The role of polyhydroxyalkanoate biosynthesis by *Pseudomonas aeruginosa* in rhamnolipid and alginate production as well as stress tolerance and biofilm formation

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*Pseudomonas aeruginosa* is capable of synthesizing polyhydroxyalkanoic acids (PHAs) and rhamnolipids, both of which are composed of 3-hydroxydecanoic acids connected by ester bonds, as well as synthesizing the biofilm matrix polymer alginate. In order to study the influence of PHA biosynthesis on rhamnolipid and alginate biosynthesis, as well as stress tolerance and biofilm formation, isogenic knock-out mutants deficient in PHA biosynthesis were generated for *P. aeruginosa* PAO1 and the alginate-overproducing *P. aeruginosa* FRD1. A gentamicin-resistance cassette was inserted replacing the 3′ region of *phaC1*, the whole of *phaZ* and the 5′ region of *phaC2*. Gas chromatography/mass spectrometry analysis showed that PHA accumulation was completely abolished in both strains. Interestingly, this gene replacement did not abolish rhamnolipid production. Thus, as previously suggested, the PHA synthase is not directly involved in rhamnolipid biosynthesis. In the PHA-negative mutant of mucoid FRD1 alginate biosynthesis was not affected, whereas in the PHA-negative PAO1 mutant an almost threefold increase in biosynthesis was observed compared to the wild-type. Consistently, PHA accumulation in FRD1 contributed only 4–7% of cell dry weight, which is fourfold less than in PAO1. These data suggest that PHA biosynthesis and alginate biosynthesis are in competition with respect to a common precursor. The surface attachment and biofilm development of the PHA-negative mutants were also compared to those of wild-type strains in glass flow-cell reactors. PHA-negative mutants of *P. aeruginosa* PAO1 and FRD1 showed reduced attachment to glass. However, the PAO1 PHA-negative mutant, in contrast to the wild-type, formed a stable biofilm with large, distinct and differentiated microcolonies characteristic of alginate-overproducing strains of *P. aeruginosa*.

The stress tolerance of PHA-negative mutants with respect to elevated temperature was strongly impaired. These data indicated a functional role for PHA in stress response and tolerance.

**INTRODUCTION**

A wide variety of micro-organisms accumulate polyhydroxyalkanoic acids (PHAs), mostly polyhydroxybutyrate, as metabolic storage materials, which are deposited as intracellular water-insoluble inclusions (Griebel et al., 1968; Rehm & Steinbüchel, 1999). Most fluorescent pseudomonads belonging to rRNA homology group I, such as *Pseudomonas aeruginosa* and *Pseudomonas putida*, are able to synthesize and accumulate large amounts of PHA consisting of various 3-hydroxy fatty acids with carbon chain lengths ranging from 6 to 14 carbon atoms (MCL, medium chain length) as carbon and energy storage compounds (Huismann et al., 1989).

The composition of PHA depends on the PHA synthases (PhaC), the carbon source and the metabolic routes involved (Rehm & Steinbüchel, 1999; Rehm, 2003). It has been confirmed that purified PHA<sub>MCL</sub> synthases, the key enzymes of PHA biosynthesis, from *P. aeruginosa* exhibit *in vitro* enzyme activity with (R)-3-hydroxydecanoyl-CoA as substrate (Amara & Rehm, 2003; Qi et al., 2000; Ren et al.,...
This substrate is derived from fatty acid de novo biosynthesis by transacylase-catalysed conversion of (R)-3-hydroxyacyl-ACP to the corresponding CoA thioester. The transacylase PhaG from P. putida, which catalyses the transfer of the (R)-3-hydroxydecanoyl moiety from the ACP thioester to CoA, has recently been identified and characterized (Fiedler et al., 2000; Hoffmann et al., 2002; Rehm et al., 1998). Meanwhile, phaG genes were isolated and characterized from Pseudomonas oleovorans and P. aeruginosa, and evidence was obtained that this transacylase-mediated pathway is widespread among pseudomonads (Hoffmann et al., 2000a, b). Beside the intracellular accumulation of PHA P. aeruginosa is capable of producing various exoproducts, such as exoenzymes, pyocyanine, the exopolysaccharide alginate and rhamnolipids.

Rhamnolipids are glycolipidic biosurfactants, which reduce water surface tension and emulsify oil. The rhamnolipids produced by P. aeruginosa in liquid cultures are mainly rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (monorhamnolipid) and rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (dirhamnolipid) (Ochsner & Reiser, 1995). Rhamnolipid biosynthesis proceeds through transfer of two rhamnose moieties from TDP-L-rhamnose to the respective ACP thioester (Maier et al., 1995). The transacylase PhaG from P. oleovorans has recently been identified and characterized (Fiedler et al., 2000). This enzyme transfers the (R)-3-hydroxydecanoyl moiety from the ACP thioester to CoA, has recently been identified and characterized (Fiedler et al., 2000). Meanwhile, phaG genes were isolated and characterized from Pseudomonas oleovorans and P. aeruginosa, and evidence was obtained that this transacylase-mediated pathway is widespread among pseudomonads (Hoffmann et al., 2000a, b). Beside the intracellular accumulation of PHA P. aeruginosa is capable of producing various exoproducts, such as exoenzymes, pyocyanine, the exopolysaccharide alginate and rhamnolipids.

Biofilm formation is thought often to represent a protective mode of growth which may enhance bacterial survival under conditions of environmental stress (Webb et al., 2003). Recently it was found that rhamnolipids play a major role in the architecture of biofilms produced by P. aeruginosa and that the formation of water channels is strongly dependent on the presence of rhamnolipids (Davey et al., 2003). PHA mobilization is known to enhance stress tolerance in P. oleovorans (Ruiz et al., 2001). In this study, the link between biofilm formation, stress tolerance and PHA accumulation was investigated in P. aeruginosa for the first time. Isogenic knock-out mutants of P. aeruginosa strains were generated by replacing the PHA synthase genes, hence abolishing the capability of PHA accumulation. Comparative analysis of the alginate-overproducing strain FRD1, the wild-type PAO1 and these knock-out mutants allowed us to investigate the role of PHA biosynthesis in rhamnolipid and alginate biosynthesis as well as biofilm formation and stress tolerance.

**METHODS**

**Bacterial strains, plasmids and growth of bacteria.** The pseudomonad and *Escherichia coli* strains, and the plasmids used in this study are listed in Table 1. *E. coli* was grown at 37°C in complex Luria–Bertani (LB) medium. Pseudomonas aeruginosa was grown at 37°C in 300 ml baffled flasks containing 50 ml of LB medium, PPGAS medium [0·02 M NH4Cl; 0·02 M KCl; 0·12 M Tris/HCl; 0·0016 M MgSO4; 0·5% (w/v) glucose; 1% (w/v) peptone] or mineral salts medium (MM) containing 0·05% (w/v) ammonium chloride and carbon source as indicated. If required antibiotics were added at appropriate concentrations. The antibiotic concentrations used for P. aeruginosa were as follows: 300 μg carbenicillin ml⁻¹, 250 μg gentamicin ml⁻¹, 150 μg tetracycline ml⁻¹, 300 μg kanamycin ml⁻¹. The antibiotic concentrations used for *E. coli* were as follows: 50 μg kanamycin ml⁻¹, 100 μg ampicillin ml⁻¹.

For cultivation of biofilms, M9 medium containing 48 mM Na2HPO4, 22 mM KH2PO4, 9 mM NaCl, 19 mM NH4Cl, 2 mM MgSO4, 100 μM CaCl2, and 5 mM glucose was used. All chemicals were purchased from Sigma-Aldrich.

**Isolation, analysis and manipulation of DNA.** DNA sequences of new plasmid constructs were confirmed by DNA sequencing according to the chain-termination method using the model 4000L automatic sequencer LI-COR (MWG-Biotech). All other genetic techniques were performed as described by Sambrook et al. (1989).

**Plasmid construction.** The isogenic *phaC1-phaZ-phaC2* gene knock-out mutant was obtained by simultaneous replacement of 1237 bp of the 3′ end of *phaC1*, the whole of *phaZ* and 1286 bp of the 5′ end of *phaC2* with a gentamicin-resistance cassette. PCR and the suicide vector pEX100T were employed as outlined in Fig. 1 to obtain the respective construct. Primers used for PCR were: for the *phaC1* 5′ end, PaphaC1-NEV and PaphaC1-CB; for the *phaC2* 3′ end, PaphaC2-NB and PaphaC2-CEV; and primers for the gentamicin-resistance cassette (Table 1). The isogenic mutant was verified by PCR using one primer which binds upstream of the *phaC1* start codon and a second primer binding close to the *phaC2* stop codon. The PCR product was analysed by restriction site mapping and direct DNA sequencing.

**Gas chromatography/mass spectrometry analysis of polyesters and fatty acids in cells.** PHAs and fatty acids were quantitatively and qualitatively analysed by gas chromatography/mass spectrometry (GC/MS). Liquid cultures were centrifuged at 10 000 g for 15 min; then the cells were washed twice in saline (0·9%, w/v, NaCl) and lyophilized overnight. Lyophilized cell material was subjected to methanolysis in the presence of 15% (v/v) sulfuric acid at 100°C for 5 h. The resulting methyl esters of the constituent 3-hydroxyalkanoic acids were assayed by GC according to Brandl et al. (1988). GC/MS analysis was performed by injecting
3 μl of sample into a Hewlett Packard 6890 gas chromatograph/mass spectrometer using a 0.5 μm diameter Permporph PEG 25 Mx capillary column 60 m in length according to Brandl et al. (1988).

Analysis of rhamnolipids. The orcinol assay (Chandrasekan & Bemiller, 1980) was used to directly assess the amount of rhamnolipids in the sample. A 33 μl sample of the culture supernatant was extracted twice with 1 ml diethyl ether. The ether fractions were pooled and evaporated to dryness, and 0.1 ml of a solution containing 0.1 M orcinol (in 53 % (v/v) H2SO4) was added; after heating for 30 min at 80 °C, the samples were cooled for 15 min at room temperature and the absorbance was measured at 421 nm. The concentrations of rhamnolipids were indicated by comparing the data with those obtained with rhamnose standards between 0 and 50 μg ml−1.

Uronic acids assay. The concentrations of uronic acid-positive material, including alginate, were determined directly from 2-propanol precipitates of supernatant fractions of planktonic P. aeruginosa cultures, using 2-hydroxydiphenyl as a reagent for colorimetric assay described (Moller et al., 1998). Channels were inoculated with 0.5 ml of early-stationary-phase cultures containing approximately 1 × 109 cells ml−1 and incubated without flow for 1 h at room temperature. Flow was then started with a mean flow velocity in the flow-cells of 0.2 mm s−1, corresponding to a laminar flow with a Reynolds number of 0.02. Biofilms were stained with SYTO 9 (molecular probes) and visualized using a confocal laser scanning microscope (CLSM) (Olympus). To analyse the effect of heat stress on biofilms, biofilms grown for 72 h were incubated at 50 °C for 20 min. Biofilms were then stained using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes). The two stock solutions of the stain (SYTO 9 and propidium iodide) were diluted to 3 μl ml−1 in biofilm medium and injected into the flow channels. Live SYTO 9-stained cells and dead propidium-iodide-stained cells were visualized with a CLSM (Olympus, Fluoview500) using fluorescein isothiocyanate and tetramethyl rhodamine isocyanate optical filters, respectively.

The attachment assay was carried out on glass coverslips attached to flow-cells (see above). Overnight cultures (16 h at 37 °C) were adjusted to an OD600 of 2.0 and inoculated into the flow-cells at 37 °C. Cells were allowed to adhere for 1 h before flow was switched on to remove unattached cells, and cells were counted under light microscopy using an eyepiece grid. Mean adhesion values for each strain were determined from three replicate flow-cells from three different cultivations.

**RESULTS**

**Generation of the phaC1-phaZ-phaC2 knock-out mutant of P. aeruginosa**

To investigate the functional role of PHA synthase and particularly PHA biosynthesis in P. aeruginosa with respect...
Fig. 1. Schematic representation of the generation of *P. aeruginosa* mutants with PHA synthase genes knocked out.
Table 2. Accumulation of PHA from gluconate by strains PAO1 and FRD1 of P. aeruginosa and their respective mutants

Cultivations were performed under PHA-accumulating conditions on mineral salts medium containing 1-5% (w/v) sodium gluconate and 0-05% (w/v) ammonium chloride. Cells were grown for 48 h at 37°C. Experiments were done in triplicate and PHA content is reported as mean ± standard deviation. PHA content and composition of comonomers were analysed by GC. 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydecanoate; ND, not detectable.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PHA content (%, w/w, of CDW)</th>
<th>Composition of PHA (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3HHx</td>
<td>3HO</td>
</tr>
<tr>
<td>FRD1</td>
<td>4-7 ±0-3</td>
<td>15</td>
</tr>
<tr>
<td>FRD1 ΔphaC1-Z-C2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PAO1</td>
<td>20 ±3</td>
<td>14</td>
</tr>
<tr>
<td>PAO1 ΔphaC1-Z-C2</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 3. Rhamnolipid and alginate production by various P. aeruginosa strains

Rhamnolipid concentration is expressed as mg rhamnose in rhamnolipids per ml culture supernatant. Alginate concentration is given as uronic acid concentration in culture supernatants. Cells were grown in PPGAS medium for 48 h at 37°C. The PPGAS medium contained 0.5% (w/v) glucose as the carbon source. Experiments were done in triplicate (three independent cultivations) and results are reported as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rhamnolipid (µg rhamnose ml⁻¹)</th>
<th>Alginate (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>77-6 ±2</td>
<td>5-6 ±3</td>
</tr>
<tr>
<td>PAO1 ΔphaC1-Z-C2</td>
<td>58-4 ±10</td>
<td>13-9 ±3</td>
</tr>
<tr>
<td>FRD1</td>
<td>8 ±1</td>
<td>185 ±8</td>
</tr>
<tr>
<td>FRD1 ΔphaC1-Z-C2</td>
<td>25-6 ±5</td>
<td>185 ±11</td>
</tr>
</tbody>
</table>

**Analysis of phaC1-phaZ-phaC2 knock-out mutants of P. aeruginosa with respect to PHA, rhamnolipid and alginate synthesis**

Since precursors for PHA and rhamnolipid biosynthesis are derived from fatty acid de novo biosynthesis and acetyl-CoA serves as a precursor for alginate biosynthesis as well as for PHA biosynthesis, mutants of *P. aeruginosa* were analysed with respect to rhamnolipid and alginate biosynthesis. For PHA biosynthesis analysis, cells were cultivated under PHA-accumulating conditions on MM medium containing 1-5% (w/v) sodium gluconate and 0.05% (w/v) ammonium chloride. For rhamnolipid and alginate biosynthesis analysis, cells were cultivated in PPGAS medium containing 0.5% (w/v) glucose as the carbon source. GC/MS analysis clearly revealed that no PHA was accumulated by the mutants. However, strain FRD1 accumulated PHA contributing only 4-7% (w/w) of cell dry weight (CDW) (Table 2). In comparison, strain PAO1 accumulated PHA contributing about 20% (w/w) of CDW under these conditions. The composition of the PHA was similar in the two strains (Table 2). Rhamnolipid production increased by about threefold in the ΔphaC1-Z-C2 FRD1 mutant, whereas rhamnolipid production was decreased to 75-26% in the ΔphaC1-Z-C2 PAO1 mutant, when compared with wild-type strains FRD1 and PAO1, respectively (Table 3). Alginate production increased by about threefold in the ΔphaC1-Z-C2 PAO1 mutant and remained unaffected in the ΔphaC1-Z-C2 FRD1 mutant when compared with non-mutated PAO1 and FRD1, respectively (Table 3).
PHA deficiency and biofilm formation

The ΔphaC1-Z-C2 PAO1 mutant formed a more structured biofilm than the wild-type PAO1, with large, distinct and differentiated microcolonies (Fig. 2). This is in contrast to the wild-type, which formed much smaller microcolonies, and had largely dispersed after 5 days. In the mucoid P. aeruginosa strain FRD1, there were no major outward differences between the wild-type and the mutant strains in this experiment. The biofilms generally had a ‘looser’ structure, with more heterogeneity and clustering than the non-mucoid strain, which is consistent with observations of biofilm development in other mucoid P. aeruginosa strains (Hentzer et al., 2001; Nivens et al., 2001).

Cell counts after the initial attachment of each of the strains within the flow-cells showed that both of the mutant strains exhibited reduced attachment to the glass flow-cell after a 1 h attachment period: attachment values (cells mm\(^{-2}\)) were 2295 ± 215 and 724 ± 62 for PAO1 and its mutant, respectively, and 21,914 ± 1036 and 7552 ± 623 for FRD1 and its mutant, respectively. The mutants showed an approximately threefold reduction in attachment to glass. These differences are highly significant by ANOVA. The mucoid strains also showed much higher adhesion than the non-mucoid PAO1.

Role of PHA accumulation in stress tolerance

Planktonic mutant and wild-type cells of P. aeruginosa PAO1 and FRD1 were subjected to heat stress under starvation conditions. Early-stationary-phase cells, cultivated...
under PHA-accumulating conditions, were challenged by heat shock at 50 ºC (Fig. 3). Both mutant strains showed a decreased heat tolerance as indicated by decreased survival after exposure to 50 ºC. Strain FRD1 and its PHA-negative mutant were less heat-tolerant than strain PAO1 and its PHA-negative mutant, respectively. After only 10 min at 50 ºC the number of viable cells dropped significantly with almost no viable cells detectable for strain FRD1 and its mutant (Fig. 3). In addition, the effect of heat stress on biofilms was investigated by incubating biofilms at 50 ºC for 20 min. In both strains the PHA-negative mutant was more sensitive towards killing by heat (Fig. 4).

**DISCUSSION**

In this study we constructed for the first time a knock-out mutant deficient in PHA biosynthesis in a pseudomonad. This was achieved in *P. aeruginosa* in order to evaluate the role of the PHA synthase and PHA biosynthesis in rhamnolipid synthesis, alginate production, stress tolerance and biofilm formation, considering the type-strain PAO1 and the mucoid strain FRD1 for comparative analysis. Isogenic knock-out mutants were generated for both strains by insertional inactivation of the *phaC1-phaZ-phaC2* gene region encoding the two PHA synthases and the intracellular PHA depolymerase, respectively (Fig. 1). Thus, mutants of strains PAO1 and FRD1 that were both PHA synthase- and PHA-negative were constructed.

GC/MS analysis with respect to PHA biosynthesis revealed that the mutants were not able to accumulate PHA, as was expected considering their deficiency in the key enzyme of PHA biosynthesis, the PHA synthase (Table 2). Interestingly, the alginate-overproducing strain FRD1 accumulated PHA that contributed only 4-7 % of the CDW, which presumably reflects the strong carbon flux towards alginate biosynthesis. Recently, it was found that the decrease in PHA accumulation in strain FRD1 was not due to impaired expression of the PHA synthase gene (Hoffmann & Rehm, 2004). The PHA-negative mutant of FRD1 did not exhibit an effect on alginate production (Table 3). The change in carbon flux appeared to be more obvious in the PHA-negative *P. aeruginosa* strain PAO1, which showed an almost threefold increase in alginate production, indicating in this strain both biosynthesis pathways are competing with respect to acetyl-CoA. Acetyl-CoA enters the fatty acid *de novo* biosynthesis pathway to provide a precursor for PHA biosynthesis or into the citric acid cycle for alginate biosynthesis. Since rhamnolipid biosynthesis relies on the availability of the precursor, 3-β-hydroxydecanoyl-3-β-hydroxydecanoate, which was thought to be synthesized by the PHA synthase (Deziel *et al.*, 2003; Rehm *et al.*, 2001), rhamnolipid production by the PHA-synthase-negative mutants was investigated. The mutants were still capable of rhamnolipid production, suggesting that none of the PHA synthases are directly involved in rhamnolipid biosynthesis. Recently, evidence was presented indicating that RhlA is required for
production of 3-β-hydroxydecanoyl-3-β-hydroxydecanoate (Deziel et al., 2003). The deficiency in PHA biosynthesis had opposite effects on the amount of rhamnolipid produced by the two respective mutants, for unknown reasons (Table 3).

Interestingly, the PHA-negative mutant of PAO1 established a more complex and differentiated biofilm than the wild-type. One possibility is that the pronounced architecture of the PHA-negative mutant of PAO1 may result from the threefold increase in alginate production observed in this mutant as compared to the wild-type strain (Table 3). Alginate overproduction is known to affect P. aeruginosa mutant as compared to the wild-type strain (Table 3). A more complex and differentiated biofilm than the wild-type, and the absence of this reserve polymer serving as a carbon source may mediate starvation stress. Because biofilm formation is thought to offer favourable conditions that protect against such environmental stresses, alginate overproduction and the formation of a more structured biofilm may be advantageous to nutrient-starved cells that do not have PHA reserves. Our data may therefore indicate a link between PHA accumulation and biofilm formation. Currently, it is not clear why attachment of the two PHA-negative mutants to a glass surface was significantly impaired.

Synthesis and degradation of PHAs are part of a cycle in which the acyl-CoA precursors are converted by different metabolic pathways into PHAs. The depolymerization produces acyl-CoAs and acetyl-CoA, which is metabolized in the citric acid cycle as a source of carbon and energy (Kessler et al., 1998). Recently, a rise in the ATP and ppGpp levels was found in P. oleovorans concomitant with PHA availability, while this phenomenon was not observed in a mutant strain unable to degrade the polymer (Ruiz et al., 2001). In addition, planktonic cells of the wild-type showed increased survival compared to the PHA-negative strain. The appearance of a round cellular shape, characteristic of bacteria growing under starvation conditions, was delayed in the wild-type in comparison to the mutant strain. Percentage survival at the end of ethanol and heat challenges was always higher in the wild-type than in the PHA-negative mutant (Ruiz et al., 2001).

In this study, the influence of PHA in P. aeruginosa was analysed for the first time with regard to bacterial stress-resistance as well as biofilm formation. Stress tolerance studies with respect to heat treatment of planktonic cells revealed that the PHA-negative mutants were more sensitive than their respective non-mutated strains (Fig. 3). Similarly, heat stress applied to biofilms showed that the PHA-negative mutants of strains PAO1 and FRD1 were more sensitive compared to their respective non-mutated strains (Fig. 4). These data indicate that PHA plays an important role in the stress response in P. aeruginosa in planktonic cells as well as in biofilms. Accordingly, it is likely that PHA availability in P. aeruginosa enhances the ATP and ppGpp levels as was found in P. oleovorans, and ppGpp has been shown in E. coli to induce expression of the rpoS gene (Gentry et al., 1993), which encodes a sigma factor involved in regulation of stress tolerance. It has been shown that an rpoS-negative mutant of PAO1 shows decreased stress-resistance in biofilms (Cochran et al., 2000).

Overall, the data suggested that PHA accumulation in P. aeruginosa is in competition with alginate biosynthesis and plays an important role in stress tolerance and biofilm formation. Moreover, the PHA synthase is not directly involved in rhamnolipid biosynthesis. The molecular link between PHA accumulation and stress tolerance remains to be determined and is currently being investigated.

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REFERENCES


