasin gene.

Sequence analysis showed that PhaF consist of two distinct domains. The N-terminal domain is homologous to PhaI proteins of several *Pseudomonas* strains and is therefore thought to be responsible for the attachment of PhaF to the PHA granule surface. The C-terminal domain contains nine copies of the AAKP motif typical of histone H1-like proteins. Members of this protein family, which includes the *Bordetella pertussis* BpH2 (Goyard, 1996; Scariato *et al.*, 1995) and the *Chlamydia trachomatis* Hc2 (Hackstadt *et al.*, 1993) are known for their DNA binding ability. However, its best-characterized member is the *Pseudomonas aeruginosa* AlgP regulatory protein. AlgP is involved in the complex regulation mechanism controlling the alginate production in response to environmental signals, and is thought to facilitate the bending or looping of the *algD* promoter under certain conditions, thereby enhancing these interactions (Deretic *et al.*, 1992; Deretic *et al.*, 1990; Medvedkin *et al.*, 1995). The AlgP ability to bend or loop DNA is reminiscent of the role played by the tails of H1 histones, which interact with the linker DNA between nucleosomes and participate in the folding of the eukaryotic chromatin fibers into higher-order structures (Allan *et al.*, 1986).

Regulation of alginate production in response to environmental changes appears to be similar to the regulation of polyhydroxyalkanoates, which suggests that the synthesis of both alginates and PHAs could be regulated by similar mechanisms. It has indeed been shown that PhaF is involved in the regulation of the *pha* gene cluster in *P. putida* GPo1 (Prieto *et al.*, 1999). It could therefore be a component of the regulatory system that responds to changes in the direct environment of the bacteria.

Both the Pc1 and the Pi promoters of *P. putida* GPo1 appear to be repressed by PhaF and no transcripts of genes under their control could be observed in a nitrogen-limited growth
medium supplemented with glucose or citrate as carbon source. A role of PhaF in repression was further supported by gene transcription experiments in the _phaF_-knockout _P. putida_ GPG-Tc6, which showed that under the same growth conditions, transcripts of _phaC1Z_, controlled by the _Pc1_ promoter, and of _phaIF_, controlled by the _Pi_ promoter, were seen in Northern blots. Supplementing cultures with octanoate as carbon source induces no repression of these two promoters in the wild-type strain. In this case, all genes are expressed and under nitrogen limited growth conditions PHA granules are formed. A model explaining this behaviour was proposed in which PhaF binds to promoter regions upstream of the _phaC1_ and _phaI_ genes until suitable conditions for PHA accumulation are present, thereby regulating the expression of genes from the _pha_ cluster (Prieto _et al._, 1999).

Demonstrating the ability of PhaF to bind DNA could provide new information about this regulation process and validate the proposed model. Therefore, the interaction between the PhaF phasin and DNA was investigated by gel shift assays. These assays were carried out using both DIG and $\gamma^{32}$P radioactively labeled _Pc1_ and _Pi_ promoter DNA. If binding of these DNA fragments by the PhaF phasin occurs, a modification of their migration speed through a polyacrylamide gel should be detectable.
2.2. Material and methods

2.2.1. Bacterial strains and culture conditions

The wild-type strain \textit{P. putida} GPo1 and the tetracycline resistant \textit{phaF}-negative mutant \textit{P. putida} GPG-Tc6 were cultured overnight at 30°C with vigorous shaking in 0.2N E2 minimal medium, a modified E2 medium (Lageveen \textit{et al.}, 1988) containing 5 times less nitrogen source, or in 0.1N M63 minimal medium, a modified M63 medium (100 mM KH$_2$PO$_4$, 15 mM (NH$_4$)$_2$SO$_4$, 1.7 µM FeSO$_4$, 1 mM MgSO$_4$) containing 10 times less nitrogen source and supplemented with MT (10 µM FeSO$_4$, 10 µM MnCl$_2$, 10 µM CoSO$_4$, 10 µM CaCl$_2$, 1 µM CuCl$_2$, 1 µM ZnSO$_4$). In both media, 15 mM octanoate was added as carbon source. When needed for selection, the antibiotic tetracycline (12.5 µg/ml) was added.

2.2.2. DNA manipulations

DNA manipulations and other molecular biology techniques were essentially performed as described before (Sambrook \textit{et al.}, 1989). The Pc1 promoter region was amplified by PCR using either \textit{P. putida} GPo1 genomic DNA or the pHA1 plasmid, a pBCKS+ based vector carrying a 6 kb EcoRI fragment containing the \textit{pha} locus of \textit{P. putida} GPG-Tc6 (Prieto \textit{et al.}, unpublished results) as template and the primers EcoRIPc1 and BamHIPc1 (Table 1). Similarly, the Pi promoter region was amplified using either \textit{P. putida} GPo1 genomic DNA or the pPF3 plasmid (Prieto \textit{et al.}, 1999) as template and the primers EcoRI Pi and BamHIPi (Table 1).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRIPc1</td>
<td>5’-AATCCAGGGGAATTCTCCTGCCTGCTGACTC-3’</td>
</tr>
<tr>
<td>BamHIPc1</td>
<td>5’-AACGACGGGATCCATCTACGACGCTCCGTCC-3’</td>
</tr>
<tr>
<td>EcoRI Pi</td>
<td>5’-CTTCAGGAATTCCCAGATGACCAGAT-3’</td>
</tr>
<tr>
<td>BamHIPi</td>
<td>5’-TTTGCCATGGCTCTGTACCTCATGCTC-3’</td>
</tr>
</tbody>
</table>

Table 1: Sequence of the PCR primers used for amplification of the \textit{Pseudomonas putida} GPo1 Pc1 and Pi promoters.
2.2.3. PHA granule isolation and analysis

For cultures grown on 0.2N E2 minimal medium, osmotically sensitive cells were obtained using a modified spheroplasting technique (Witholt et al., 1976) from which lysozyme was excluded. 200 ml cell cultures with densities of about 1.5 g (cdw)/l were harvested by centrifugation and resuspended in 200 mM Tris-HCl pH 8.0 to a density of about 7 g (cdw)/l. After addition of 1 volume of 200 mM Tris-HCl / 1 M sucrose pH 8.0, 0.02 volume of 100 mM EDTA pH 7.6 and 2 volumes of water, the cells, having a density of about 1.75 g (cdw)/l, were incubated on ice for 1 hour and pelleted by centrifugation for 30 minutes at 10'000 rpm and 4 °C. The pellets were then resuspended in 1 volume of water, giving a cell density of about 7 g (cdw)/l, and passed twice through a French Pressure cell at 1'500 kPa. The cell extracts were then layered on 5 volumes of 20% sucrose and centrifuged for 2 hours at 12'000 rpm and 4 °C. The top layer was then diluted in 10 volumes of cold water, centrifuged for 30 minutes at 10'000 rpm and 4 °C, and the pellet was resuspended in 15 mM Tris buffer to a concentration of about 100 mg wet granules per ml. Based on a PHA content of the cells of 40%, this represents a granule yield of 70 to 80%.

Cultures grown on 0.1N M63 minimal medium were harvested by centrifugation and resuspended in 15 mM Tris pH 8.0 buffer. The cells were then disrupted by three passages through a French Pressure cell at 1'500 kPa. PHA granules and cell debris were separated by density centrifugation. The cell extract was layered on 55% glycerol and centrifuged at 10'000 rpm for 30 minutes (Stuart et al., 1995). After centrifugation, the PHA granules located at the interphase of both layers, were carefully pipetted out and washed three times with 15 mM Tris pH 8.0 buffer.

The purified granules were kept at 4°C and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) methods (Sambrook et al., 1989).

2.2.4. Protein release and analysis

After isolation of PHA granules from cultures grown on 0.2N E2 minimal medium, the proteins were released from the granule surface by shaking the isolated PHA granules in the presence of 0.1% rhamnolipids at 4°C during 30 minutes. The treated granules were removed by centrifugation at 4°C and 12'000 rpm for 30 minutes (de Roo et al., 2003).
In order to recover granule-free PhaF from granules isolated from cultures grown on 0.1N M63 minimal medium, the granules were frozen in a dry ice/ethanol bath and thawed at room temperature before being centrifuged at 14'000 rpm for 15 minutes. The pellets were then resuspended in 15 mM Tris pH 8.0 buffer. After several freezing and thawing cycles, PhaF was found in the supernatant of the granules.

Aliquots of released proteins were kept at -20°C and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) methods (Sambrook et al., 1989).

2.2.5. DIG labeling of DNA

3’-end DIG labeling of the Pc1 promoter region DNA was performed according to the DIG gel shift assay kit instructions using DIG-11-ddUTP and a terminal transferase (Boehringer Mannheim). The efficiency of the labeling was evaluated by chemiluminescent detection of dilutions of the labeled DNA spotted on a dry positively charged Nylon membrane.

2.2.6. Radioactive labeling of DNA

The DNA fragments were labeled using $\gamma^{32}$P-ATP. 100 ng of DNA were mixed with 2 µl of 10x T4 kinase buffer, 1.5 µl $\gamma^{32}$P-ATP (15 µCi) and 2 µl T4 polynucleotide kinase (50 U) and brought to a final volume of 20 µl with water. The mixture was then incubated for 45 minutes at 37°C. Unbound $\gamma^{32}$P-ATP was removed by running the sample through a PCR purification column (Roche). A 1 µl sample of the eluted product was then used for thin layer chromatography (TLC) in 1 N HCl. A film was exposed 30 seconds over the TLC plate to visualize the efficiency of the labeling.

2.2.7. Gel shift assays

Gel shift assays using DIG labeled Pc1 promoter region DNA were performed according to the instructions supplied with the DIG Gel Shift Kit from Boehringer Mannheim. 4 µl binding buffer, 1 µl poly [d(I-C)], 1 µl L-lysine, 2 µl DIG-labeled oligonucleotide and 1 µl binding protein were mixed together to a final volume of 20 µl. After incubation at room
temperature for 15 minutes, the samples were loaded on a 6% polyacrylamide native gel and electrophoresed at 120V and 4°C in 0.25x TBE buffer. After migration, semi-dry electroblotting during 1.5 hours was used to transfer the DNA to a positively charged nylon membrane (Boehringer Mannheim). The bands were detected by chemiluminescence via antidioxigenin-AP Fab fragments and CSPD (Boehringer Mannheim).

For gel shift assays using radioactively labeled DNA, protein samples were diluted with water to a final volume of 6.5 µl. After addition of 1 µl 10x TPGlut buffer (1 M potassium Glutamate; 400 mM HEPES pH 8; 100 mM MgCl₂), 1 µl 5 mg/ml BSA and 1 µl 2mM DTT, 0.5 µl (~1 ng) labeled DNA were mixed with the sample, bringing it to a final volume of 10 µl. The protein-DNA mixes were incubated 20 minutes at room temperature. 2 µl of loading buffer were added to each sample before loading on a 4% polyacrylamide native gel. The gel was run at 120V until the buffer front was at about 1 cm from the bottom of the gel and then dried under vacuum. An Agfa Curix blue HC-S Plus X-ray film was then exposed overnight.
Chapter 2

2.3. Results

In order to investigate the interaction of free PhaF proteins with DNA, gel shift assays were performed using two DNA labeling methods: DIG and $\gamma^{32}$P.

2.3.1. Assay using DIG labeling

The PhaF proteins used for DIG-labeling assays were released from granules isolated from *P. putida* GPo1 grown on 0.2N E2 minimal medium using 0.1% rhamnolipids (de Roo *et al.*, 2003). In order to evaluate the efficiency of the assay, the DIG gel shift assay kit used provides a control reaction composed of the Oct2A octamer transcription factor and a 39 bp long DNA fragment containing the Oct2A 5’-ATGCAAAT-3’ binding site (Kemler *et al.*, 1989). As expected, the control DNA showed a shift when incubated with the Oct2A factor. In this case, only a small amount of labeled DNA was still present at the same height than the control DNA treated without Oct2A factor. However, no shift could be observed when incubating the labeled Pcl promoter DNA with the PhaF protein. The absence of a visible band shift with the Pcl promoter region and PhaF may be due to the size of the labeled fragment (579 bp), which did not migrate far enough on the gel in comparison to the control DNA. Furthermore, even with longer migration times, it may not be possible to see a band shift if the interaction between the protein and the DNA is not strong enough and the complex is disrupted by the assay, or if only a low amount of labeled DNA actually shifts due to an excess of labeled DNA in comparison to active PhaF proteins.

<table>
<thead>
<tr>
<th>Amount</th>
<th>Oct2A</th>
<th>Control DNA</th>
<th>Free PhaF</th>
<th>Pcl DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng</td>
<td>25-75</td>
<td>0.8</td>
<td>50</td>
<td>10-20</td>
</tr>
<tr>
<td>molecules</td>
<td>3.1-9.2*10^9</td>
<td>1.9*10^{10}</td>
<td>1.16*10^{12}</td>
<td>1.6-3.2 *10^{10}</td>
</tr>
</tbody>
</table>

Table 2: Amounts of DNA and protein present in the gel shift assay. The protein amount given for free PhaF is estimated based on protein gels (0.05 g/l). One molecule of control DNA is 39 bp long and therefore weighs 4.22*10^{-19} g, while Oct2A being a 49 kDa protein, one molecule weighs 8.13*10^{-20} g. Similarly, one molecule of Pcl DNA is 579 bp long and therefore weighs 6.26*10^{-19} g, while PhaF being a 26 kDa protein, one molecule weighs 4.32*10^{-20} g.
Table 2 gives the estimated number of molecules present in the assay. The ratio of binding proteins to DNA fragment can then be calculated based on these numbers. This ratio varies from 16 to 48 for the control reaction, while it lays between 36 and 72 in the case of the PhaF phasin and Pc1 DNA. It can therefore be expected that enough PhaF phasin is present in this assay for a DNA band shift to occur in case of binding, provided most of the free phasins are still active.

### 2.3.2. Assays using radioactive labeling

In order to increase the detection limit of the shifted DNA band, gel shift assays using $\gamma^{32}$P radioactively labeled DNA were performed. The PhaF proteins used in these were released from granules isolated from *P. putida* GPo1 grown on 0.1N M63 minimal medium by freezing and thawing cycles. A negative control sample, a protein preparation lacking PhaF, was prepared from granules isolated from the PhaF-negative mutant *P. putida* GPG-Tc6 that was treated in the same way.

![Figure 1: SDS-PAGE of crude extract from *P. putida* GPo1 and *P. putida* GPG-Tc6, and of protein released by freeze/thaw cycles from isolated PHA granules. Panel A: Crude extracts from cultures grown on M63 minimal medium. GPo1: *P. putida* GPo1 crude extract; GPG-Tc6: *P. putida* GPG-Tc6 crude extract. Panel B: Proteins released from PHA granules. Gra: Granules before protein release treatment; Sup: Supernatant of the treated granules; Pel: Pellet of the treated granules; G: Treatment of *P. putida* GPo1 granules; T: Treatment of *P. putida* GPG-Tc6 granules.](image-url)
Figure 1 illustrates typical crude extract of cultures grown on M63 minimal medium (panel A) and shows the freeze/thaw-released PhaF protein preparations used for these gel shift assays (panel B).

Gel shift assays with two different DNA fragments were carried out: one with the 579 bp long Pc1 promoter region upstream of the PHA polymerase C1, and one with the 427 bp long Pi promoter region upstream of the granule associated protein PhaI. Both *P. putida* GPo1 and *P. putida* GPG-Tc6 crude extracts and proteins released from GPo1 and GPG-Tc6 granules were used.

The choice to use crude extract was made because production of PhaF mRNA was observed when cells are grown in minimal medium without nitrogen limitation (Prieto *et al.*, 1999). It is therefore reasonable to assume that the PhaF protein is also produced under these conditions. Several dilutions of the crude extracts and the released protein preparations were used to carry out the assay. As can be seen in figure 2, no shifts could be observed with the Pc1 or the Pi promoter regions. Since it is possible that the 10x TPGlut incubation buffer does not provide suitable conditions for a binding to occur, it was replaced by a 10x buffer consisting of 200 mM Tris pH 7.5, 20 mM β-mercaptoethanol and 10 mM EDTA in glycerol. The assay was repeated under these new conditions using the labeled Pi promoter region, but did not result in any band shift (results not shown).

Table 3 gives the estimated number of molecules present in the assay for the least and most diluted samples. Based on these numbers, it can be calculated that the ratio of PhaF proteins to DNA fragment varies from 14’438 to 21 for the crude extract samples, and from 3’619 to 53 for the free PhaF phasin. Although not all PhaF phasin might be active, and even with an overestimation of these numbers by a factor 100, enough PhaF phasin should be present in this assay for a DNA band shift to occur in case of binding.

<table>
<thead>
<tr>
<th>Amount</th>
<th>DNA</th>
<th>Crude extract</th>
<th>Crude extract</th>
<th>Free PhaF</th>
<th>Free PhaF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>least diluted</td>
<td>most diluted</td>
<td>least diluted</td>
<td>most diluted</td>
</tr>
<tr>
<td>ng</td>
<td>1</td>
<td>1000</td>
<td>2</td>
<td>250</td>
<td>5</td>
</tr>
<tr>
<td>molecules</td>
<td>1.6-2.2*10^9</td>
<td>2.31*10^13</td>
<td>4.63*10^10</td>
<td>5.79*10^14</td>
<td>1.16*10^11</td>
</tr>
</tbody>
</table>

Table 3: Amounts of DNA and protein present in the gel shift assay. The protein amounts are given for PhaF and are estimated based on figure 1. One molecule of Pc1 DNA is 579 bp long and therefore weighs 6.26*10^-19 g, while one molecule of Pi DNA is 427 bp long and therefore weighs 4.62*10^-19 g. PhaF being a 26 kDa protein, one molecule weighs 4.32*10^-20 g.
Results presented in chapter 3 have shown that DNA fragments are bound by native PHA granules regardless of their nucleotide sequence. Furthermore, the presence of magnesium in the reaction mixture was shown to be essential and sufficient for binding to occur. Based on this information, gel shift assays with PhaF proteins released by treatment with rhamnolipids and the 39 bp long DIG-labeled DNA fragment bound by the Oct2A factor were carried out with magnesium in the reaction mixture.

Figure 3 shows the proteins released from granules isolated from *P. putida* GP01 grown in 0.2NE2 minimal medium used in these assays. As a negative control, a sample of
protein released from granules obtained with the \textit{phaF}-negative mutant \textit{P. putida} GPG-Tc6 was used.

Figure 3: Protein released from \textit{P. putida} GPo1 and \textit{P. putida} GPG-Tc6 PHA granules using rhamnolipids. \textbf{M}: Marker; \textbf{Gra}: PHA granules before rhamnolipids treatment; \textbf{Sup}: Supernatant after rhamnolipids treatment; \textbf{Pel}: Pellet after rhamnolipids treatment; \textbf{G}: Treatments of \textit{P. putida} GPo1 granules; \textbf{T}: Treatment of \textit{P. putida} GPG-Tc6 granules.

As can be seen in figures 4, no band shift could be observed with the PhaF protein in the presence or absence of magnesium, while the control reaction was positive.

Figure 4: Gel Shift Assay using Oct2A target DIG-labeled DNA. \textbf{0}: Labelled DNA only; \textbf{Oct2A}: Labelled DNA with the Oct2A factor; \textbf{GPo1}: Protein released from \textit{P. putida} GPo1 granules; \textbf{GPG-Tc6}: Protein released from \textit{P. putida} GPG-Tc6 granules; -: Without addition of magnesium; \textbf{Mg}^{++}: With addition of magnesium.
This result was confirmed by repeating the experiment both with the same protein preparation and after the preparation of new protein fractions released from *P. putida* GPo1 and *P. putida* GPG-Tc6 granules.

Table 4 gives the estimated number of molecules present in the assay. The ratio of binding proteins to DNA fragment can then be calculated based on these numbers. This ratio varies from 16 to 48 for the control reaction, while it is between 121 and 242 if the control DNA is used with PhaF. It can therefore be expected that enough PhaF phasin is present in this assay for a DNA band shift to occur in case of binding, provided enough of the free phasins are still active.

<table>
<thead>
<tr>
<th>Amount</th>
<th>Oct2A</th>
<th>Control DNA</th>
<th>Free PhaF</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng</td>
<td>25-75</td>
<td>0.8</td>
<td>100-200</td>
</tr>
<tr>
<td>molecules</td>
<td>3.1-9.2*10^{11}</td>
<td>1.9*10^{10}</td>
<td>2.3-4.6*10^{12}</td>
</tr>
</tbody>
</table>

Table 4: Amounts of DNA and protein present in the gel shift assay. The protein amount given for free PhaF is estimated based on figure 3 (0.1-0.2 g/l). One molecule of control DNA is 39 bp long and therefore weighs 4.22*10^{-19} g, while Oct2A being a 49 kDa protein, one molecule weighs 8.13*10^{-20} g. PhaF being a 26 kDa protein, one molecule weighs 4.32*10^{-20} g.
Chapter 2

2.4. Discussion

Based on the amino acid sequence of the PhaF C-terminal domain, which shows homologies to histone H1-like proteins, a DNA binding activity was expected for the PhaF phasin of *P. putida* GPo1. Gel shift assays were therefore carried out using either DIG-labeled DNA or γ\(^{32}\)P-labeled DNA in order to verify this expectation. Unfortunately, no visible DNA shift was observed on the resulting gels, even though a sufficient amount of proteins per DNA fragment should be present in all the assays (tables 2-4).

A preliminary experiment using a DIG-labeled Pc1 promoter DNA fragment showed that poor separation of shifted and unshifted bands might occur when using small gels with large DNA fragments. In order to circumvent this problem, larger gels were prepared. At the same time a lower detection limit for the band shift was obtained by using γ\(^{32}\)P-labeled DNA probes. Finally, the method applied to release the proteins from the PHA granule surface was changed to freezing-thawing cycles to avoid a possible degradation or denaturation of the PhaF phasin by the rhamnolipids treatment during isolation.

Despite these modifications, no band shift could be observed after incubation of labeled Pc1 or Pi DNA fragments with *P. putida* GPo1 crude extract or PhaF phasins released from its PHA granules (figure 2). As a negative control, crude extract and protein released from PHA granules isolated from the *phaF*-negative mutant *P. putida* GPG-Tc6 were used. As these preparations differ from the *P. putida* GPo1 samples only by the absence of the PhaF phasin, it should be possible to attribute differences in the band shift pattern to interactions of PhaF with the DNA. However, the shifts seen in both panels C and D of figure 2 are probably due to other DNA binding proteins such as DNA polymerases. Indeed, these shifts are not only present when crude extract containing PhaF is used (panel C), but also in the absence of PhaF (panel D). In this case, the shifts appear even clearer than when PhaF is present. The observed shifts are therefore not due to interactions of the PhaF phasin with the labeled DNA, and other host proteins are likely to be responsible. Furthermore, it can be seen in both panels that using proteins released from the granules, no band shifts are obtained. This further indicates the involvement of proteins that are not bound to the PHA granule in the shifts observed with the crude extracts.

The gel shift assays with radioactively labeled DNA were carried out with the 10x TPGlut buffer. However, a necessary component for the binding may be missing in TPGlut or
one of its components could be preventing this binding. Unfortunately, no DNA binding could be observed by replacing the TPGlut buffer by a buffer of different composition.

The results presented in Chapter 3 indicate that magnesium is necessary for the binding of any DNA fragment by native PHA granules. However, no band shift was observed when using the TPGlut buffer, which does also contain magnesium. Furthermore, new gel shift assays carried out with DIG-labeled DNA in the presence of magnesium only did not produce any visible band shift with the analyzed samples either.

Several factors could be responsible for the failure to observe binding. The protein may have been inactivated during the release process, a further component necessary for the binding of free PhaF may be missing from the reaction buffer, or one of the released components may prevent binding of PhaF to DNA. Furthermore, the electric field applied during electrophoresis may disrupt the binding of DNA by the free PhaF protein. In the case of native PHA granules, a similar behavior was also observed and is described in chapter 3. In that particular case however, it is not clear whether the separation of the protein-DNA complex was due to the inability of the PHA granule to enter the agarose gel or due to the application of the electric field.

In order to investigate the possible inactivation of the PhaF protein by the release process, a method to isolate the phasin from crude extracts of non PHA producing cells should be developed and gel shift assays using these protein preparations should be carried out. Such a method could be based on a His-tag fusion, but should ultimately provide native proteins. Furthermore, the influence of the electric field on the PhaF-DNA complex could be investigated using DNAse I footprinting.

The model postulated by Prieto et al. (Prieto et al., 1999) proposed a binding of DNA by free PhaF phasins in order to regulate part of the pha gene cluster. When PHA starts to accumulate, PhaF binds to the granule surface, which was expected to interfere with binding to specific DNA regions. However, this assumption might be wrong since neither specific nor unspecific binding of DNA by free PhaF phasins could be shown. A PhaF-dependant binding of DNA by native PHA granules was however observed (chapter 3). Therefore the model developed by Prieto and coworkers needs to take these new elements into account.
2.5. References


Chapter 3

DNA binding by native PHA granules harboring PhaF phasins on their surface

Summary

The *P. putida* GPo1 PhaF phasin contains characteristic motifs of the histone-H1-like protein family in its C-terminal domain and is expected to possess DNA binding activity. However, no such activity was observed with PhaF proteins released from PHA granules (Chapter 2). Therefore, investigations of the DNA binding capacity of native PHA granules harboring PhaF were carried out. Experiments showed binding of $\gamma^{32}$P-labeled DNA fragments containing the Pc1 or the Pi promoter region to PHA granules having PhaF on their surface. Further experiments carried out with the $\lambda$BstEII and 2-log DNA markers showed that this binding is not source, sequence or size specific. The presence of magnesium ions in the reaction buffer was found to be necessary for binding. Calculations showed that the ratio of base pairs per PhaF phasin varies from 0.5 to 53.1, presumably depending on the amount of active PhaF on the PHA granule surface.
3.1. Introduction

Polyhydroxyalkanoates (PHAs) are accumulated by *P. putida* GPo1 as discrete intracellular granules to serve as an internal reserve of carbon and energy. These granules are surrounded by a layer consisting of different proteins and phospholipids, which forms an interphase between the hydrophobic polymer material and the hydrophilic cytoplasm of the bacterial cell (Preusting *et al.*, 1991).

The proteins found on the surface of the PHA granules include the PhaF and PhaI phasins, the PhaC1 and PhaC2 polymerases, the PhaZ depolymerase, as well as an acyl-CoA synthetase and a leucine aminopeptidase (de Roo *et al.*). Among these granule-bound proteins, the two most abundant are the PhaI and PhaF phasins, migrating on a sodium dodecyl sulfate polyacrylamide gel at a size of 18 kDa and 35 kDa respectively. DNA sequence analysis has shown homologies between the granule-bound phasin PhaI and the N-terminal domain of PhaF (Prieto *et al.*, 1999), suggesting that this domain could be responsible for the granule binding ability of PhaF. Little is know about the role of either phasin, but they might be involved in PHA granule formation and stability. This is for instance the case in the PHB producing strain *Ralstonia eutropha*, where overexpression of its PhaP phasin results in many small granules while its absence leads to the formation of a single large granule (Wieczorek *et al.*, 1995).

Analysis of granule size and number in *P. putida* GPo1 has not shown any changes in the absence of the PhaF phasin (Prieto *et al.*, 1999), but the lack of PhaI on the granule surface resulting from the knocking-out of the regulatory protein PhaD might be involved in the observed increase in the number of granules and the decrease of their size. However, it remains unknown whether this phenotype is related to the absence of only PhaD, only PhaI or both (Klinke *et al.*, 2000).

It has been demonstrated that PhaF is involved in gene regulation, especially in transcriptional regulation of the *pha* gene cluster, encoding the proteins responsible for PHA metabolism (Prieto *et al.*, 1999). The C-terminal domain of PhaF, consisting of 112 amino acids, contains AAKP motifs that are characteristic of members of the histone H1-like protein family, and shows similarity to the AlgP protein, a histone H1-like protein of *Pseudomonas aeruginosa* involved in the regulation of alginate production. The presence of these proline-containing repeated motifs and the high positive net charge of PhaF (pI=10.52) could explain
the slower electrophoretic mobility observed in SDS-gels, which is a typical behaviour of proteins with repeated motifs rich in proline (Bühler, 1998; Deretic et al., 1990). Indeed, on 12 % SDS-gels, PhaF appears to be about 35 kDa whereas its calculated size is 26 kDa.

Based on this homology with AlgP and histone H1-like proteins, attempts were made to demonstrate DNA binding by free PhaF using gel shift assays. However, these attempts remained unsuccessful, possibly due to a loss of DNA binding activity of the PhaF phasins during their release from the PHA granule surface (Chapter 2).

To circumvent this potential problem and to study the interaction of PhaF with DNA in vitro, two different assays using native PHA granules harboring PhaF on their surface were developed. In the first assay, two putative binding DNA sequences for the PhaF protein were studied. These sequences, located upstream of phaC1 (Pc1 promoter region) and upstream of phaI (Pi promoter region), were labeled with $\gamma^{32}$P-ATP and their binding by native PHA granules was quantified. The second assay was based on the detection by ethidium bromide staining of the amount of DNA present in the supernatant and the pellet of centrifuged PHA granule and DNA mixes. The use of this method confirmed the DNA binding activity previously measured and allowed the identification of the elements necessary for this binding to occur.
3.2. Material and methods

3.2.1. Bacterial strains and culture conditions

The wild-type strain *P. putida* GPo1 and the tetracycline resistant *phaF*-negative mutant *P. putida* GPG-Tc6 were cultured under the conditions described in Chapter 2.

3.2.2. PHA granule isolation and analysis

Isolation and analysis of PHA granules from both strains was carried out according to the methods described in Chapter 2.

3.2.3. DNA manipulations and radioactive labeling

DNA manipulations, labeling of DNA fragments with $\gamma^{32}$P-ATP and other molecular biology techniques were essentially performed as described in Chapter 2. The $\lambda$BstEII marker was generated by overnight digestion at 60°C of 200 µl of $\lambda$ DNA with 10 µl BstEII (New England BioLabs) in a final volume of 900 µl. The 2-log marker used was purchased from New England BioLabs.

3.2.4. DNA binding assays using native PHA granules

In order to investigate the binding of DNA by native PHA granules harboring or lacking PhaF proteins on their surface, the following assays were used.

1 ml binding mixes were prepared by diluting 5 ng $\gamma^{32}$P-labeled DNA and unlabeled competitor DNA (1 mg/ml) in TPGlut buffer (100 mM potassium glutamate; 40 mM HEPES pH 8; 10 mM MgCl$_2$) containing 500 µg/ml BSA and adding 100 µl PHA granules isolated from *P. putida* GPo1 or *P. putida* GPG-Tc6. The mix was incubated during 30 minutes at room temperature to allow binding. The granules were then pelleted by centrifugation at 14'000 rpm for 10 minutes and the radioactivity present in 500 µl of the supernatant was assayed by scintillation. For each promoter and each granule type, the assay was carried out in triplicates in order to minimize handling errors. The amounts of counts recorded in each
supernatant were averaged and the value obtained was normalized against that of the control.

When no labeling of the DNA was performed, the DNA fragments were incubated at room temperature with PHA granules isolated from *P. putida* GPo1 or *P. putida* GPG-Tc6. After centrifugation for 10 minutes at 14'000 rpm part of the supernatant was loaded on a 1% agarose gel in TAE. After migration, ethidium bromide stained DNA bands were observed under UV light.
3.3. Results

*P. putida* GPo1 and *P. putida* GPG-Tc6 accumulate PHA in large hydrophobic granules when grown on fatty acids. Several proteins needed for PHA metabolism, such as the PHA polymerases or the PHA depolymerase, are bound to the granule surface. However, the two main proteins visible on a SDS-PAA gel of granules isolated from *P. putida* GPo1 are the phasins PhaI (18 kDa) and PhaF (35 kDa). In *P. putida* GPG-Tc6, only PhaI is present.

Preliminary assays showed a possible binding of the Pc1 promoter region DNA by PHA granules isolated from *P. putida* GPo1 within 48 hours of the experiment, but these results could not be reproduced with granules stored at 4°C for a week. SDS-PAGE analysis of PHA granules and N-terminal sequencing of the proteins bound to their surface showed C-terminal degradation products for PhaF (de Roo *et al.*), suggesting that degradation of PhaF is responsible for the observed loss of the DNA binding ability.

3.3.1. DNA-binding ability

To compare the DNA binding ability of native PHA granules, which harbor PhaF phasins on their surface, and granules lacking PhaF, assays with PHA granules isolated from cultures grown on 0.1N M63 minimal medium and radioactively labeled DNA were performed using the Pi promoter region and the Pc1 promoter region. These regions are located upstream of the genes encoding the PhaC1 PHA polymerase and the PhaI phasin, respectively.

Figure 1 shows the results obtained when fixed amounts of \( \gamma^{32} \)P labeled Pc1 and Pi promoter DNA fragments were incubated with PHA granules isolated from the wild-type strain *P. putida* GPo1 and its phaF-negative mutant *P. putida* GPG-Tc6. As a control, an assay was carried out in the absence of granules.

In the control assays, both set to 100 %, the standard deviation was 25.4 % with the Pc1 promoter and 13.3 % with the Pi promoter. Granules isolated from *P. putida* GPo1 bound most of the added DNA, since only 11.3±3.6 % of the Pc1 and 25.2±14.0 % of the Pi promoters remained in the supernatant. Granules isolated from *P. putida* GPG-Tc6 did not bind DNA, all of which remained in the supernatant.
Figure 1: Binding of DNA by PHA granules in the presence and absence of the PhaF phasin. Granules isolated from *P. putida* GPo1 and *P. putida* GPG-Tc6 were incubated with two radioactively labeled DNA fragments containing promoters of the *pha* cluster. The radioactivity remaining in the supernatant after centrifugation was measured by scintillation counting.

Based on this experiment, 10 mg of PHA, containing 18.9 µg of PhaF, bound 4.4 ng of Pc1 or 3.7 ng of Pi DNA. These results suggested that the molar ratio of DNA to PhaF in the DNA-PHA granule complexes was in the order of $7.8 \times 10^{-3}$ to $9.3 \times 10^{-3}$ basepairs per granule-bound PhaF molecule.

**3.3.2. Specificity of the DNA binding**

To more carefully determine the stoichiometry and specificity of DNA binding by PHA granules in the presence of granule-bound PhaF phasins, assays were carried out using increasing amounts of unlabeled salmon sperm DNA as competitor to the labeled Pc1 and Pi promoters. In both cases, the radioactivity measured in the supernatant of the assays increased with increased competitor DNA concentration. With the Pc1 promoter, addition of 100 µl
competitor DNA was necessary to release all the labeled DNA (figure 2), while only 10 µl were necessary with the Pi promoter (figure 3).

Figure 2: Binding of labeled Pc1 promoter DNA by PHA granules in the presence and absence of granule-bound PhaF proteins. Granules containing PhaF (isolated from P. putida GPo1) and lacking PhaF (isolated from P. putida GPG-Tc6) were incubated with fixed amounts of radioactively labeled Pc1 promoter DNA fragments and varying amounts of salmon sperm DNA as competitor. The radioactivity remaining in the supernatant after centrifugation was measured.

As already seen in figure 1, in the absence of unlabeled competitor DNA, about 90 % of the labeled Pc1 promoter is bound to the granules isolated from P. putida GPo1 (11.3±3.6 % is recovered) and none of the same DNA bound to granules isolated from P. putida GPG-Tc6 (104.6±29.3 % is recovered). Addition of 1 µl competitor DNA did not change this result significantly (10.3±0.8 % and 72.3±4.2 % of the Pc1 promoter remained in the supernatant after binding to granules isolated from P. putida GPo1 and P. putida GPG-Tc6, respectively). It is interesting that addition of 1 µl salmon sperm DNA to granules isolated from P. putida GPG-Tc6 decreased the amount of the labeled DNA in the supernatant, the opposite of what
might have been expected if some DNA had been bound to the granules in the absence of cold DNA. This was not the case, and it is possible that there is some non-specific DNA binding to PHA granules, with small amounts of labeled DNA then interacting with the excess unlabeled DNA. If this were the case, it would not explain how addition of 10 to 100 µl unlabeled DNA would reduce such initial interactions (84.5±9.7 % and 90.7±2.9 % of the label DNA remained in the supernatant after addition of 10 and 100 µl unlabeled DNA to granules isolated from *P. putida* GPG-Tc6). Increasing the amount of unlabeled DNA to 10 µl reduced the binding of Pc1 DNA to granules isolated from *P. putida* GPo1 to about 73 % (26.5±5.7 % of the labeled DNA remained in the supernatant). A further increase to 100 µl competitor DNA decreased the binding of Pc1 DNA to granules isolated from *P. putida* GPo1 to about 11 % (89.1±9.1 % of the labeled DNA was then present in the supernatant).

![Figure 3: Binding of labeled Pi promoter DNA by PHA granules in the presence and absence of granule-bound PhaF proteins. Granules containing PhaF (isolated from *P. putida* GPo1) and lacking PhaF (isolated from *P. putida* GPG-Tc6) were incubated with fixed amounts of radioactively labeled Pi promoter DNA fragments and varying amounts of competitor DNA. The radioactivity remaining in the supernatant after centrifugation was measured.](image-url)
The data obtained with the Pi promoter (figure 3) does not differ significantly from that obtained with the Pc1 promoter (figure 2). The only striking point is that the increase in the amount of labeled DNA measured in the supernatant occurs at lower competitor DNA concentrations. Without competitor, only 75% of the labeled Pi DNA is bound to granules isolated from \( P. \ putida \) GPo1, and none is bound to granules isolated from \( P. \ putida \) GPG-Tc6 (25.2±14.0 % and 101.8±7.6 % of the labeled DNA was found in the supernatant, respectively). With 1 µl competitor DNA, binding of the labeled DNA to the granules isolated from \( P. \ putida \) GPo1 decreased to 63 %, while there was no binding to the granules isolated from \( P. \ putida \) GPG-Tc6 (37.0±5.7 % and 103.7±5.3 % of the labeled DNA was found in the supernatant, respectively) and with 10 µl of competing unlabeled DNA, only 4.7 % of the labeled DNA binds to granule isolated from \( P. \ putida \) GPo1, while none binds to the control granules (95.3±8.6 % and 104.2±9.7 % of the labeled DNA was found in the supernatant, respectively). As expected, addition of 100 µl competitor DNA has little additional effect, with 3.5 % of the label still binding to granules isolated from \( P. \ putida \) GPo1 and none to the control granules (96.5±6.4 % and 111.3±8.0 % of the labeled DNA was found in the supernatant, respectively).

The above experiments indicate that the granules isolated from \( P. \ putida \) GPo1 bind about 0.44 ng Pc1 DNA per mg PHA, or 0.50 ng Pc1 DNA per µg PhaF protein, equivalent to \( 9.3*10^{-3} \) basepairs per PhaF molecule. The corresponding numbers for Pi DNA are 0.37 ng Pi DNA per mg PHA, 0.42 ng Pi DNA per µg PhaF protein and \( 7.8*10^{-3} \) basepairs per PhaF molecule. Large amounts of non-specific DNA are necessary to displace the Pc1 and Pi DNAs, the former requiring higher amounts than the latter. This might be due to differences in the specificity of DNA-granule interactions, but it could also be due to variations in the number of active PhaF phasins bound to the PHA granule.

### 3.3.3. Binding of unlabeled DNA

The assay used to demonstrate binding of DNA by native PHA granules harboring PhaF was modified to measure binding of unlabeled DNA. To achieve this, granules isolated from cultures grown on 0.2NE2 minimal medium and DNA were incubated together. After centrifugation the supernatant and pellet were separated and loaded on a 1% agarose gel. Detection was then possible by staining with ethidium bromide and observing the gel under UV light.
Figure 4: Binding of DNA by PHA granules in the presence of PhaF and disruption of the granule-DNA complex during electrophoresis. Panel A: 30 µl granules isolated from *P. putida* GPo1 or *P. putida* GPG-Tc6 and 20 µl Pi DNA were incubated together for 20 minutes at room temperature. 25 µl of the mix were kept and the remainder was centrifuged for 5 minutes at 15'000 rpm and room temperature. 20 µl of the supernatant was collected. The pellet was resuspended in 20 µl water. Panel B: 10 µl Pi DNA were mixed with 15 µl granules and incubated for 30 minutes at room temperature. After 5 minutes centrifugation at 15'000 rpm and room temperature, 20 µl of the supernatant were collected. 10 µl samples were loaded on the gel. **GPo1**: Granules isolated from *P. putida* GPo1; **GPG-Tc6**: Granules isolated from *P. putida* GPG-Tc6; **λB**: λBstEII DNA marker; **Mix**: Granules mixed with Pi DNA; **Pel**: Pellet after centrifugation of granules mixed with Pi DNA; **Sup**: Supernatant of granules with Pi DNA; **Con**: Pi DNA control.

Using this protocol, not only the binding of DNA by granules harboring PhaF was observed, but also the disruption of the granule-DNA complex during electrophoresis. Figure 4 shows the DNA recovered in the supernatant and the pellet of DNA/granule mixes using granules from *P. putida* GPo1 and *P. putida* GPG-Tc6, as well as the unseparated mix. Both panels show that when Pi DNA is incubated with granules isolated from *P. putida* GPo1 it remains associated with the pellet after centrifugation of the incubation mix: no DNA is found in the corresponding supernatant. When mixed with granules isolated from *P. putida* GPG-Tc6 the Pi DNA is found in the supernatant after centrifugation, and only small amounts are present in the corresponding pellet. Interestingly, the granule-bound DNA migrated out of the slots while the granules stayed in the loading well. This indicates that the electrophoresis, the buffer composition, or another parameter of the assay separates bound DNA from the PhaF containing granules. This effect was independent of the applied voltage as it was observed at
both 100V and 10V. The results shown in panels A and B were obtained with two different batches of granules.

### 3.3.3.1. Binding of DNA by granule mixes

In order to rule out a possible entrapment of DNA by pelleted granules without a specific binding of DNA due to PhaF, an assay was carried out where the total volume of granules was kept constant by mixing different amounts of granules isolated from *P. putida* GPo1 and *P. putida* GPG-Tc6 (figure 5). 0 to 5 µl granules from *P. putida* GPo1 were mixed with 5 to 0 µl granules from *P. putida* GPG-Tc6 to obtain a fixed final volume of 5 µl granules. This mix was then incubated with Pi promoter DNA, the granules were pelleted by centrifugation, and DNA bands stained with ethidium bromide were observed under UV light after migration on a 1% agarose gel. The increase in the ratio of granules isolated from *P. putida* GPo1 to granules isolated from *P. putida* GPG-Tc6 resulted in a decrease in the amount of DNA present in the supernatant; That is, the disappearance of DNA from the supernatants correlates with the PhaF content of the granule mix. This result excludes a general coprecipitation of DNA with PHA granules, which would have left the same amount of DNA in the supernatant, regardless of the composition of the granule mix.

![Figure 5: Binding of DNA by granule mixes.](image)

**Figure 5:** Binding of DNA by granule mixes. 5 µl mixes with different ratios of granules isolated from *P. putida* GPo1 and *P. putida* GPG-Tc6 were incubated together with 10 µl Pi DNA and 10 µl water at room temperature during 30 minutes. The mix was then centrifuged for 10 minutes at 15'000 rpm and room temperature. 10 µl of the supernatant was collected and loaded on the gel. **LB:** λBstEII DNA marker; **C:** Control with no granules.

### 3.3.3.2. Binding of unrelated DNA of different sizes

Figure 6 shows the binding of unrelated DNA fragments of different sizes by PHA granules containing PhaF phasins. The fragments were obtained from two different DNA
markers, \( \lambda \)BstEII and 2-log. With \( \lambda \)BstEII, all marker fragments are retained by the granules, whereas no effect is seen with the 2-log marker. The binding does not display any preference for sizes. Of the various alternative explanations, differences in the buffer of both DNA samples appears the most likely choice to influence the binding ability of PhaF.

**Figure 6: Binding of unrelated DNA of different sizes.** 10 µl granules isolated from *P. putida* GPo1 or *P. putida* GPG-Tc6 were incubated together with 10 µl DNA marker at room temperature during 30 minutes. The mix was then centrifuged for 10 minutes at 15'000 rpm and room temperature. 15 µl supernatant and 5 µl pellet were then loaded on the gel. Both the \( \lambda \)BstEII DNA marker (panel A) and the 2-log DNA marker (panel B) were used. **GPo1**: Granules isolated from *P. putida* GPo1; **GPG-Tc6**: Granules isolated from *P. putida* GPG-Tc6; **C**: 5 µl \( \lambda \)BstEII DNA marker (panel A) or 10 µl 2-log DNA marker (panel B); **Sup**: Supernatant from DNA marker and granules mix; **Pel**: Pellet from DNA marker and granules mix.

**3.3.3.3. Identification of the minimal buffer required for DNA binding**

In order to identify whether or not a necessary component is missing in the DNA buffer used with the 2-log DNA marker, both markers were purified by phenol:chlorophorm extraction and redissolved in water. Using these preparations, no binding could be observed (figure 7).

Based on the previous experiments, it is known that NEB buffer 3, which was used to digest \( \lambda \) DNA with BstEII, should allow binding; that DyNAzyme DNA Polymerase buffer with MgCl\(_2\), which was used for PCRs of the Pi promoter, should allow binding and that Tris-HCl/EDTA, in which the 2-log DNA Marker is dissolved, does not allow binding. MgCl\(_2\) is one of the components of the first two buffers that is absent in the 2-log DNA sample, therefore its effect on the binding of DNA by PhaF-harboring granules was investigated using
marker DNA resuspended in different buffers. The results obtained with NEB buffer 3, 2.5 mM MgCl₂ and DyNAzyme DNA polymerase buffer lacking MgCl₂ are shown in figure 8.

Figure 7: Binding of marker DNA resuspended in water. λBstEII and 2-log DNA markers were purified by phenol:chloroform extraction and resuspended in water. 10 µl granules isolated from P. putida GPo1 or P. putida GPG-Tc6 were incubated together with 10 µl marker DNA at room temperature during 30 minutes. The mix was then centrifuged for 10 minutes at 15'000 rpm and room temperature. 15 µl supernatant and 5 µl pellet were then loaded on the gel. **Sup:** Supernatant from DNA marker and granules mix; **Pel:** Pellet from DNA marker and granules mix; **GPo1:** Granules isolated from P. putida GPo1; **GPG-Tc6:** Granules isolated from P. putida GPG-Tc6.

When incubated with NEB buffer 3 and 2.5 mM MgCl₂, the marker DNA is found in the pellets and not in the supernatants and thus fully retained by the PhaF-harboring granules. Incubation in water or with DyNAzyme DNA polymerase buffer lacking MgCl₂ prevents retention of DNA by the granules. The presence of MgCl₂ therefore appears to be necessary and sufficient for the binding of DNA by PHA granules containing PhaF proteins. Since Cl⁻ ions are present in the 2-log DNA marker, but no binding is observed, it can reasonably be assumed that Mg²⁺ is the necessary cofactor. The C-terminal of PhaF consists mostly of alanine, lysine, proline and arginine (46 %, 17 %, 16 % and 7 % respectively) and contains no aspartic acid or glutamic acid. It is therefore positively charged and a possible binding of DNA to the C-terminal of PhaF can thus not result from an ionic effect occurring via magnesium.
Figure 8: Binding of marker DNA resuspended in various buffers. λBstEII marker DNA was cleaned by phenol:chloroform extraction and resuspended in 9 µl water. 1 µl water, NEB buffer 3, 50mM MgCl₂ or DyNAzyme DNA polymerase buffer lacking MgCl₂ were added. 10 µl granules isolated from P. putida GPo1 were incubated together with 10 µl marker DNA at room temperature during 30 minutes. The mix was then centrifuged for 10 minutes at 15'000 rpm and room temperature. 10 µl supernatant and 10 µl pellet were then loaded on the gel. Sup: Supernatant of the DNA marker and granule mix; Pel: Pellet of the DNA marker and granule mix; H₂O: Samples with addition of water; NEB3: Samples with addition of NEB buffer 3; MgCl₂: Samples with addition of MgCl₂; Pol: Samples with addition of DyNAzyme DNA polymerase buffer lacking MgCl₂.

3.3.3.4. Influence of incubation time on DNA binding

The influence of the incubation time on the amount of DNA bound by PhaF-harboring granules was investigated by mixing granules isolated from P. putida GPo1 with 2-log marker DNA and MgCl₂ and incubating the mix at room temperature for up to 12 minutes before centrifugation for 10 minutes at 15'000 rpm and 4°C. The supernatant (figure 9, panel A) and pellet (figure 9, panel B) were then loaded on a 1% agarose gel and the DNA present in each fraction detected by ethidium bromide staining. The sample at time 0 was obtained by centrifuging a tube with DNA and buffer in the bottom and granules in the cap.

The amount of DNA recovered in the supernatant of the assay decreases with longer incubation times (panel A). The appearance of this DNA in the pellet is visible in panel B. In panel A, only the last lane does not display any visible DNA band, indicating that 10 to 12 minutes are necessary for the granules to bind the DNA in the assay.

Based on the data available from New England Biolabs regarding the 2-log DNA marker, it can be calculated that 1 µg DNA contains $1.58 \times 10^{12}$ molecules, forming $9.3 \times 10^{14}$
base pairs. Furthermore 1 mg wet weight of granules has a dry weight of 0.35 mg (Kraak et al., 1997). Based on the evaluation made by Kraak et al., about 1.8 % of this mass is proteins for granules isolated from P. putida GPo1 containing 30 % PHA (Kraak, 1998), and observation of SDS gels of PHA granules indicates that about 30 % of the total proteins found on the granules surface are PhaF phasins. Therefore, 1 mg wet weight of granules correspond to 1.89 µg PhaF phasins. Furthermore, based on polyacrylamide gel analysis it is known that 10 µl PHA granules, having a dry weight of 0.35 mg, are necessary to obtain a PhaF band intensity similar to that obtained for the bands of 10 µl of a low molecular weight marker. The concentration of such a marker being approximately of 0.1 to 0.2 mg per ml for each protein, it can be estimated that 2.86 to 5.71 µg PhaF are present per mg PHA granule. These numbers agree with that obtained earlier, which can therefore be considered relevant.

Figure 9: Influence of incubation time on DNA binding. 5 µl granules isolated from P. putida GPo1 were incubated together with 5 µl 2-log DNA marker (1 µg) and 1 µl 50mM MgCl₂ in a final volume of 15 µl. Varying incubation times at room temperature were applied. The mix was then centrifuged for 10 minutes at 15'000 rpm and 4°C. 10 µl supernatant (panel A) and 5 µl pellet (panel B) were loaded on the gel. M: 5 µl 2-log DNA marker.

Table 1 gives the relationships between the amounts of PhaF phasins and the 2-log DNA marker bound based on figure 9. In this case, the amount of PhaF phasin molecules per 2-log DNA molecule is 14, resulting in 42.5 base pairs per phasin.
DNA Granules

\[
\begin{array}{|c|c|c|c|c|c|c|}
\hline
\text{µg molecules base pairs} & \text{µg molecules} & \text{µg molecules} & \text{g/g mo./mo.} & \text{bp/ mo.} \\
\hline
1 & 1.58 \times 10^{12} & 9.3 \times 10^{14} & 500 & 0.95 & 2.19 \times 10^{13} & 0.95 & 14 & 44.5 \\
\hline
\end{array}
\]

Table 1: Relationship between the amounts of DNA and PhaF phasins. Based on figure 9, it was calculated that 0.5 mg granules are binding to 1 µg 2-log DNA. Using those numbers, ratios between PhaF phasins and DNA amounts have been calculated. mo.: molecules; bp: base pairs.

3.3.3.5. DNA binding by fixed granule amount

In order to investigate a possible saturation of the PHA granule by DNA, varying amounts of DNA were incubated with a fixed amount of granules isolated from \textit{P. putida} GPo1 (figure 10).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure10}
\caption{Varying DNA amount with fixed granule amount. Varying amounts of a 2.5 kb DNA PCR product were incubated for 30 minutes with a fixed amount of granules isolated from \textit{P. putida} GPo1 and 1 µl 50mM MgCl\textsubscript{2} in a final volume of 20 µl. The mix was then centrifuged for 10 minutes at 15'000 rpm and 4°C. \textbf{Panel A}: 5 µl pellet using 10 µl of granules. \textbf{Panel B}: 20 µl supernatant using 1 µl of granules. \textbf{M}: 5 µl 2-log DNA marker.}
\end{figure}

Panel A of figure 10 shows that when a high amount of granules carrying the PhaF phasin is used, an increase of the DNA amount present in the assay results in a increase in the DNA pelleted with the granules. This effect is probably limited by the saturation of the PhaF-harboring granules by DNA. From that point on, no further increase of DNA should be visible.
Under the conditions of the assay presented in panel A, no DNA was recovered in the supernatants (results not shown).

In panel B of figure 10 the amount of granules used was ten times lower than in panel A. In that case, the PHA granules are rapidly saturated by DNA. A further increase in the amount of DNA incubated with the granules resulted in accumulation of this DNA in the supernatant. Under these conditions, only a small amount of DNA was present in the pellets (results not shown).

3.3.3.6. Influence of granule amounts on quantity of DNA bound

In order to determine the amount of granules necessary to bind a given quantity of DNA, the amount of granules in the assay was varied from 0 to 50 µl in the presence of 50 ng Pc1 promoter DNA. This increase in the amount of granules resulted in a decreasing amount of DNA recovered in the supernatant. According to figure 5, between 0.5 and 2 mg granules are necessary to bind 50 ng DNA.

![Figure 11: Influence of granule amount on quantity of DNA bound. Varying amounts of granules isolated from *P. putida* GPo1 were incubated together with 10 µl Pc1 DNA (50 ng) in a final volume of 60 µl for 45 minutes at room temperature. The mix was then centrifuged for 10 minutes at 15'000 rpm and room temperature. 20 µl of the supernatant was collected and loaded on the gel. **λB**: λBstEII DNA marker.](image)

The Pc1 promoter region being 579 bp long, one molecule has a weight of $6.26 \times 10^{-19}$ g. The 50 ng used represent therefore $7.98 \times 10^{10}$ molecules, or $4.62 \times 10^{13}$ base pairs. In the case of figure 11, there are 0.95 to 3.78 µg PhaF phasins present for each 50 ng of DNA bound. Table 2 shows the relationship between the amounts of DNA and PhaF phasins necessary for binding in this assay.
Table 2: Relationship between the amounts of DNA and PhaF phasins. Based on figure 11, it was calculated that 0.5 to 2 mg granules are binding to 50 ng Pc1 promoter DNA. Using those numbers, ratios between PhaF phasins and DNA amounts have been calculated. mo.: molecules; pb: base pairs.

In this assay, the amount of PhaF phasin molecules per Pc1 promoter DNA molecule is between 273 and 1092, representing from 0.5 to 2.1 base pairs per phasin.

3.3.3.7. DNA binding by increasing granule amount

Using a DNA marker allows the use of a higher DNA amount compared to the use of Pi or Pc1 promoter DNA PCR products, 1 to 2 µg instead of 50 to 100 ng, the quantity of
DNA being spread between the 0.1 and 10 kilobase bands on the agarose gel used for detection. Figure 12 shows the binding of all the bands of the 2-log DNA marker if sufficient amounts of granule are present.

Since 100 mg wet weight of the granules from *P. putida* GPo1 were resuspended in 1 ml, it can be calculated that 1 µg of the 2-log DNA marker is bound by 1 to 1.5 mg granules from *P. putida* GPo1. Similarly between 0.4 and 0.6 mg granules were necessary to bind 1 µg 2-log DNA marker in the experiment presented in panel B of figure 12. As seen earlier, 1 mg wet weight granules correspond to 1.89 µg PhaF phasins and 1 µg DNA contains 1.58 * 10^{12} molecules, forming 9.3 * 10^{14} base pairs.

Table 3 gives the relationships between the amount of 2-log DNA marker and PhaF phasins base on the data of figure 12.

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</table>

**Table 3: Relationship between the amounts of DNA and PhaF phasins.** Based on figure 12, it was calculated that 0.4 to 1.5 mg granules are binding to 1 µg 2-log DNA. Using those numbers, ratios between PhaF phasins and DNA amounts have been calculated. mo.: molecules; pb: base pairs.

In this assay, the amount of PhaF phasin molecules per 2-log DNA molecule is between 11 and 42, representing from 14.2 to 53.1 base pairs per phasin.
3.4. Discussion

In order to investigate the ability of the PhaF phasin of *P. putida* GPo1 to bind DNA, PHA granules were isolated from *P. putida* GPo1 after overnight growth on octanoate. To prevent further degradation of the granule-bound proteins, the recovered granules were constantly kept on ice and stored at 4°C when necessary. However, preliminary tests made in order to develop a reliable DNA binding assay showed that results obtained with newly isolated granules could not be reproduced with the same granule batch after storage at 4°C for one week. This observation coincides with the appearance of degradation products on the granule surface that were identified as originating from the PhaF phasin by N-terminal sequencing (de Roo et al.). Unfortunately, this degradation of the PhaF phasin reduces the time span in which the isolated granules are usable. This phenomenon was not investigated further. A modification of the isolation procedure or storage conditions may prevent this degradation.

Prieto and coworkers reported that the C-terminus of the PhaF phasin contains nine copies of the AAKP motif characteristic of histone H1-like proteins, suggesting that PhaF belongs to this protein family. Furthermore, as the N-terminus of PhaF shows homology to the PhaI phasin, it is likely that this domain is responsible for the binding of PhaF to the PHA granule. To investigate the possible binding of DNA by the C-terminal domain of PhaF, an assay was set up in which the retention of $\gamma^{32}$P radioactively labeled DNA fragments by PHA granules was measured. PHA granules isolated from the wild-type strain *P. putida* GPo1 and its *phaF*-negative mutant *P. putida* GPG-Tc6 were incubated with either the Pcl or the Pi promoter region. After centrifugation, less radioactivity could be found in the supernatant when using wild-type granules than when using granules lacking PhaF (figure 1). Thus, binding of DNA to granules may be mediated by PhaF, its two-domain structure enabling PhaF to simultaneously bind PHA granules and DNA. Despite the fact that the release of the labeled DNA by PhaF-harboring granules occurs at lower competitor DNA concentrations for the Pi promoter (figure 3) than for the Pcl promoter (figure 2), no conclusions can be drawn from these experiments regarding the DNA binding specificity. Indeed, the granules used in both assays were obtained in two different granule isolation experiments and therefore had similar yet not equal concentration and quality.

Using a simplified assay in which unlabelled DNA and granule mixes were separated by centrifugation and the DNA concentration in the supernatant and the pellet was analyzed
by electrophoresis, it was possible to show that DNA binding occurred regardless of the nature of the DNA fragment used. Indeed, all fragments generated by the digestion of λ phage DNA by BstEII were recovered in the pellet when using granules isolated from *P. putida* GPo1 (figure 6, panel A). The commercially available 2-log DNA marker, which consists of DNA fragments ranging from 100 to 10'000 bp, was, however, not bound by the wild-type granules (figure 6, panel B). This DNA bound to these granules only after resuspending phenol:chlorophorm extracted DNA in a buffer containing magnesium (figure 8). Thus, the presence of Mg$^{2+}$ ions is necessary and sufficient for binding of DNA by PHA granules harboring the PhaF protein.

The data presented in figure 11 shows the increase in the amount of DNA bound by increasing granule quantities. Based on these data, it was calculated that 273 to 1092 molecules PhaF were necessary to bind one molecule of Pc1 promoter DNA, resulting in the binding of 0.5 to 2.1 base pairs per PhaF molecule. A similar experiment carried out using 2-log DNA instead of Pc1 promoter DNA and summarized in figure 12 shows that one molecule of DNA was bound in the presence of 11 to 42 PhaF molecules. In this case, the number of base pairs bound by one PhaF molecule is between 14.2 and 53.1. Finally in figure 9, presenting the increase of DNA bound by a fixed amount of granules in time, it was shown that 10 to 12 minutes were necessary to bind one molecule of the 2-log marker DNA to 14 PhaF molecules, equivalent to the binding of 42.5 base pairs per PhaF molecule. The incubation times used in our other experiments are not expected to significantly affect these numbers since all the experiments were carried out with incubation times longer than 15 minutes. It is indeed expected that once all the active PhaF phasins are involved in the DNA binding, no further changes occur with time. Comparing these numbers, a fairly big difference is observed between figure 11 and figures 9 and 12. This difference should, however, not be ascribed to the different target DNAs used. Instead, it is more likely to be due to the state and age of the granule preparations used for each assay. While the assays shown in figures 12 and 9 were carried out at the same time and with the same PHA granule preparation, the one shown in figure 11 was made with a different preparation. Taking the amount of PhaF phasins per granule to be constant, this difference is probably due to a lower number of active PhaF phasins on the granule surfaces. It has been observed that the activity of the PhaF phasins decreases rapidly and that repetition of an assay on a later day requires higher granule amounts in order to yield the same result. Furthermore, the amount of active PhaF phasins on the PHA granule surface could vary between isolations. This can be due to variations in the
growth stage of the cultures upon harvesting as well as to the granule isolation procedure itself. In order to obtain more reliable data about the kinetics of the DNA binding, it would therefore be necessary to further standardize the granule isolation process and the granule storage conditions to ensure the presence of a constant high level of active PhaF phasins.

The number of base pairs bound per PhaF molecules is comparable to that bound by histone H1 proteins. Although DNA binding by histone H1 is relatively non-sequence specific and not well understood, it was reported that the 110 amino acid long carboxy-terminal domain of histone H1 bind from 15 to 80 base pairs (Clark et al., 1988; Sevall, 1988). Studies carried out with histone H1\(^0\) showed that turbidity of the assay solution, which is due to conformational change of DNA, occurs at 30 base pairs per protein, while saturation is obtained with 10 base pairs per protein (Mamoon et al., 2002). The carboxy-terminal domain of histone H1, to which the C-terminal domain of PhaF is homologous, is lysine-rich and does not fold in solution, presumably due to electrostatic repulsion between the lysine residues (Mamoon et al., 2002). It is this domain that is responsible for the ability of histone H1 to change the DNA structure from B-DNA to \(\Psi\)-DNA, a twisted, tightly packaged assembly of DNA (Mamoon et al., 2002). Medvedkin and coworkers showed that the repeated AAKP motif found in the \textit{P. aeruginosa} AlgR3 and AlgP proteins consists of a single turn of \(\alpha\)-helix followed by a bend imposed by the proline residue, the resultant coiled-coil forming a right-handed superhelix (Medvedkin et al., 1995). This structure is able to bind to the major groove of B-DNA. Replacement of the proline in every fifth AAKP motif by threonine and alanine, which yields the longer AAKTA block, results in an even better fit to the major groove. The C-terminal domain of PhaF being homologous to AlgP, it can be imagined that it adopts a similar structure, sticking out of the PHA granule. Although no evidence has been found for a conformational change in the DNA structure in the presence of PhaF, possibly resulting in a spatial compression of the DNA, such an event would increase the space available for the PHA granules in the bacterial cell.

Considering that a \textit{P. putida} cell weighs \(5 \times 10^{-13}\) g (Huisman, 1991), of which 30\% is PHA and that there are on average 2 granules per cells, an average PHA granule weighs \(7.5 \times 10^{-14}\) g. If 1.8 \% of the granule mass is proteins, 30\% of which being PhaF phasins, it can be calculated that there are \(4.05 \times 10^{-16}\) g PhaF phasins per granule. One PhaF phasin has a molecular mass of 26 kDa, or \(4.32 \times 10^{-20}\) g, and there are therefore 9’384 PhaF phasins on the surface of one PHA granule. Based on the assumption that a PHA granule is a sphere with a diameter of 0.5 \(\mu\)m (Fuller et al., 1992), its surface area is 78’539’750 Å\(^2\). There is therefore
one PhaF phasin for each 8370 Å². Supposing that the PhaF phasins are evenly spaced on the granule surface, there would therefore be about 91.5 Å between each phasin. Interestingly, Preusting and coworkers reported a faint pattern on the granule surface consisting of a nearly rectangular array with an angle of 84° and a spacing of 73±2 Å (Preusting et al., 1991). Whether this pattern is or is not produced by the presence of the PhaF phasins on the surface of the granules remains unknown, but its presence or absence on the surface of granules isolated from a phaF negative mutant should be investigated in order to corroborate or refute this hypothesis.

Although DNA binding by free PhaF proteins has not been observed so far, it was shown that DNA is bound to PHA granules if PhaF is present on the granule surface. Furthermore, it has been shown that this binding is not specific to a particular DNA sequence, but occurs regardless of the DNA fragment used, as long as magnesium ions are present in the reaction. There are several possibilities that could explain this binding of DNA by PHA granules: coprecipitation, aspecific binding to naked PHA, binding to phospholipids around the outer surface of the granule, or binding to one of more granule-bound proteins (figure 13).

**Figure 13: Possible explanations for binding of DNA by PHA granules.** A: coprecipitation of the DNA with the PHA granules, B: aspecific binding of DNA to the PHA granule, C: binding of DNA to phospholipids, D: binding of DNA to granule-bound proteins.

Coprecipitation of the DNA with PHA granules, aspecific binding to the PHA granule and binding to the phospholipid monolayer surrounding the PHA granule are not suitable to explain the observed results, since the DNA binding occurs only in the presence of PhaF. If
one of these three possibilities were correct, DNA binding should also be observed with granules lacking PhaF. This is not the case, as can be seen in figure 5. Furthermore, if interactions with DNA and phospholipids did occur, these should also happen with other cellular membranes, in particular the inner face of the cytoplasmic membrane. However, no such interactions have been observed so far, and only selective binding of specific membrane proteins to DNA have been reported, mostly in relation with DNA replication (Firshein, 1989; Newman et al., 2000).

In the case of DNA binding to a granule-bound protein, the obvious candidates are the PhaF and PhaI phasins, which represents the two most abundant proteins on the PHA granule surface (30% and 60% respectively), as well as the PhaZ depolymerase and the PhaC1 and PhaC2 polymerases, found on the granule surface only in small amounts. Of these proteins, PhaF is the most likely candidate since it is the only one to show homologies to proteins known to bind DNA. The C-terminal domain of PhaF is homologous to histone-H1 like proteins (Prieto et al., 1999) and possesses several copies of a motif known to produce a DNA binding tertiary structure (Medvedkin et al., 1995). We have quantified the amount of DNA that binds to granules and determined how many bases bind per PhaF molecules, should this binding occur between PhaF and DNA. The numbers obtained are in the same range as those reported for the histones H1 and H1\(^0\) (Clark et al., 1988; Mamoon et al., 2002; Sevall, 1988). Given the above, the binding of DNA by native granules observed in this chapter is most likely to result from an interaction of the C-terminal domain of PhaF with DNA molecules.

This simultaneous binding of PHA granules and unspecific DNA by PhaF tends to contradict the model proposed by Prieto et al. (Prieto et al., 1999), in which PhaF is bound either to DNA or to a PHA granule. A possible reason for PhaF to simultaneously bind PHA granules and DNA could be to bring the bacterial cell in a resting state by a global inhibition of transcription, as is the case with the bacterial histone Hc1 (Barry III et al., 1993), or a participation of PhaF in the cell division process. PhaF might be involved in the partitioning of the granules between both daughter cells by attaching the PHA granules to the DNA strands as they are separated, thus providing an even partitioning of the PHA granules between the two new cells. Furthermore, the division of a cell containing PHA granules lacking PhaF could be problematic, since the granule represents a significant portion of the volume of the cell.
Chapter 3

3.5. References


Chapter 4

Activity study of promoters from the *Pseudomonas putida* GPo1 *pha* gene cluster

Summary

Expression levels of genes belonging to the *pha* cluster were investigated using *lacZ* fusion as reporters. Single copies of the *lacZ* reporter gene under the control of the *phaC1*, *phaC2*, *phaI* and *phaF* promoters were introduced in the wild-type strain *Pseudomonas putida* GPo1 and its knockout mutants *P. putida* GPo1001, *P. putida* GPo1-XFT and *P. putida* GPo1-XIT, which lack *phaD*, *phaF* and *phaI* respectively. In the wild-type strain, the Pc2 promoter does not appear to be active in media containing octanoate, and the ß-galactosidase activity resulting from the *lacZ* expression with the Pc1 promoter remained about 50 times lower than that obtained with the Pf or the Pi promoters in the presence of octanoate. Furthermore, a strong influence of PhaI on all promoters of the *pha* gene cluster was observed. In the *phaI* knockout, the activities obtained from the Pc1, Pc2 and Pi promoters was significantly higher than in the wild-type, while that obtained from the Pf promoter was one order of magnitude lower. The lack of PhaD resulted in lower activities with the Pi and Pf promoters, and that of PhaF in a decreased activity with the Pf promoter.
4.1. Introduction

Six proteins have been identified to be responsible for the accumulation of polyhydroxyalkanoates (PHAs) in *P. putida* GPo1: two polymerases, PhaC1 and PhaC2; a depolymerase, PhaZ; two phasins, PhaF and PhaI; and a regulatory protein, PhaD. The genes encoding these proteins are found in the *phaC1ZC2DFI* gene cluster depicted in figure 1, and four promoters, Pc1, Pc2, Pf and Pi, have been identified, located upstream of *phaC1*, *phaC2*, *phaF* and *phaI* respectively.

The exact role of the two phasins PhaF and PhaI, and of the regulatory protein PhaD remain unknown, but all three proteins appear to be involved in the regulation of genes belonging to the *pha* cluster. Indeed, based on experiments carried out using a *phaF* knockout mutant of *P. putida* GPo1, a model has been proposed in which PhaF interacts with the Pc1 and Pi promoters to repress the transcription of the *phaC1* and *phaIF* mRNA in cultures grown in E2 minimal medium containing either citrate or glucose as carbon source (Prieto *et al.*, 1999). Furthermore, Klinke *et al.* observed that smaller and more numerous PHA granules are accumulated in the *phaD* knockout strain *P. putida* GPo1001. In addition, analysis of the proteins present at the surface of the granules isolated from this knockout revealed the absence of the PhaI phasin (Klinke *et al.*, 2000).

**Figure 1: Organization of the *pha* gene cluster in *Pseudomonas putida* GPo1.** *phaC1*: PHA polymerase C1 (1680 bp); *phaZ*: PHA depolymerase (852 bp, 64 bp downstream of *phaC1*); *phaC2*: PHA polymerase C2 (1683 bp, 120 bp downstream of *phaZ*); *phaD*: regulatory protein (615 bp, 14 bp downstream of *phaC2*); *phaF*: phasin (768 bp, 11 bp downstream of *phaI*); *phaI*: phasin (420 bp). 126 bp separate the stop codons of *phaD* and *phaF*. The boxes above the genes represent the promoter regions.

In order to better understand the role of these proteins in the PHA accumulation process, *lacZ* reporters were constructed for the four known promoters of the *pha* gene cluster using the pUT mini-Tn5 system which allows stable random insertions in the genome of gram-negative bacteria (de Lorenzo *et al.*, 1990; Herrero *et al.*, 1990). The reporters were inserted in the wild-type strain *P. putida* GPo1, and its knockout mutants *P. putida* GPo1001,
P. putida GPo1-XFT and P. putida GPo1-XIT, which lack phaD, phaF and phaI, respectively.
4.2. Material and methods

4.2.1. Bacterial strains and culture conditions

*E. coli* strains were grown at 37°C in complex Luria-Bertani (LB) medium (Sambrook et al., 1989), while *P. putida* strains were grown at 30°C in the same medium. To study the influence of specific carbon sources, the *P. putida* strains were grown in E2 minimal medium (Lageveen et al., 1988) or in its nitrogen deficient variant, 0.2NE2, when production of PHA was desired. If necessary, antibiotics were added at the following concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; tetracycline, 15 µg/ml. Screening for the loss of β-galactosidase activity was done by adding 100 µg/ml IPTG and 15 µg/ml XGAL to the medium.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant genotype or phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em>:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH10B</td>
<td>Host for <em>E. coli</em> plasmids</td>
<td>(Hanahan, 1983)</td>
</tr>
<tr>
<td>HB101</td>
<td>Host for helper plasmids RK600</td>
<td>(Sambrook <em>et al.</em>, 1989)</td>
</tr>
<tr>
<td>CC118λpir</td>
<td>Host for pUT-derived plasmids</td>
<td>(Herrero <em>et al.</em>, 1990)</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em>:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPo1</td>
<td>Wild-type strain</td>
<td>(Schwartz <em>et al.</em>, 1973)</td>
</tr>
<tr>
<td>GPo1001</td>
<td>Tc&lt;sup&gt;c&lt;/sup&gt;, pha&lt;sup&gt;D&lt;/sup&gt;</td>
<td>(Klinke <em>et al.</em>, 2000)</td>
</tr>
<tr>
<td>GPo1-XFT</td>
<td>Tc&lt;sup&gt;c&lt;/sup&gt;, pha&lt;sup&gt;F&lt;/sup&gt;</td>
<td>Chapter 6</td>
</tr>
<tr>
<td>GPo1-XIT</td>
<td>Tc&lt;sup&gt;c&lt;/sup&gt;, pha&lt;sup&gt;I&lt;/sup&gt;</td>
<td>Chapter 6</td>
</tr>
<tr>
<td>GPo1-Pc2</td>
<td>GPo1 with Pc1::lacZ</td>
<td>(Prieto <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>GPo1-Pf</td>
<td>GPo1 with Pf::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>GPo1-Pi</td>
<td>GPo1 with Pi::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>GPo1001-Pc1</td>
<td>GPo1001 with Pc1::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>GPo1001-Pc2</td>
<td>GPo1001 with Pc2::lacZ</td>
<td>This study</td>
</tr>
<tr>
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<td>GPo1001 with Pf::lacZ</td>
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<td>GPo1-XFT-Pc1</td>
<td>GPo1-XFT with Pc1::lacZ</td>
<td>This study</td>
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<td>This study</td>
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<tr>
<td>GPo1-XFT-Pf</td>
<td>GPo1-XFT with Pi::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>GPo1-XFT-Pi</td>
<td>GPo1-XFT with Pi::lacZ</td>
<td>This study</td>
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### Table 1: Strains and plasmids used.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Relevant genotype or phenotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>RK600</td>
<td>Helper plasmid, tra&lt;sup&gt;+&lt;/sup&gt;, mob&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(Figurski et al., 1979)</td>
</tr>
<tr>
<td>pUT-Km</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;; mini-Tn5 delivery plasmid</td>
<td>(de Lorenzo et al., 1990)</td>
</tr>
<tr>
<td>pUJ9</td>
<td>lacZα reporter construction plasmid</td>
<td>(de Lorenzo et al., 1990)</td>
</tr>
<tr>
<td>pPG132</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;; Pc1::lacZ reporter, pUT-Km derivative</td>
<td>(Prieto et al., 1999)</td>
</tr>
<tr>
<td>pUT-Km-Pc2</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;; Pc2::lacZ reporter, pUT-Km derivative</td>
<td>This study</td>
</tr>
<tr>
<td>pUT-Km-Pf</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;; Pf::lacZ reporter, pUT-Km derivative</td>
<td>This study</td>
</tr>
<tr>
<td>pUT-Km-Pi</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;; Pi::lacZ reporter, pUT-Km derivative</td>
<td>This study</td>
</tr>
<tr>
<td>pUJ9-Pc2</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; Pc2::lacZ fusion construct</td>
<td>This study</td>
</tr>
<tr>
<td>pUJ9-Pf</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; Pf::lacZ fusion construct</td>
<td>This study</td>
</tr>
<tr>
<td>pUJ9-Pi</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; Pi::lacZ fusion construct</td>
<td>This study</td>
</tr>
</tbody>
</table>

**4.2.2. DNA manipulation**

DNA manipulation and other molecular biology techniques were essentially performed as described before (Sambrook et al., 1989). The promoter regions Pc2, Pf and Pi were amplified by PCR from genomic DNA using the primers given in table 2 and inserted in pUJ9 to yield pUJ9-Pc2, pUJ9-Pf and pUJ9-Pi respectively.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI Pc2</td>
<td>5′-GGTGGAAATTCGGCGGCAAGATGGGCTACTA-3′</td>
</tr>
<tr>
<td>BamHI Pf</td>
<td>5′-AAAGGATCCATGGCAACACTCCCTCGTCTA-3′</td>
</tr>
<tr>
<td>EcoRI Pf</td>
<td>5′-TGCCGAATTCGACTGTAAGAAAAAGG-3′</td>
</tr>
<tr>
<td>BamHI Pf</td>
<td>5′-CTTCTTGGGATCCATCCCTCGTCTC-3′</td>
</tr>
<tr>
<td>EcoRI Pi</td>
<td>5′-CTTCAGGAATTTCCCGCATGACCCAGAT-3′</td>
</tr>
<tr>
<td>BamHI Pi</td>
<td>5′-TTTGCATGGCTCTGTACCTCATGCTC-3′</td>
</tr>
</tbody>
</table>

**Table 2: Sequences of the PCR primers used for amplification.**
Transformation of *P. putida* strains with the reporter constructs was performed by triparental mating. 1 ml overnight cultures of a *P. putida* strain, *E. coli* HB101 [RK600] or *E. coli* DH10B carrying one of the reporter construct was resuspended in 500 µl 0.9% NaCl. 100 µl of the donor, helper and acceptor strains were then mixed together and pipetted on a sterile filter placed on a LB plate. After 30 minutes incubation at room temperature, the plate was placed at 30°C for 3 hours. Subsequently the cells present on the filter were resuspended in 1 ml LB, centrifuged for 1 minute at 14’000 rpm, resuspended again in a small volume LB and plated on E2 plates containing 15 µg/ml tetracycline and 0.2% citrate as carbon source. The plates were then incubated at 30°C for two days.

### 4.2.3. *o*-nitrophenyl-β-D-galactopyranoside assay

The activity of the promoters in shake flasks cultures was determined by measuring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG). For each sample, the optical density of the culture at 600 nm was measured. The sample was then diluted at least 2x in buffer Z (60 mM Na₂HPO₄.2H₂O, 40 mM NaH₂PO₄.2H₂O, 10 mM KCl, 1 mM MgSO₄.7H₂O, 50 mM β-mercaptoethanol, pH7) to a final volume of 1 ml. The cells were lysed by addition of one drop 0.1% SDS and two drops chloroform and vortexing for 10 seconds. The reaction was started by the addition of 200 µl of a 13.3 mM ONPG solution (per liter: 4 g ONPG, 10.5 g K₂HPO₄, 4.5 g KH₂PO₄, 1.0 g (NH₄)₂SO₄, 0.5 g Na₃citrate.2H₂O, pH 7.0). Incubation at 28°C was carried out until the formation of a yellow color was visible (OD420 between 0.1 and 0.5). The reaction was then stopped by addition of 500 µl 1M Na₂CO₃ and the reaction time recorded. After centrifugation for 1 minute at 15’000 rpm, the optical density of 1 ml supernatant was measured at 420 nm. The *lacZ* activity is given by the following formula:

\[
U/\text{mg cell} = (\text{OD}_{420} \times V) / (0.4677 \times \varepsilon \times \Delta t \times v \times l \times \text{OD}_{600})
\]

where the molar extinction coefficient for the chromogen *o*-nitrophenol \(\varepsilon = 4.6 \text{ ml/µmole x cm}\), the assay volume \(V = 1.7 \text{ ml}\) and the length of the light path \(l = 1 \text{ cm}\). \(v\) is the volume of cell culture used in ml and \(\Delta t\) is the reaction time in minutes.
4.3. Results

To investigate the activity of promoters of the *pha* gene cluster, the Pc2, Pf and Pi promoter regions were amplified by PCR and inserted as EcoRI-BamHI fragments upstream of the *lacZ* gene of pUJ9 (de Lorenzo *et al.*, 1990). The resulting plasmids, pUJ9-Pc2, pUJ9-Pf and pUJ9-Pi respectively, were sequenced in order to ensure that no mutations had occurred during the amplification of the promoter regions. The 4.3 kb *NcoI* cassettes containing the fusions were then subcloned into the mini-Tn5 delivery plasmid pUT-Km (de Lorenzo *et al.*, 1990) (figure 2). The resulting constructs were named pUT-Km-Pc2, pUT-Km-Pf and pUT-Km-Pi.

A single copy of each of the reporter genes was inserted in the chromosome of the wild-type strain *P. putida* GPo1, the *phaD* knockout *P. putida* GPo1001, the *phaF* knockout *P. putida* GPo1-XFT and the *phaI* knockout *P. putida* GPo1-XIT by triparental mating using *E. coli* CC18λpir strains harboring pPG132, pUT-Km-Pc2, pUT-Km-Pf or pUT-Km-Pi as donors. Recombinants were selected on E2 minimal medium containing 0.2% citrate as carbon source, 50 µg/ml kanamycin and 15 µg/ml tetracycline if necessary.

![Figure 2: Construction of the pUJ9-Px helper plasmids and the pUT-Km-Px reporter vectors.](image)

A: Map of the pUJ9 vector with the insertion point of the different promoters; B: Map of the pUT-Km vector with the insertion point of the different promoter-*lacZ* reporter constructs.
The activity of the Pc1, Pc2, Pf and Pi promoters in *P. putida* GPo1 was determined by measuring the β-galactosidase activity resulting from a single copy of one of the promoter-*lacZ* fusions inserted in the bacterial genome.

**Figure 3:** *P. putida* GPo1 *lacZ* expression by the Pc1, Pc2, Pf and Pi promoter in 0.2NE2 medium containing 15 mM octanoate. **A:** β-galactosidase activity of the *lacZ* gene expressed by the Pc1 and Pc2 promoters in *P. putida* GPG-132 and GPo1-Pc2 respectively; **B:** β-galactosidase activity of the *lacZ* gene expressed by the Pf and Pi promoters in *P. putida* GPo1-Pf and GPo1-Pi respectively. The dashed line indicates the start of the nitrogen limitation, based on the cessation of growth.

Figure 3 shows that in the wild-type strain *P. putida* GPo1, the final specific activity of LacZ expressed from the Pc1 promoter is about 20 mU/mg cells, whereas that of the Pc2 promoter is almost undetectable. The Pf and Pi activities are 45 times higher, at around 900 mU/mg cells.

**4.3.1. Activity of *pha* promoters in *P. putida* GPo1 *pha* mutants**

To probe the effect of different *pha* promoters on PHA protein expression, the experiment described above was carried out with several *pha* mutants.

In *P. putida* GPo1001, which lacks *phaD*, the β-galactosidase activity resulting from the *lacZ* expression by the Pc2 promoter remained close to zero, as was the case with the wild-type (figure 4). The activity obtained with the Pc1 promoter was higher than in the wild-type, at about 35 mU/mg cells. However, the most interesting effect of the PhaD absence,
visible in figure 4B, is the decrease of the β-galactosidase activity obtained with the Pf and Pi promoters by an order of magnitude, resulting in values of only 85 and 20 mU/mg cells respectively.

![Figure 4](image1)

**Figure 4:** *P. putida* GPo1001 lacZ expression by the Pc1, Pc2, Pf and Pi promoter in 0.2NE2 medium containing 15 mM octanoate. **A:** β-galactosidase activity of the *lacZ* gene expressed by the Pc1 and Pc2 promoters in *P. putida* GPo1001-Pc1 and GPo1001-Pc2 respectively; **B:** β-galactosidase activity of the *lacZ* gene expressed by the Pf and Pi promoters in *P. putida* GPo1001-Pf and GPo1001-Pi respectively. The dashed line indicates the start of the nitrogen limitation, based on the cessation of growth.

![Figure 5](image2)

**Figure 5:** *P. putida* GPo1-XFT lacZ expression by the Pc1, Pc2, Pf and Pi promoter in 0.2NE2 medium containing 15 mM octanoate. **A:** β-galactosidase activity of the *lacZ* gene expressed by the Pc1, Pc2 and Pf promoters in *P. putida* GPo1-XFT-Pc1, GPo1-XFT-Pc2 and GPo1-XFT-Pf respectively; **B:** β-galactosidase activity of the *lacZ* gene expressed by the Pi promoters in *P. putida* GPo1-XFT-Pi respectively. The dashed line indicates the start of the nitrogen limitation, based on the cessation of growth.
The activities obtained with the Pc1 and Pc2 promoters after knocking out the \textit{phaF} open reading frame are similar to those obtained in the absence of PhaD (figure 5A). The $\beta$-galactosidase activity resulting from the Pc2 promoter is under 1 mU/mg cells, and that from the Pc1 promoter about 30 mU/mg cells. The results obtained with the Pf and Pi promoters in \textit{P. putida} GPo1-XFT are, however, different from those observed in the \textit{phaD} knockout. The activity obtained with the Pi promoter decreased to about 650 mU/mg cells (figure 5B), and that obtained with the Pf promoter decreased to a level as low as 25 mU/mg cells (figure 5A), which is 36 times lower than in the wild-type.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{\textit{P. putida} GPo1-XIT \textit{lacZ} expression by the Pc1, Pc2, Pf and Pi promoter in 0.2NE2 medium containing 15 mM octanoate. \textbf{A}: $\beta$-galactosidase activity of the \textit{lacZ} gene expressed by the Pc1, Pc2 and Pf promoters in \textit{P. putida} GPo1-XIT-Pc1, GPo1-XIT-Pc2 and GPo1-XIT-Pf respectively; \textbf{B}: $\beta$-galactosidase activity of the \textit{lacZ} gene expressed by the Pi promoters in \textit{P. putida} GPo1-XIT-Pi respectively. The dashed line indicates the start of the nitrogen limitation, based on the cessation of growth.}
\end{figure}

The absence of PhaI has strong effects on the activity of all pha promoter constructs, as is apparent in figure 6. Apart from Pf, the activity of which is decreased by one order of magnitude when compared to that in the wild-type, all promoters exhibit an increased activity in \textit{P. putida} GPo1-XIT. LacZ expression from the Pc1 promoter reaches an activity of about 125 mU/mg cells, while the Pc2 promoter driven expression becomes detectable, with an activity around 15 mU/mg cells. Finally the Pi promoter activity is strongly enhanced, reaching more than 5’000 mU/mg cells.

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4.3.2. Influence of different growth media

The influence of different growth media on Pc1, Pc2, Pf and Pi promoter activity was investigated by growing the strains carrying the reporter fusions in complex Luria-Bertani (LB) medium, in E2 minimal medium containing 0.2% citrate, 2% gluconate or 15 mM octanoate, or in the nitrogen-limited 0.2NE2 minimal medium containing 15 mM octanoate.

Figure 7 shows that the Pc1 promoter driven expression is in general the highest in LB or E2 minimal medium containing citrate. The only situation where this is not the case is in the phaI knockout mutant *P. putida* GPo1-XIT, where the expression levels seen in octanoate containing media are higher. In the wild-type strain *P. putida* GPo1 and all its knockout mutants, the expression observed in E2 containing gluconate is the lowest.

The β-galactosidase activities resulting from the *lacZ* expression by the Pc2 promoter are similar to those obtained by the Pc1 promoter in LB and E2 containing either citrate or gluconate as carbon source. This activity is however less than 2 mU/mg cells in octanoate containing media, unless phaI is missing.

Pf expression decreases by at least one order of magnitude in octanoate containing media as soon as one of the phaD, phaI or phaF genes is knocked out. Furthermore, in the phaF knockout strain *P. putida* GPo1-XFT, its expression in LB and E2 minimal medium containing citrate is lowered by a factor 2, and in the phaI knockout strain *P. putida* GPo1-XIT, it is doubled in E2 media containing gluconate.

Finally, in octanoate containing media, the β-galactosidase activities resulting from the *lacZ* expression by the Pi promoter is greatly decreased in the phaD knockout strain *P. putida* GPo1001, but increases in the phaI knockout strain *P. putida* GPo1-XIT. In this strain, the Pi expression is doubled in cultures grown in LB or E2 minimal medium containing citrate or gluconate.

*Figure 7: Influence of media on LacZ expression by the Pc1, Pc2, Pf and Pi promoters.* The β-galactosidase activity obtained from each reporter expressed in the wild-type *P. putida* GPo1 (first row), the phaD knockout *P. putida* GPo1001 (second row), the phaF knockout *P. putida* GPo1-XFT (third row) and the phaI knockout *P. putida* GPo1-XIT (fourth row) are given. **LB**: Luria-Bertani rich medium; **Cit**: E2 minimal medium containing 0.2% citrate; **Glu**: E2 minimal medium containing 2% gluconate; **Oct**: E2 minimal medium containing 15 mM octanoate; **N. Oct**: 0.2NE2 minimal medium containing 15 mM octanoate.
4.4. Discussion

In order to study the activity of the four known *pha* gene cluster promoters, named Pc1, Pc2, Pf and Pi according to the gene upstream of which they are located, they were fused to the *lacZ* gene and introduced as single copy chromosomal insertions into the genome of the wild-type strain *P. putida* GPo1 and the knockout strains *P. putida* GPo1001, *P. putida* GPo1-XFT and *P. putida* GPo1-XIT using a mini-Tn5 transposon delivery system.

Study of the ß-galactosidase activities resulting from reporters inserted in the wild-type strain *P. putida* GPo1 showed that the Pc2 promoter activity remained under 2 mU/mg cells throughout the entire cultivation (figure 3A). Regarding the Pc1, Pf and Pi promoters, figure 3 shows a lag phase during the first five hours of cultivation, before the activity starts rising. After 12 hours, the activity stays at a relatively constant level. The levels of activity of the Pf and Pi promoters reached values around 900 mU/mg cells after the 12 first hours. These values are about 45 times higher than those obtained for the Pc1 promoter (20 mU/mg cells). The decrease in activity observed for the Pc1 promoter in figure 3A is probably due to a higher activity resulting from the inoculation culture. It could, however, also be an artifact due to the very low cell concentration, and hence the less accurate culture density measurements.

In order to better understand the role of the PhaD regulatory protein as well as that of the PhaF and PhaI phasins in the PHA accumulation process, similar assays were performed with *P. putida* GPo1001, *P. putida* GPo1-XFT and *P. putida* GPo1-XIT knockout mutants carrying one of the reporter constructs as a single copy chromosomal insert. The absence of the PhaD regulatory protein resulted not only in a slight increase of the Pc1 promoter activity, but more interestingly, in a significant decrease of the activity obtained with the Pf and Pi promoters (figure 4). In their study on the role of *phaD*, Klinke and coworkers reported that in the *phaD* knockout strain *P. putida* GPo1001, PhaI could no longer be detected on the PHA granule surface (Klinke et al., 2000). However, they could not determine whether this was due to the absence of *phaI* expression or if a protein-protein interaction between PhaI and PhaD was necessary for this binding to occur. The results shown in figure 4 give LacZ expression levels of 85 mU/mg cells for the Pf promoter and 20 mU/mg cells for the Pi promoter. This clearly indicates that PhaD affects *phaI* expression at the transcription level, preventing PhaI production to a certain extent. An interaction of PhaI and PhaD at the protein level is therefore unlikely.
In *P. putida* GPo1-XFT, the lack of PhaF appears to have no significant influence on the activity levels of the Pc1 and Pc2 promoters, and that of the Pi promoter is decreased by about one third (figure 5). However, the strongest influence resulting from the absence of PhaF is on its own promoter. The activity of the Pf promoter is strongly reduced at only 25 mU/mg cells. The PhaF protein therefore seems not only to strengthen the PhaI production, but also to be necessary for its own production.

Figure 6 shows the results of the absence of PhaI on the *pha* promoter driven expression. Unlike for the absence of PhaD and PhaF, the lack of PhaI leads to higher activity of both the Pc1 and the Pc2 promoters. Furthermore, the Pf activity is decreased to about 75 mU/mg cells in *P. putida* GPo1-XIT while that of the Pi promoter is increased to values above 5'000 mU/mg cells. The PhaI phasin therefore seems to play an important role in the down regulation of the Pc1, Pc2 and Pi promoters, and to act on its own DNA coding sequence in order to enhance the PhaF production.

The effects of the activities of the Pc1, Pc2, Pf and Pi promoters resulting from the knockout of *phaD, phaF* or *phaI* can be summarized based on the data given in figure 7. Table 3 gives the factor by which the activity of the different promoters is increased for each medium and for each mutant when compared to the wild-type strain.

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<tr>
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<td>0.52</td>
<td>0.77</td>
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**Table 3:** Relative activity of four *pha* promoters as a function of *pha* knockouts and growth media. The ratio of promoter activity in each of the knockout strains, versus promoter activity in the parent strain *P. putida* GPo1, is indicated. The values with a gray background represent situations where a decrease in relative promoter activity has occurred. The bold values indicate factors representing a significant effect, and the values in italics indicate factors obtained based on the very low activities recorded for the Pc2 promoter in octanoate containing media. **LB:** Luria-Bertani; **Cit:** E2 containing 0.2% citrate; **Glu:** E2 containing 2% gluconate; **Oct:** E2 containing 15 mM octanoate; **NOct:** 0.2NE2 containing 15 mM octanoate.
Figure 8: Regulation of the pha genes. A: In the absence of octanoate; B: In the presence of octanoate, the Pi driven expression increases and PhaI blocks the Pc2 promoter; C: PhaD is expressed and increases the activity of Pf and Pi; D: PhaF contributes to increasing the Pf activity while PhaI inhibits the Pi promoter; E: a balance between activation and repression effects is reached, where Pc1 is active at a low level, Pc2 is turned off, and Pf and Pi are active at high levels.
When looking at the values given in table 3, the activities of the different promoters given in figure 7 should be taken into account. For instance, the values obtained with the Pc2 promoter in the presence of octanoate were generally very low, and therefore errors made in the activity measurements have a large influence on the factor calculated. In this particular case, rather than considering an activity increase, it is more likely that the Pc2 promoter does not lead to expression under normal conditions, whereas it does in the phaI knockout strain. Furthermore, since the lack of PhaD in P. putida GPo1001 results in a 10 to 45 times decrease of the Pf and Pi activities when compared to P. putida GPo1, if the Pc2 promoter is indeed not active in these two strains, it can be concluded that phaD possesses its own promoter. Table 3 shows that the most significant changes due to the lack of PhaD, PhaF or PhaI occur when octanoate is present. A possible reason for this is the higher activity of the Pf and Pi promoters in media containing octanoate and the presence of more PhaF and PhaI phasins in the wild-type strain resulting from it.

Figure 8 shows a model explaining how the pha genes could be regulated by the presence or absence of octanoate. Panel A illustrates the situation in the absence of octanoate. The first effect of the presence of octanoate is given in panel B, where the Pi activity increases, and the produced PhaI phasin apparently blocks the Pc2 promoter. This in turn possibly permits the synthesis of PhaD, driven by a putative Pd promoter (panel C). The presence of PhaD further enhances the activity of the Pi and Pf promoters, and therefore the synthesis of the PhaI and PhaF phasins. The PhaF phasin then activates its own promoter, whereas PhaI down regulates Pi, as can be seen in panel D. Finally, in panel E, a balance between the different activation and repression effects of PhaD, PhaF and PhaI on the promoters of the pha gene cluster is reached. In this state, Pc1 has low activity, Pc2 is almost completely repressed and Pf and Pi are both highly active.

In this model, the Pc2 promoter is active only when no or small amounts of PHA are present. This ensures that a basal level of PHA polymerase is present in the bacteria at all times, thus allowing a rapid response to the disappearance of a crucial nutrient. Soon after the start of the PHA accumulation, the Pi promoter driven expression increases, providing high amounts of PhaI phasins to cover the growing PHA granule surface. At this stage, the absence of PhaI could possibly result in the accumulation of smaller granules, and therefore in a less efficient PHA production. Similarly, the increased activity of the Pf promoter results in the presence of a significant amount of PhaF phasins on the PHA granule surface. As this amount increases with the granule size, more genomic DNA can be bound, increasing the inhibition of
global transcription. With the accumulation of a high amount of PHA, the cell reaches a resting state where it can remain until growth conditions are once again normal. When returning to normal growth, the accumulated PHA is slowly degraded, releasing the PhaF phasins in the cytoplasm, thereby lowering the inhibition of global transcription that led to the resting state.
4.5. References


Chapter 5

Conclusions and outlook

The aim of this work was to investigate the potential DNA binding ability of the C-terminal domain of the PhaF phasin. As the *pha* accumulation process is a complex system involving several proteins in addition to the PHA polymerases and the PHA depolymerase, the influence of the PhaF and PhaI phasins and that of the PhaD regulatory protein on the *pha* promoters was also investigated in order to better understand the role of these proteins.
5.1. Interaction of the PhaF protein with DNA

The C-terminal domain of the *P. putida* GPo1 PhaF phasin contains several copies of the AAKP motif typical of histone H1-like proteins. Therefore PhaF was suspected to share the characteristic DNA binding ability of this protein family. The regulation model of Prieto and coworkers proposes that free PhaF binds DNA, thus inhibiting the Pc1 and Pi promoters. The presence of PHA granules would prevent this inhibition via the binding of the PhaF phasin to the PHA granule (Prieto *et al.*, 1999). To confirm or refute this idea studies were carried out with free PhaF proteins and whole granules. Using free PhaF phasins, obtained by releasing these from the PHA granule surface by freeze/thaw cycles or by treatment with rhamnolipids, no DNA binding could be observed (chapter 2). However, when the PhaF phasin remained associated with PHA granules, DNA binding activity could be observed when magnesium was present (chapter 3). In these experiments, it is the binding of DNA by PHA granules that is measured. In principle, this binding could be mediated by any of the components of the PHA granule, to wit: the surface bound lipids, one or several of the surface bound proteins, or naked PHA strands exposed on the granule surface. These options are discussed in chapter 3. Briefly, binding of DNA to granule bound lipid head groups is unlikely, since the same lipid head groups are present on the intracellular face of the cytoplasmic membrane, and binding of DNA to the cytoplasmic membrane is specific and protein mediated (Firshein, 1989; Newman *et al.*, 2000). Binding of naked aliphatic PHA to charged polar DNA is not expected, and was not observed when DNA was added to boiled granules. This leaves binding to one or more proteins on the granule surface. Candidates include PhaC1, PhaC2, PhaZ, PhaF and PhaI. Of these PhaF shows the expected behavior: there is DNA binding to PhaF containing granules, and no DNA binding when this protein is knocked out. Finally, given the high homology of the C-terminal domain of PhaF with that of AlgP (67 %), as well as the work of Medvedkin and coworkers on DNA binding by synthesized \((\text{AAKP})_n\) and \((\text{AAKP})_4\text{AAKTA(AAKP)}_4\) oligomers (Medvedkin *et al.*, 1995), the available data strongly favor binding of DNA to PHA granules via the PhaF phasin.

DNA binding to granule-bound PhaF is at odds with the model proposed by Prieto and coworkers. This model is based on the idea that free PhaF binds specifically to the Pc1 and Pi promoters, while granule-bound PhaF does not bind to these two promoters, thereby allowing their expression. However, chapter 3 shows that there was binding of DNA to PhaF containing granules regardless of the size, origin or sequence of the DNA fragments used.
This result refutes the idea of specific DNA binding to the Pc1 and Pi promoters, as well as the idea that DNA binding does not occur when PhaF is bound to the PHA granule.

**A:** Growth is not limited

![Diagram showing growth is not limited](image)

**B:** Growth starts to be limited

![Diagram showing growth starts to be limited](image)

**C:** Growth is limited

![Diagram showing growth is limited](image)

**Figure 1: Model for the interaction of PhaF with PHA granules and DNA.** Under conditions favorable for PHA accumulation, granule-bound PhaF binds to DNA and gradually brings the bacterial cell to a resting state. **A:** Bacterial growth is not limited, and no PHA accumulation occurs. Low amounts of PhaF are found in the cytoplasm; **B:** Beginning of the limited growth conditions. PHA starts to be accumulated and the production of PhaF increases. PhaF binds to the PHA granule and to DNA; **C:** Bacterial growth is limited and large amounts of PHA are present in the cells. A large amount of PhaF binds both the PHA granule and DNA.

The above data can be explained by the model shown in figure 1. In this model, the PhaF phasin acts as a global inhibitor of transcription and brings the bacterial cell to a resting state when growth conditions are limited. When there is no limitation, the bacterium does not produce PHA and the PhaF phasin is found only in low amounts, in the cytoplasm (panel A).
As soon as growth conditions deteriorate and an essential nutrient (typically nitrogen) limitation occurs, PHA accumulation is triggered, the production of PhaF phasin increases and phasins bind to the PHA granule surface. The granule-bound PhaF proteins then bind DNA (panel B). As the amount of PHA increases, the amount of granule-bound PhaF rises, and more DNA is bound, thereby gradually inhibiting global transcription and effectively bringing the bacterium to a resting state (panel C).

Based on the estimates of chapter 3, cells which have accumulated 30 % PHA contain about 20’000 granule-bound PhaF molecules, and between 15 and 50 base pairs of DNA are bound by each PhaF molecule. This represents a total of 300’000 to 1’000’000 bp bound. The size of the Pseudomonas putida genome being approximately 6’100’000 bp, 5 to 15 % of the whole genome could therefore be covered by PhaF phasins. In E. coli the expression of the 18 kDa Chlamydia trachomatis Hc1 protein to about 6 % of the total soluble protein fraction, or about 200’000 Hc1 proteins, results in a complete condensation of the chromosomal DNA (about 5’200’000 bp) (Barry III et al., 1992). Although based on these two calculations the amount of Hc1 proteins is ten times higher than that of granule-bound PhaF phasins, an effect of PhaF on global transcription remains possible, considering that a complete coverage of the genomic DNA might not be necessary. According to the model of figure 1, PhaF inhibition of global transcription would increase with increasing PHA in the cell, and decrease as PHA is consumed. This could explain why a long lag phase is observed before the recovery of a normal growth rate when PHA producing cells are transferred from media which limit growth to media without nutrient limitations (unpublished observations in our laboratory).

This model should be confirmed by assaying global transcription in the P. putida GPo1 wild-type strain and its phaF-negative mutant P. putida GPo1-XFT in order to determine whether or not an inhibition occurs and whether this inhibition is linked to the presence of PhaF. In addition, the effect of the presence or absence of PhaF on the lag phase observed by growth media change should be investigated.

5.2. Activity of promoters of the pha gene cluster

The role of PhaD, PhaF and PhaI in the PHA accumulation process was investigated using β-galactosidase-based reporter constructs inserted in the chromosome of P. putida GPo1 and the phaD, phaF and phaI knockout mutants. The activity of the Pc1, Pc2, Pf and Pi
promoters were measured and a model was presented in chapter 4 to explain the regulation of the *pha* genes in the presence of octanoate.

In this model (figure 2), the presence of octanoate triggers an increase of the Pi promoter driven expression, resulting in the synthesis of more PhaI phasins, which appears to block the Pc2 promoter driven expression. The activity of this promoter being repressed, the PhaD protein can be synthesized via its own promoter. The activity of the Pf and Pi promoters is further reinforced by the presence of PhaD, and higher amounts of PhaF and PhaI phasins are produced. Both phasins influence their own promoters, PhaF positively and PhaI negatively, until a stable activity is obtained for all the *pha* promoters. At this point, the Pf and Pi promoters drive expression at high levels, the Pc1 promoter at a rather low level, and the Pc2 promoter is almost completely repressed. The numerous effects of PhaD, PhaF and PhaI on the different *pha* promoters show that the regulation of the PHA accumulation process is very complex in *P. putida* GPo1. It is likely that this complexity does not only result from the effects of PhaD, PhaF and PhaI, but also from other factors affecting the activity of the promoters of the *pha* gene cluster.

It is interesting to note that despite the complexity of the PHA accumulation process in *P. putida* GPo1, the introduction of a PHA synthase gene in a suitable *E. coli* strain is sufficient to obtain reasonable levels of PHA accumulation (up to 20%). Of course, in order for this accumulation to occur, PHA precursors need to be available for the PHA synthase. Considering the evolution of PHA accumulation, this is likely to be a very ancient process, as it is found in most genera of eubacteria and in members of the family Halobacteriaceae of the Archaea (Rehm, 2003). However, the only proteins that are shared between most, if not all of these strains are the PHA synthase and depolymerase. Other proteins, such as regulators or phasins, were only identified in defined subcategories of bacteria.

As it appears that only the PHA synthase is necessary for PHA accumulation and only the PHA depolymerase is needed for degradation of the stored material, one can ask why these other proteins emerged. One possible explanation is that PHA initially served as an osmotically neutral carbon and energy storage material only, and with time additional functions were linked to the PHA accumulation process. These functions could for instance include the control of the granule size and number, control of granule surface properties, or the control of the global transcription level via proteins such as PhaF, as proposed in figure 1. Some of these additional functions could have evolved to increase the tolerance of the bacteria to PHA granules, thus allowing more PHA to be accumulated.
Figure 2: Regulation of the pha genes. A: In the absence of octanoate; B: In the presence of octanoate, the Pi driven expression increases and PhaI blocks the Pc2 promoter; C: PhaD is expressed and increases the activity of Pf and Pi; D: PhaF contributes to increasing the Pf activity while PhaI inhibits the Pi promoter; E: a balance between activation and repression effects is reached, where Pc1 is active at a low level, Pc2 is turned off, and Pf and Pi are active at high levels.
Although the phasins are not necessary for PHA accumulation, it remains of interest to understand the influence of the different additional proteins that are part of this process; and that independently of the bacterial strain in which they are found. Indeed, they may well provide elegant ways to circumvent present or future problems encountered in recombinant bacteria or plants in which only a PHA synthase is expressed, and thus contribute to increase the amount of PHA that can be accumulated in these mutants.

5.3. Outlook

Further work should be carried out in order to corroborate or refute the different hypotheses proposed based on the work presented in the thesis. To find out whether or not PhaF phasins are involved in granule partitioning during cell division, microscopic observation of dividing bacteria containing PHA granules with and without PhaF should be undertaken. Similarly, an effect of PhaF on global transcription should be further investigated. This could be done for instance by testing the wild-type strain and the phaF-knockout for survival under various conditions such as UV exposure, drought or temperature variations. The survival rates should be measured for cultures grown in different media, so that the influence of PhaF is investigated in the presence or absence of PHA granules or octanoate. Furthermore, overexpression and purification of PhaF should be undertaken to obtain pure PhaF phasins. Gel shift assays or DNA protection assays could then be carried out to fully elucidate the DNA binding ability of free PhaF phasins.
5.4. References


Chapter 6

Technical appendix

Aim

This appendix describes the construction of the knockout strains *Pseudomonas putida* GPo1-XFT and *P. putida* GPo1-XIT, the identification of the Pc2 promoter as well as interesting preliminary results obtained with the Pc1 promoter. The author is aware of the fact that these results should be confirmed and extended by further experimental work.
6.1. Generation of *Pseudomonas putida* GPo1 *phaF* and *phaI* knockouts

To investigate the role of the *phaF* and *phaI* genes, knockouts were constructed by gene-replacement using the pEX18Ap vector (Hoang *et al.*, 1998). This vector allows a site-specific insertion of a DNA marker in the genome of *Pseudomonas* strains by crossover recombination. It possesses an origin of replication for *E. coli* strains and an origin of transfer to facilitate its transfer into *Pseudomonas* strains by triparental mating. It lacks an origin of replication for *Pseudomonas* and can only survive if a single crossover recombination occurs. Double crossover recombination events can be selected for using the *Bacillus subtilis* gene *sacB* present on the pEX18Ap vector, which renders sucrose toxic (Schweizer, 1992).

6.1.1. Bacterial strains and culture conditions

The construction of the two knockouts was performed using the strains and plasmids listed in table 1. *E. coli* strains were grown at 37°C in complex Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). *P. putida* strains were grown at 30°C in LB medium. After triparental mating, transconjugants were selected on E2 plates (Lageveen *et al.*, 1988) containing 0.2% citrate as carbon source. To favor double crossover recombination, the *P. putida* GPo1 mutants were grown on LB plates supplemented with 5% sucrose (Schweizer, 1992). For PHA granule isolation, the *P. putida* GPo1 mutants were grown overnight at 30°C in 50 ml shake flasks. These cultures were carried out with the 0.2NE2 nitrogen-limited medium described in Chapter 2 and 15 mM octanoate as carbon source.

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### Table 1: Strains and plasmids used to generate \( \text{phaF} \) and \( \text{phaI} \) knockouts of \( P. \text{putida} \) GPo1.

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<tr>
<td>pEX18Ap</td>
<td>Ap(^r); knockout plasmid</td>
<td>(Hoang et al., 1998)</td>
</tr>
<tr>
<td>pIds</td>
<td>pCR2.1Topo with region downstream of ( \text{phaI} )</td>
<td>This study</td>
</tr>
<tr>
<td>pIus</td>
<td>pCR2.1Topo with region upstream of ( \text{phaI} )</td>
<td>This study</td>
</tr>
<tr>
<td>pFds</td>
<td>pCR2.1Topo with region downstream of ( \text{phaF} )</td>
<td>This study</td>
</tr>
<tr>
<td>pFus</td>
<td>pCR2.1Topo with region upstream of ( \text{phaF} )</td>
<td>This study</td>
</tr>
<tr>
<td>pXI</td>
<td>pEx18Ap with regions upstream and downstream of ( \text{phaI} )</td>
<td>This study</td>
</tr>
<tr>
<td>pXF</td>
<td>pEX18Ap with regions upstream and downstream of ( \text{phaF} )</td>
<td>This study</td>
</tr>
<tr>
<td>pXFT</td>
<td>pEX18Ap with regions upstream and downstream of ( \text{phaI} ) and Tc gene</td>
<td>This study</td>
</tr>
<tr>
<td>pXIT</td>
<td>pEX18Ap with regions upstream and downstream of ( \text{phaF} ) and Tc gene</td>
<td>This study</td>
</tr>
</tbody>
</table>

Where appropriate, antibiotics were added at the following concentrations: ampicillin, 100 \( \mu \)g/ml; kanamycin, 50 \( \mu \)g/ml; tetracycline, 15 \( \mu \)g/ml. Screening for the loss of \( \beta \)-galactosidase activity was done by adding 100 \( \mu \)g/ml IPTG and 15 \( \mu \)g/ml XGAL to the medium or by restriction analysis.

### 6.1.2. DNA manipulation

DNA manipulations and other molecular biology techniques were essentially performed as described before (Sambrook et al., 1989). Triparental mating was performed as described in Chapter 4. The amplification of the DNA region upstream of \( \text{phaF} \) was done with the Fusfw and Fusrv primers (table 2), and that of the downstream region with Fdsfw and Fdsrv. Similarly, the amplification of the DNA region upstream of \( \text{phaI} \) was done with
the Iusfw and Iusrv primers, and that of the downstream region with Idsfw and Idsrv. All four fragments were amplified using \textit{P. putida} GPo1 genomic DNA as template.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Fusfw</td>
<td>5'–CGCTGCAGGCCGCCGGAAAGCACG–3'</td>
</tr>
<tr>
<td>Fusrv</td>
<td>5'–CTTCTTGGGATCCATCCTGCTC–3'</td>
</tr>
<tr>
<td>Fdsfw</td>
<td>5'–CCGGATCCCTGATCTGATACCGG–3'</td>
</tr>
<tr>
<td>Fdsrv</td>
<td>5'–ACCGGGAATTCGTGCTGCCAACAG–3'</td>
</tr>
<tr>
<td>Iusfw</td>
<td>5'–GAGCACGCACATCTAGAGGTG–3'</td>
</tr>
<tr>
<td>Iusrv</td>
<td>5'–TGGATCCATGCTCTGACCTCATGCTC–3'</td>
</tr>
<tr>
<td>Idsfw</td>
<td>5'–AAAGGGATCCTGTAGAAAAGGACGC–3'</td>
</tr>
<tr>
<td>Idsrv</td>
<td>5'–AGATCAGGGTACCCGCTGTCG–3'</td>
</tr>
<tr>
<td>Tcfw</td>
<td>5'–CCCGTCCTGTGGATACTCTACGCCG–3'</td>
</tr>
<tr>
<td>Tcrv</td>
<td>5'–GCGTCCGGCGGTAGATCCACAGGAGCC–3'</td>
</tr>
<tr>
<td>pUTfw</td>
<td>5'–CTAGACTGTCTCTTGGATCGAGTCCTTGCG–3'</td>
</tr>
<tr>
<td>pUTrv</td>
<td>5'–AATTCTGACTCTTATACACAGTGCGGC–3'</td>
</tr>
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<td>phaFfw</td>
<td>5'–GGAGAGCCATATGGCTGGCAAGAAGAAT–3’</td>
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<tr>
<td>phaFrv</td>
<td>5'–GGCGACGCAGGATCCAGGATCCAGGACGG–3’</td>
</tr>
<tr>
<td>phaIfw</td>
<td>5'–GAGCATGAGGAATCCAGCAGGCCAAGAAGAAT–3’</td>
</tr>
<tr>
<td>phaIrv</td>
<td>5'–ATTCTTCTTGCCAGGATCTCCTC–3’</td>
</tr>
</tbody>
</table>

\textbf{Table 2: Sequences of the PCR primers used for amplification.}

The plasmid pUT-Tc contains a tetracycline resistance gene that is known to be expressed well in \textit{P. putida} GPo1. It possesses a strong T7 terminator. The tetracycline resistance cassette from this vector was cloned in pCR2.1Topo by PCR using the pUTfw and pUTrv primers, yielding pCR2.1Topo-Tc. The \textit{BamHI} restriction site present in the tetracycline resistance gene was removed by a single point silent mutation, replacing the ATT codon encoding for an isoleucine by an ATA codon. This Quik-Change PCR was performed using the Tcfw and Tcrv primers and resulted in the pTCB plasmid.
6.1.3. Colony blotting

Probes for blotting were obtained by PCR using 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 100 µM dTTP, and 100 µM DIG labeled dUTP. The phaF probe was obtained from *P. putida* GPl genomic DNA using the primers phaFfw and phaFrv. Similarly, the phaI probe was obtained using the primers phaffw and phaIr. The tetracycline probe was obtained from pTCB using the primers pUTfw and pUTrv.

Colonies present on plates grown overnight at 30°C were lifted using a positively charged Nylon membrane. DNA was bound to the membrane by drying the filter at 120°C for 30 minutes.

Prehybridization was carried out by incubating the membrane for 1 hour at 68 °C in hybridization buffer (5x SSC / 0.1% N-lauroylsarcosine / 0.02% SDS / 2% blocking reagent). The DNA probe was denatured in a boiling water bath for 10 minutes and immediately chilled on ice. The membrane was incubated overnight at 68°C in hybridization buffer containing 5 µl of the denatured probe. After hybridization, the membrane was first washed twice for 5 minutes at room temperature in 2x SSC containing 0.1% SDS, and then twice 15 minutes at 68°C in 0.5x SSC containing 0.1% SDS.

To detect the bound DIG labeled probe, the membrane was equilibrated for 1 minute in buffer 1 (100 mM maleic acid, 150 mM NaCl, pH 7.5). It was then incubated for 30 minutes in buffer 1 containing 1 % (w/v) blocking reagent (buffer 2). Anti-DIG alkaline phosphatase was diluted 1:10000 times in buffer 2 and the membrane was incubated in this new buffer for another 30 minutes. The membrane was then washed twice 15 minutes in buffer 1 containing 0.3% Tween 20. After washing, the membrane was equilibrated in buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl2) for 2 minutes. The membrane was placed in a hybridization bag and 5 µl CSPD diluted in 500 µl buffer was added. The bag was placed at 37°C for 15 minutes before exposure to an X-ray film.

Old probes were stripped from the blots by rinsing the membranes with water, incubating them twice for 15 minutes at 37°C in 0.2 M NaOH containing 0.1% SDS and once for 5 minutes at room temperature in 2x SSC buffer.
6.1.4. Construction of *P. putida* GPo1 *phaF* and *phaI* knockouts

In order to create knockouts of the *phaF* and *phaI* genes of *P. putida* GPo1 by double crossover recombination, regions upstream and downstream of the targeted gene were amplified by PCR. In the case of *phaF*, the entire open reading frame was replaced by a tetracycline resistance gene. The amplified regions, Fus and Fds, are shown in figure 1.

**Figure 1: Double crossover region for the generation of the *phaF* knockout of *P. putida* GPo1.** A: Layout of the *pha* gene cluster and location of the Fus and Fds regions amplified by PCR. B: DNA sequence at the start and stop codon of *phaF*. The *phaF* start codon is given in bold and its stop codon is underlined. The bases shown in italics are the *BamHI* sites flanking the tetracycline cassette.

In the case of *phaI*, replacing the entire open reading frame was not possible because this would result in the deletion of the Pf promoter region, thus preventing expression of the PhaF phasin. We therefore decided to insert the tetracycline resistance at the start codon of the *phaI* gene and to add a stop codon in frame with the rest of the *phaI* sequence, leaving the Pf promoter unchanged (figure 2). The two amplified regions are denoted Ius and Ids.

**Figure 2: Double crossover region for the generation of the *phaI* knockout of *P. putida* GPo1.** A: Layout of the *pha* gene cluster and location of the Ius and Ids regions amplified by PCR. B: DNA sequence at the start codon of *phaI*. The *phaI* start codon is given in bold. The bases shown in italics are the *BamHI* sites flanking the tetracycline cassette represented by #.
Figure 3: Construction of pXF and pXI. A: The Fus and Fds fragments were inserted into pEX18Ap to yield pXF. B: The Ius and Ids fragments were inserted into pEX18Ap to yield pXI.
After amplification and insertion in the pCR2.1Topo vector, resulting in the pFus, pFds, pIus and pIds plasmids, the four fragments were sequenced to ensure that point mutations had not been produced by the PCR. The pFus plasmid was then cut with PstI and BamHI and the pFds plasmid with BamHI and EcoRI, and both fragments were inserted into a PstI-EcoRI digested pEX18Ap vector resulting in the plasmid pXF (figure 3A). Similarly, the plus plasmid was digested with XbaI and BamHI and the plds plasmid with BamHI and Asp718 and both fragments inserted into an XbaI-Asp718 digested pEX18Ap vector resulting in the plasmid pXI (figure 3B).

The pTCB plasmid was then digested with BamHI and the fragment containing the tetracycline resistance cassette was inserted in the BamHI site of pXF and pXI, yielding pXFT and pXIT respectively (figure 4).

Figure 4: Construction of pXFT and pXIT. The 2.2 kb tetracycline resistance cassette from pTCB was inserted as a BamHI fragment into pXF and pXI, yielding pXFT and pXIT respectively.
The pXFT and pXIT knockout vectors were introduced in *P. putida* GPO1 by triparental mating. Between 10 and 15 colonies were obtained for each of the two constructs. These were then replated several times on LB plates containing 5% sucrose and 15 µg/ml tetracycline in order to favor double crossover recombination events, thereby removing functional copies of the targeted knockout gene and the remaining part of the knockout vector. About 50 colonies of each knockout were obtained.

6.1.5. Analysis of both mutant by colony blotting

Screening for mutants in which double crossover recombination had taken place was done by colony blots. For each desired knockout, 25 of the colonies obtained after several rounds of plating on sucrose-containing LB plates were selected, and their DNA was crosslinked to a positively charged Nylon membrane and hybridized with a tetracycline probe to make sure that at least a single crossover event had occurred. These blots show that all colonies contained the tetracycline resistance gene. The tetracycline probe was then stripped from the blots and the membranes were hybridized with either the *phaF* or the *phaI* probe. One of the putative *phaF* knockout colonies appeared not to hybridize with the *phaF* probe, indicating a possible double crossover recombination.

All of the putative *phaI* knockout colonies hybridized with the *phaI* probe. In this case however, we did not expect to find colonies that do not hybridize, since the complete *phaI* open reading frame was not removed. It was rather expected that a colony resulting from a double crossover recombination and hence containing only one copy of *phaI* would give a weaker signal than a colony where a single crossover recombination occurred. Based on this assumption, three double crossover candidate colonies could be selected for further analysis.

6.1.6. Analysis of PHA granules isolated from both mutants

In order to confirm the knockout of the *phaF* or *phaI* gene, the colonies selected above were grown overnight in 50 ml 0.2NE2 medium containing 15 mM octanoate. The PHA granules accumulated by these mutants were then isolated and analyzed by electrophoresis on a 12% SDS polyacrylamide gel as described in chapter 2 (figure 5).
Figure 5: Analysis of the proteins found on the surface of PHA granules isolated from putative *P. putida* GPo1 knockouts. **XFT**: putative *P. putida* GPo1-XFT; **XIT1**: putative *P. putida* GPo1-XIT #1; **XIT2**: putative *P. putida* GPo1-XIT #2; **XIT3**: putative *P. putida* GPo1-XIT #3.

Figure 5 shows that the PhaF protein is absent for the putative *phaF* knockout *P. putida* GPo1-XFT mutants selected by Southern blotting, confirming that a double crossover event had indeed occurred.

The analysis of the putative *phaI* knockout mutants was more difficult. In all selected mutants, the amount of PHA was significantly lower than in the wild-type, making the granule isolation more difficult. This resulted in lower amounts of protein visible on the protein gel. Of the three samples originating from putative *phaI* knockouts, the first (XIT1) still has both the PhaF and the PhaI proteins and is most likely from a colony were only a single crossover event happened. The second (XIT2) and third (XIT3) putative *phaI* knockouts, however, both appear to lack PhaF and PhaI. The putative *P. putida* GPo1-XIT #3 was further investigated by PCR.

**6.1.7. Analysis of the *phaI* knockout by PCR**

Several polymer chain reactions were carried out to confirm the insertion of the tetracycline cassette in the *pha* gene cluster in the *P. putida* GPo1-XIT knockout and to verify whether a double crossover had taken place (figure 6).
Amplification of the *phaI* and *phaF* open reading frames of *P. putida* GPo1 and *P. putida* GPo1-XIT are shown in figure 6A. While *phaF* is amplified in both cases, *phaI* is only amplified with *P. putida* GPo1 genomic DNA. This was expected since the *phaI* forward primer used was designed to bind the original DNA sequence, and not the modified *phaI* start sequence present in the *P. putida* GPo1-XIT knockout.

A DNA fragment containing the *phaI* gene and its promoter region was therefore amplified using the phaIr and the EcoRIPi (5′-CTTCAGGAATTCGCCATGACCCAGAT-3′) primers. In this case, the presence of the tetracycline cassette should increase the size of the amplified fragment by 2.2 kb, resulting in a 3 kb DNA fragment. Furthermore, digesting this DNA fragment with *BamHI* should yield the 2.2 kb tetracycline cassette and two fragments of 420 bp and 427 bp. The 3 kb DNA fragment is visible in lane 1 of figure 6B, and the *BamHI* digested fragment in the lane 2. In both cases, the expected fragments were found, confirming the expected gene arrangement. The 1.35 kb band visible in lane 1 of figure 6B most likely results from an unspecific DNA amplification as it is too large to represent the *phaI* gene and its promoter.
6.1.8. Growth of the wild-type and its knockouts

The growth of the wild-type strain *P. putida* GP01 and its *phaD*, *phaF* and *phaI* knockouts in E2 and 0.2NE2 media containing 15 mM octanoate was followed in 50 ml shake flasks (figure 7).

![Figure 7: Growth of the wild-type *P. putida* GP01 strain and the *phaD*, *phaF* and *phaI* knockouts. A: Growth in E2 minimal medium containing 15 mM octanoate; B: Growth in 0.2NE2 minimal medium containing 15 mM octanoate.](image)

In E2 minimal medium, where no limitation is present and hence no PHA is accumulated, all four strains reach cell densities of 1.5 to 2 g/l. In contrast, in 0.2NE2 medium nitrogen is limiting, and PHA accumulation is observed. While the *P. putida* GP01 wild-type and its *phaF* knockout mutant *P. putida* GP01-XFT reach a final cell density of 1.3 g/l, the *phaD* knockout mutant *P. putida* GP01001 and the *phaI* knockout mutant *P. putida* GP01-XIT only reach 0.6 g/l. All four strains grow similarly during the first 8 hours, at which point the *phaD* and *phaI* knockouts stop growing. Observation of the four strains under the microscope showed that unlike the wild-type *P. putida* GP01 and the *phaF* knockout, the *phaD* and *phaI* knockouts apparently accumulate less PHA. This could explain the difference in growth behavior observed, since the measured cell density is influenced by the presence of the PHA granules inside the bacterial cells (Kraak, 1998). The further increase in cell density observed for the wild-type strain *P. putida* GP01 and the *phaF* knockout *P. putida* GP01-XFT is therefore most likely due to PHA accumulation.
6.1.9. Discussion

The generation of *P. putida* GPo1 mutants lacking either the *phaI* or the *phaF* gene was carried out to obtain strains suitable to study the influence of the PhaI and PhaF phasins on the activity of the different *pha* gene cluster promoters.

The generation of the *phaF* negative mutant was achieved by replacement of the target gene by a resistance marker via double crossover recombination. Removing the complete open reading frame was possible since no other gene encoding in the same direction is found after *phaF*. This was not the case for the *phaI* knockout, where a complete removal of the open reading frame would also have removed the Pf promoter. There, it was necessary to knock out the gene without replacing a DNA fragment. This was achieved by inserting a resistance marker with terminator at the start of the *phaI* open reading frame, leaving the rest of the DNA sequence intact. The resistance marker used was obtained from a mini-Tn5 derived plasmid used to randomly insert single copies of a gene cassette into the genome of Gram-negative bacteria lacking the π protein. One of the advantages of using a resistance marker coming from this construct is that it possesses strong terminators on both sides of the resistance gene, preventing undesired read-through from the resistance gene promoter.

Screening of the putative knockouts was performed by colony blotting. However, this did not yield satisfactory results, especially in the case of the *phaI* knockout. Due to the procedure used, most of the *phaI* open reading frame is still present in the knockout, and hybridization of the *phaI* probe was therefore expected. Under these conditions, differentiating between single and double crossover events was difficult. Considering the intensity of the detected probe on the blot to be proportional to the number of copies of *phaI*, it was nevertheless possible to select a few colonies for further investigation.

A rather straightforward method to ensure the absence of PhaF or PhaI in the mutants is to check the proteins present on the surface of the PHA granules they accumulate. This analysis showed that the mutant selected as a *phaF* knockout did indeed lack the PhaF phasin. In the case of the three putative *phaI* knockouts the isolation of the PHA granules was however more difficult due to a lower amount of PHA accumulated; only two of them were lacking PhaI on the granule surface. This result was expected since the colony blot screening was not as effective as for the *phaF* knockout. The lack of PhaF on the granules isolated from this *phaI* knockout mutant was however unexpected since the Pf promoter had been retained.
during the knockout generation process. To make sure that no undesired mutation occurred in the *pha* gene cluster, analysis of DNA fragments from various PCR amplification was carried out and the expected genotype was confirmed. The absence of the PhaF phasin from the PHA granule surface in the *phaI* knockout is therefore most likely due to an effect of PhaI on the activity of the Pf promoter or to a necessary interaction of PhaI with PhaF to allow its binding to the PHA granule. A disruption of the *phaF* open reading frame along with that of the *phaI* open reading frame could however still be possible and this explanation should not be completely ruled out. Complementation of both knockouts with vectors containing *phaF* or *phaI* under an inducible promoter should help to resolve this issue.

Comparison of the two knockout strains with the wild-type *P. putida* GPo1 and its *phaD* knockout *P. putida* GPo1001 (Klinke et al., 2000) showed normal growth in E2 minimal medium containing 15 mM octanoate. However, in the nitrogen-limited 0.2NE2 medium, containing 15 mM octanoate as carbon source, a difference is visible. While the increase on OD$_{600}$ of *P. putida* GPo1001 and *P. putida* GPo1-XIT stops after 8 to 10 hours, that of *P. putida* GPo1 and *P. putida* GPo1-XFT continues. As it is known that intracellular PHA increases the observed optical density (Kraak, 1998), the increase in cell density of figure 7 is possibly due to normal PHA accumulation in *P. putida* GPo1 and *P. putida* GPo1-XFT, and decreased PHA accumulation in *P. putida* GPo1001 and *P. putida* GPo1-XIT. In order to investigate if such a difference in PHA accumulation exists, the quantity of PHA present in the cell at the various time points should be assayed. Furthermore, observations of the cultures by electron microscopy should be carried out to investigate the PHA granule size and number since they might be influenced by the absence of PhaF or PhaI.
6.2. The Pc2 promoter of *Pseudomonas putida* GPo1

In *P. putida* GPo1, the existence of at least 3 promoters in the *pha* gene cluster has been shown by Prieto and coworkers (Prieto *et al.*, 1999). These promoters are located upstream of *phaC1*, *phaF* and *phaI* and are denoted Pc1, Pf and Pi respectively. Although it is known that Pc1 is responsible for the expression of the PhaC1 polymerase and the PhaZ depolymerase, no promoters have thus far been identified that regulate the expression of the PhaC2 polymerase and the PhaD protein.

Sequence analysis has shown the existence of two inverted repeats directly downstream of *phaZ* and *phaD* (Huisman *et al.*, 1991), the presence of actual terminators at these locations has not been confirmed thus far. Furthermore, in his analysis of the mRNA pattern of the *pha* locus, Bühler was not able to establish whether or not the *phaC2* gene is expressed by the same mRNA as *phaC1*. Indeed, no hybridization bands could be obtained with mRNA transcripts isolated from *P. putida* GPo1 and the *phaC2* probe, thus leaving the question of the existence of a terminator downstream of *phaZ* and that of a Pc2 promoter open (Bühler, 1998). An indication that *phaC2* does have its own promoter was obtained using the pETQ2 plasmid (Ren, 1997). This vector contains *phaC2* and its presumed upstream promoter region. When transferred to a suitable host such as *E. coli* JMU193 or JMU194, this plasmid allows PHA accumulation that can only result from an expression of the polymerase encoded by *phaC2* (Ren, 1997). We therefore carried out experiments to test for the presence of a Pc2 promoter by assaying for PHA production in *E. coli* JMU194 and the PHA-deficient *P. putida* GPp104 mutant harboring pETQ2 and a plasmid derived from it containing the Ω terminator.

6.2.1. Bacterial strains and culture conditions

*E. coli* strains were grown at 37°C in complex Luria-Bertani (LB) medium (Sambrook *et al.*, 1989), while *P. putida* strains were grown at 30°C in the same medium. If necessary, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml.
<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant genotype or phenotype</th>
<th>Source</th>
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<td>JMU194</td>
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<td>(Rhie et al., 1995)</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em>:</td>
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<td></td>
</tr>
<tr>
<td>GPp104</td>
<td>PHA- mutant</td>
<td>(Huisman et al., 1991)</td>
</tr>
<tr>
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<td>Source</td>
</tr>
<tr>
<td>pJRD215</td>
<td><em>Km</em>&lt;sup&gt;r&lt;/sup&gt;, <em>Sm</em>&lt;sup&gt;r&lt;/sup&gt;, RSF1010 <em>ori</em>, p15A <em>ori</em>, <em>mob&lt;sup&gt;+&lt;/sup&gt;</em></td>
<td>(Davison et al., 1987)</td>
</tr>
<tr>
<td>pETQ2</td>
<td><em>Km</em>&lt;sup&gt;r&lt;/sup&gt;, <em>Sm</em>&lt;sup&gt;r&lt;/sup&gt;, <em>phaC2</em> containing pJRD215</td>
<td>(Ren, 1997)</td>
</tr>
<tr>
<td>pETQ2Ω</td>
<td><em>Km</em>&lt;sup&gt;r&lt;/sup&gt;, <em>Sm</em>&lt;sup&gt;r&lt;/sup&gt;, <em>phaC2</em>, Ω terminator, pETQ2 derivative</td>
<td>(Ren, 1997)</td>
</tr>
</tbody>
</table>

Table 3: strains and plasmids used.

DNA manipulation and other molecular biology techniques were essentially performed as described previously (Sambrook et al., 1989).

6.2.2. PHA assay

To determine the polyester content of the bacteria, cells were grown in 0.2NE2 minimal medium supplemented with 15 mM octanoate. 0.1% yeast extract was added for *E. coli* cells cultures in order to sustain growth. After methanolation of lyophilized cell material in the presence of 15% sulfuric acid, the hydroxyacyl methyl esters obtained were analyzed by gas chromatography to determine the amount of PHA present in the cells (Lageveen et al., 1988).

6.2.3. Confirmation of the existence of a Pc2 promoter in *P. putida* GPo1

Plasmid pETQ2, carrying the *phaC2* gene and the upstream 117 bp, has been previously reported to enable *E. coli* to accumulate PHA (Ren, 1997). Even though the expression of *phaC2* could be due to the native promoter, it could also result from read-through expression of a preceding promoter present on the plasmid. Furthermore, this expression has been obtained in *E. coli* strains but was not tested in *Pseudomonas putida* GPo1. The pETQ2 and its derivative pETQ2Ω, which carries the Ω terminator in front of the putative Pc2 promoter, are based on the broad host range vector pJRD215, and can therefore
be used not only in *E. coli* but also in *P. putida* (Davison *et al.*, 1987). Both plasmids were therefore transferred to the PHA negative mutant *P. putida* GPp104 and *E. coli* JMU194, and the amount of PHA accumulated by these four transformants when grown under nitrogen limitation with 15 mM octanoate as carbon source was measured (table 4).

<table>
<thead>
<tr>
<th>Strain</th>
<th>PHA %</th>
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<tbody>
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<tr>
<td>GPp104 [pETQ2Ω]</td>
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</tbody>
</table>

Table 4: Percentage of PHA accumulated by *E. coli* JMU194 and *P. putida* GPp104 carrying pETQ2 or pETQ2Ω.

The use of pETQ2Ω in *E. coli* JMU194 results in the loss of the PHA accumulation phenotype observed with pETQ2, indicating that in this construct the *phaC2* gene is probably expressed from another promoter than its own. However, both plasmids allow PHA accumulation in *P. putida* GPp104, confirming the existence of a Pc2 promoter allowing gene expression in *Pseudomonas*, but not in *E. coli*. 
6.3. The Pc1 promoter of *Pseudomonas putida* GPo1

The Pc1 promoter region used to construct the Pc1::lacZ reporter present in the *P. putida* GPG-132 genome was amplified by PCR from the pGec405 plasmid (Prieto *et al.*, 1999). This plasmid contains an *EcoRI* fragment of the *P. putida* GPo1 genome that harbors a functional part of the *pha* gene cluster (Huisman *et al.*, 1992). The complete sequence upstream of *phaC1* was amplified, resulting in a 555 bp long Pc1 promoter region. Huisman and coworkers reported transcription start sites for *phaC1* located 124 bp and 209 bp upstream of its start codon (Huisman, 1991), and the necessity of a long promoter is therefore questionable. In addition to the identified transcription start sites, sequence analysis has shown secondary structures in the downstream half of the Pc1 promoter region, but not in the upstream region. This also suggests that the Pc1 promoter might be shorter than the sequence used in the *P. putida* GPG-132 Pc1::lacZ reporter. In order to investigate this possibility, a shortened version of the Pc1::lacZ reporter consisting of a 340 bp long Pc1 promoter region was created and the expression level of both constructs was assayed.

6.3.1. Bacterial strains and culture conditions

*E. coli* strains were grown at 37°C in complex Luria-Bertani (LB) medium (Sambrook *et al.*, 1989), while *P. putida* strains were grown at 30°C in the same medium. To study the β-galactosidase activity resulting from the reporters, the *P. putida* strains were grown in E2 minimal medium (Lageveen *et al.*, 1988). If necessary, antibiotics were added at the following concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant genotype or phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em>:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH10B</td>
<td>Host for <em>E. coli</em> plasmids</td>
<td>(Hanahan, 1983)</td>
</tr>
<tr>
<td>HB101</td>
<td>Host for helper plasmids RK600</td>
<td>(Sambrook <em>et al.</em>, 1989)</td>
</tr>
<tr>
<td>CC118λpir</td>
<td>Host for pUT-derived plasmids</td>
<td>(Herrero <em>et al.</em>, 1990)</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPo1</td>
<td>Wild-type strain</td>
<td>(Schwartz <em>et al.</em>, 1973)</td>
</tr>
<tr>
<td>GPG-132</td>
<td>GPo1 with Pc1::lacZ</td>
<td>(Prieto <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>GPo1-Pc1-short</td>
<td>GPo1 with shorter Pc1::lacZ</td>
<td>This study</td>
</tr>
</tbody>
</table>
### Table 5: Strains and plasmids used.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Relevant genotype or phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>RK600</td>
<td>Helper plasmid, <em>tra</em>&lt;sup&gt;+&lt;/sup&gt;, <em>mob</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(Figurski et al., 1979)</td>
</tr>
<tr>
<td>pUT-Km</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;; mini-Tn5 delivery plasmid</td>
<td>(de Lorenzo et al., 1990)</td>
</tr>
<tr>
<td>pUJ9</td>
<td><em>lacZ</em>α reporter construction plasmid</td>
<td>(de Lorenzo et al., 1990)</td>
</tr>
<tr>
<td>pPG132</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;; Pc1::<em>lacZ</em> reporter, pUT-Km derivative</td>
<td>(Prieto et al., 1999)</td>
</tr>
<tr>
<td>pUT-Km-Pc1-short</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;; shorter Pc1::<em>lacZ</em> reporter, pUT-Km derivative</td>
<td>This study</td>
</tr>
<tr>
<td>pUJ9-Pc1-short</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; shorter Pc1::<em>lacZ</em> fusion construct</td>
<td>This study</td>
</tr>
</tbody>
</table>

### 6.3.2. DNA manipulation

DNA manipulation and other molecular biology techniques were essentially performed as described previously (Sambrook et al., 1989). Triparental mating was performed as described in Chapter 4. The Pc1-short promoter region was amplified by PCR from genomic DNA using the primers EcoRIPc1-short (5′-TGGGCGAATTCCTCGACGAACCTG-3′) and BamHIPc1 (5′-AACGACGGGATCCATCTACGACGCTCCGTTGCTCC-3′), and inserted in pUJ9 to yield pUJ9-Pc1-short.

### 6.3.3. Construction of *lacZ* reporter gene fusions

Sequence analysis of the Pc1 promoter region showed the presence of several secondary structure elements directly upstream of *phaC1* (figure 8). Such elements are, however, not visible on the remaining part of the Pc1 promoter region and the 555 bp long Pc1 promoter used by Prieto et al. (Prieto et al., 1999) was therefore shortened to create a 340 bp long Pc1-short reporter fusion containing only these secondary structure elements.
Figure 8: Secondary structures present in the Pc1 promoter region. The position of the EcoRI Pc1-short primer used to amplify a shortened Pc1 promoter is indicated by the arrow. The potential –35/-10 and –24/-12 promoter regions are indicated, as well as a possible NifA box. Inverted repeats are marked by the IR1 and IR2 labels, and the ribosome binding site by the RBS label.

The shorter Pc1 promoter region was amplified by PCR and inserted as EcoRI-BamHI fragments upstream of the lacZ gene of pUJ9 (de Lorenzo et al., 1990). The resulting plasmid, pUJ9-Pc1-short, was then sequenced in order to ensure that no mutations had occurred during the amplification of the promoter region. The 4.3 kb NotI cassette containing the fusion was subcloned into the mini-Tn5 delivery plasmid pUT-Km (de Lorenzo et al., 1990) (figure 9). The insertion of a single copy of the reporter gene in the wild-type strain P. putida GPo1 was obtained by triparental mating and selection on E2 minimal medium containing 0.2% citrate as carbon source and 50 µg/ml kanamycin.
Figure 9: Construction of the pUJ9-Pc1-short helper plasmids and the pUT-Km-Pc1-short reporter vectors. A: Positions on the *pha* gene cluster of the Pc1 and the Pc1-short promoter fragment amplified by PCR; B: Map of the pUJ9 vector with the insertion point of the different promoters; C: Map of the pUT-Km vector with the insertion point of the different promoter-\(\beta\)-galactosidase reporter constructs.

The expression levels of both constructs in *P. putida* GPO1 were then assayed by measuring the resulting \(\beta\)-galactosidase activity using the assay described in chapter 4. The activity of the Pc1 promoter region was found to be significantly higher than that of the Pc1-short promoter region (results not shown). To make sure that the higher expression level seen with Pc1 is due to the length of the promoter and not to the insertion point of either fusion in the genome of *P. putida* GPO1, five new insertions of each promoter constructs were generated.

Measuring the \(\beta\)-galactosidase activity of each mutant showed that the mutants harboring the longest promoter region had a four-fold higher activity than those with the Pc1-short promoter region (figure 10), and the variation observed within each set of mutants could be due to the insertion points of the transposons or to the assay procedure. The observed activity decrease therefore appears to be due to the DNA sequence removed from the longer version of the promoter. This DNA fragment probably contains structural elements that are not absolutely required for expression, but enhance it. Further studies should be carried out in order to
identify and characterize these elements as well as to determine the minimal Pc1 promoter length necessary for activity.

Figure 10: Activity of the lacZ gene under the control of regions of the Pc1 promoter of different lengths. The Pc1 and Pc1-short reporter constructs were inserted in the wild-type strain *P. putida* GPo1 and the obtained transformants grown in E2 minimal medium containing 15 mM octanoate.
6.4. References


Curriculum Vitae

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Date of birth February 9, 1974
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1980-1986  Primary school in Sion (VS)
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1988-1991  High school in Sion (VS)
1991-1992  Berea High School, Berea, OH, USA
           High School Diploma
1992-1994  High school in Sion (VS)
           Federal Matura (Type C, scientific)
1994-1999  Swiss Federal Institute of Technology (ETH), Zurich
           Undergraduate studies in Biology
           Diploma of Natural Sciences (dipl. Natw. ETH)
1999-2004  Research assistant at the Institute of Biotechnology, ETH Zurich
           Preparation of this Ph.D. thesis in the group of Prof. B. Witholt
Publications


