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

Thi Hang Pham,¹ Jeremy S. Webb² and Bernd H. A. Rehm³

Correspondence

Bernd H. A. Rehm

B.Rehm@massey.ac.nz

¹Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster, Corrensstrasse 3, D-48149 Münster, Germany

²School of Biotechnology and Biomolecular Sciences and Centre for Marine Biofouling and Bioinnovation, University of New South Wales, NSW 2052, Sydney, Australia

³Institute of Molecular Biosciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

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.

Received 25 May 2004

Revised 13 July 2004

Accepted 14 July 2004

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 (Huisman *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_{MCL} 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.*,

Abbreviations: ACP, acyl carrier protein; CDW, cell dry weight; CLSM, confocal laser scanning microscope; MCL, medium chain length; PHA, polyhydroxyalkanoic acid; Rt, rhamnosyltransferase.

2000). 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 (mono-rhamnolipid) and rhamnosyl-rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate (dirhamnolipids) (Ochsner & Reiser, 1995). Rhamnolipid biosynthesis proceeds through transfer of two rhamnose moieties from TDP-L-rhamnose (Maier & Soberon-Chavez, 2000). For the synthesis of mono-rhamnolipid, the enzyme rhamnosyltransferase 1 (Rt 1) catalyses the rhamnose transfer to β -hydroxydecanoyl- β -hydroxydecanoate, while rhamnosyltransferase 2 (Rt 2) synthesizes dirhamnolipid from TDP-L-rhamnose and monorhamnolipid. Genes for biosynthesis, regulation and induction of the Rt 1 enzyme are organized in tandem in the *rhlABRI* gene cluster (Ochsner & Reiser, 1995). The gene *rhlC* encoding the Rt 2 enzyme has been described (Rahim *et al.*, 2001), and is homologous to rhamnosyltransferases involved in lipopolysaccharide biosynthesis. Recently, Campos-Garcia *et al.* (1998) identified the *rhlG* gene encoding a β -ketoacyl reductase, which appears to be involved in biosynthesis of rhamnolipids. RhlG is thought to catalyse the NADPH-dependent reduction of β -ketodecanoyl-ACP, which is an intermediate of fatty acid *de novo* biosynthesis, resulting in β -hydroxydecanoyl-ACP, a putative precursor for rhamnolipid biosynthesis. Some evidence was recently provided that RhlA is involved in synthesis of 3-(3-hydroxyalkanoxy)alkanoic acids (HAAs) (Deziel *et al.*, 2003); however, it still remains unclear whether the PHA synthase is capable of catalysing the synthesis of HAAs, which has been previously postulated (Campos-Garcia *et al.*, 1998; Deziel *et al.*, 2003; Rehm *et al.*, 2001).

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, P*phaC1*-NEV and P*phaC1*-CB; for the *phaC2* 3' end, P*phaC2*-NB and P*phaC2*-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 qualitatively and quantitatively 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 (8–10 mg) 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

Table 1. Bacterial strains, plasmids and oligonucleotides

Strain, plasmid or oligonucleotide	Relevant characteristics	Reference
Strains		
<i>Pseudomonas aeruginosa</i>		
PAO1	Prototroph, Alg ⁻	ATCC 15692
PAO1Δ <i>phaC1-Z-C2</i>	PAO1 containing chromosomal deletion of <i>phaC1-Z-C2</i> genes	This study
FRD1	Alginate-overproducing isolate	Ohman & Chakrabarty (1981)
FRD1Δ <i>phaC1-Z-C2</i>	FRD1 containing chromosomal deletion of <i>phaC1-Z-C2</i> genes	This study
<i>Escherichia coli</i>		
S17-1	<i>recA</i> ; harbours the <i>tra</i> genes of plasmid RP4 in the chromosome; <i>proA thi-1</i>	Simon <i>et al.</i> (1983)
JM109	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _k ⁻ m _k ⁺) <i>supE44 relA1 λ⁻ lac</i>	Sambrook <i>et al.</i> (1989)
Plasmids		
pEX100T	Amp ^r , <i>oriT sacB lacPOZ'</i>	Schweizer & Hoang (1995)
pGEM-Teasy	Amp ^r , <i>lacPOZ'</i>	Promega
pPS856	Amp ^r Gm ^r , <i>FRT</i>	Hoang <i>et al.</i> (1998)
pEXC1ZC2	Vector pEX100T containing the Gm cassette flanked by the 5'-end of the <i>phaC1</i> gene and 3'-end of the <i>phaC2</i> gene	This study
Oligonucleotides for PCR		
<i>PaphaC1</i> -NEV	5'-AAAGATATCGTCCCCGGTTTCGTTGCGGTC-3'	This study
<i>PaphaC1</i> -CB	5'-GTTGGATCCAGAAGCGCTTGACCGCCGCCGGG-3'	This study
<i>PaphaC2</i> -NB	5'-GGTGGATCCAGCGGGACCGCGGTGGACCTGGGC-3'	This study
<i>PaphaC2</i> -CEV	5'-GGGGATATCCGAGCATTTCGAGGATTCGGTCGCG-3'	This study

3 µl of sample into a Hewlett Packard 6890 gas chromatograph/mass spectrometer using a 0.5 µm diameter Permphase 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 333 µl sample of the culture supernatant was extracted twice with 1 ml diethyl ether. The ether fractions were pooled and evaporated to dryness, and 0.5 ml H₂O was added. To 100 µl of each sample 900 µl of a solution containing 0.19% 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 A₄₂₁ was measured. 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 directly determined from 2-propanol precipitates of supernatant fractions of planktonic *P. aeruginosa* cultures, using 2-hydroxydiphenyl as a reagent for colorimetric assay (Blumenkrantz & Asboe-Hansen, 1973). *Macrocystis pyrifera*-derived alginate (Sigma-Aldrich) was used as standard. All spectrometric measurements were performed with a Biochrom Libra S5 (Biochrom).

Heat challenge protocol. Bacteria were grown, with shaking, until the early stationary phase (OD₆₀₀, 1–1.1) in MM medium supplemented with 15 g sodium gluconate l⁻¹ at 37 °C. The cells were harvested by centrifugation (10 000 g) and rinsed twice with saline solution. The sediments were then resuspended in 1 ml saline and then, at time zero, diluted with preheated saline solution (50 °C) to 10³ cell ml⁻¹. The bacteria were counted immediately after dilution (time zero) and then every 5 min by serial dilutions onto triplicate nutrient agar plates. The variation coefficient for replicate platings was ≤20%. Stress-resistance was expressed as the percentage of survival compared to the situation shortly before stress application, which was set at 100%.

Biofilm experiments. *P. aeruginosa* PAO1 wild-type and PHA

mutant strains were grown in continuous-culture flow-cells (channel dimensions 1 × 4 × 40 mm) at room temperature as previously 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 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 $\pm$ standard deviation. PHA content and composition of comonomers were analysed by GC. 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate; ND, not detectable.

Strain	PHA content (%, w/w, of CDW)	Composition of PHA (mol%)			
		3HHx	3HO	3HD	3HDD
FRD1	4.7 $\pm$ 0.3	15	22	53	10
FRD1 Δ <i>phaC1-Z-C2</i>	ND	ND	ND	ND	ND
PAO1	20 $\pm$ 3	14	21	61	4
PAO1 Δ <i>phaC1-Z-C2</i>	ND	ND	ND	ND	ND

to rhamnolipid synthesis, alginate synthesis, stress tolerance and biofilm formation, we generated an isogenic *phaC1-phaZ-phaC2* knock-out mutant by insertional inactivation of the chromosomal *phaC1-phaZ-phaC2* gene region. To achieve this we constructed plasmid pEXC1ZC2, which contains the *phaC1-phaZ-phaC2* gene region disrupted and partially replaced by a gentamicin-resistance cassette. The gentamicin-resistance cassette replaced the major part (including the essential lipase box) of the two synthase genes as well as the entire *phaZ* gene. Plasmid pEXC1ZC2 was transferred by conjugation into *P. aeruginosa* PAO1 and *P. aeruginosa* FRD1, and gentamicin/sucrose-resistant transformants, which were putative double recombinants carrying an interrupted *phaC1-phaZ-phaC2* gene region, were selected (Fig. 1). Mutants were analysed by PCR, employing primers which bind 45 bp downstream of the *phaC1* gene start codon and at the 3'-end (1505 bp downstream of the start codon) of the *phaC1* gene. No PCR product was obtained from mutant genomic DNA, and a PCR product of about 1.5 kb was obtained from wild-type genomic DNA, indicating that the mutants indeed derived from a double recombination event in which the *phaC1-phaZ-phaC2* gene region was interrupted by a gentamicin-resistance cassette (Fig. 1). This was further confirmed by PCR using primers binding to the 5'-end of *phaC1* (PaphaC1-NEV) and the 3'-end of *phaC2* (PaphaC2-CEV), respectively. Further analysis of the resulting PCR product of about 2 kb in size, including restriction site mapping and DNA sequencing, showed that the gentamicin-resistance cassette was properly inserted in the *phaC1-phaZ-phaC2* gene region (Fig. 1).

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 Δ *phaC1-Z-C2* FRD1 mutant, whereas rhamnolipid production was decreased to 75.26 % in the Δ *phaC1-Z-C2* PAO1 mutant, when compared with wild-type strains FRD1 and PAO1, respectively (Table 3). Alginate production increased by about threefold in the Δ *phaC1-Z-C2* PAO1 mutant and remained unaffected in the Δ *phaC1-Z-C2* FRD1 mutant when compared with non-mutated PAO1 and FRD1, respectively (Table 3).

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

Rhamnolipid concentration is expressed as μ g 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 carbon source. Experiments were done in triplicate (three independent cultivations) and results are reported as mean $\pm$ standard deviation.

Strain	Rhamnolipid (μ g rhamnose ml ⁻¹)	Alginate (μ g ml ⁻¹)
PAO1	77.6 $\pm$ 2	5.6 $\pm$ 3
PAO1 Δ <i>phaC1-Z-C2</i>	58.4 $\pm$ 10	13.9 $\pm$ 3
FRD1	8 $\pm$ 1	185 $\pm$ 8
FRD1 Δ <i>phaC1-Z-C2</i>	25.6 $\pm$ 5	185 $\pm$ 11

Fig. 2. Comparison of biofilms generated by *P. aeruginosa* strains and their respective PHA-negative mutants. Biofilms were stained with SYTO 9 (molecular probes) and visualized using a CLSM. Upper panels are *xy*, top-down, confocal images; lower panels are *xz* vertical sections of the biofilm. Magnification, approximately $\times 140$.

PHA deficiency and biofilm formation

The $\Delta phaC1$ -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 $21\,914 \pm 1036$ 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

Fig. 3. Heat challenge of planktonic *P. aeruginosa* cells as described in Methods. The experiment was performed in triplicate and mean values are shown; standard deviation was <15% of the mean. The value for 100% is 10^3 cells ml^{-1} . ▲, wild-type PAO1; ●, PAO1 mutant; ◆, wild-type FRD1; ■, FRD1 mutant.

under PHA-accumulating conditions, were challenged by heat shock at 50 °C (Fig. 3). Both mutant strains showed a decreased heat tolerance as indicated by decreased survival after exposure to 50 °C. 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 °C 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 °C 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,

Fig. 4. Heat challenge of *P. aeruginosa* biofilms. CLSM images of live/dead stains of PAO1, the PAO1 mutant, FRD1 and the FRD1 mutant after heat treatment as described in Methods. Green cells are viable; red cells have a compromised cell membrane and are dead. Magnification, approximately $\times 110$.

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* biofilm architecture and function (Hentzer *et al.*, 2001). Presumably, mutant cells defective in PHA production are more sensitive to nutrient stress 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.

ACKNOWLEDGEMENTS

This study was supported by the Massey University Research Fund MURF/57348. We thank Dr H. P. Schweizer for provision of various very useful vectors.

REFERENCES

- Amara, A. A. & Rehm, B. H. A. (2003). Replacement of the catalytic nucleophile cysteine-296 by serine in class II polyhydroxyalkanoate synthase from *Pseudomonas aeruginosa* mediated synthesis of a new polyester, identification of catalytic residues. *Biochem J* **374**, 413–421.
- Blumenkrantz, N. & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Anal Biochem* **54**, 484–489.
- Brandl, H., Gross, R. A., Lenz, R. W. & Fuller, R. C. (1988). *Pseudomonas oleovorans* as a source of poly(3-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Appl Environ Microbiol* **54**, 1977–1982.
- Campos-Garcia, J., Caro, A. D., Najera, R., Miller-Maier, R. M., Al Tahhan, R. A. & Soberon-Chavez, G. (1998). The *Pseudomonas aeruginosa* *rhlG* gene encodes an NADPH-dependent beta-ketoacyl reductase which is specifically involved in rhamnolipid synthesis. *J Bacteriol* **180**, 4442–4451.
- Chandrasekan, E. V. & Bemiller, J. N. (1980). Constituent analyses of glycosaminoglycans. *Methods Carbohydr Chem* **8**, 89–96.
- Cochran, W. L., Suh, S. J., McFeters, G. A. & Stewart, P. S. (2000). Role of RpoS and AlgT in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide and monochloramine. *J Appl Microbiol* **88**, 546–553.
- Davey, M. E., Caiazza, N. C. & O'Toole, G. A. (2003). Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185**, 1027–1036.
- Deziel, E., Lepine, F., Milot, S. & Villemur, R. (2003). *rhlA* is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*, 3-(3-hydroxyalkanooyloxy) alkanic acids (HAAs), the precursors of rhamnolipids. *Microbiology* **149**, 2005–2013.
- Fiedler, S., Steinbüchel, A. & Rehm, B. H. A. (2000). PhaG-mediated synthesis of poly(3-hydroxyalkanoates) consisting of medium-chain-length constituents from non-related carbon sources in recombinant *Pseudomonas fragi*. *Appl Environ Microbiol* **66**, 2117–2124.
- Gentry, D. R., Hernandez, V. J., Nguyen, L. H., Jensen, D. B. & Cashel, M. (1993). Synthesis of the stationary-phase sigma factor σ^S is positively regulated by ppGpp. *J Bacteriol* **175**, 7982–7989.

- Griebel, R., Smith, Z. & Merrick, J. M. (1968). Metabolism of poly-beta-hydroxybutyrate. I. Purification, composition and properties of native poly-beta-hydroxybutyrate granules from *Bacillus megaterium*. *Biochemistry* 7, 3676–3681.
- Hentzer, M., Teitzel, G. M., Balzer, G. J., Heydorn, A., Molin, S., Givskov, M. & Parsek, M. R. (2001). Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *J Bacteriol* 183, 5395–5401.
- Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. & Schweizer, H. P. (1998). A broad-host-range FLP-FRT recombination system for site-specific excision of chromosomally-located DNA sequences, application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212, 77–86.
- Hoffmann, N. & Rehm, B. H. A. (2004). Regulation of polyhydroxyalkanoate biosynthesis in *Pseudomonas putida* and *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 237, 1–7.
- Hoffmann, N., Steinbüchel, A. & Rehm, B. H. A. (2000a). Homologous functional expression of cryptic *phaG* from *Pseudomonas oleovorans* establishes the transacylase-mediated polyhydroxyalkanoate biosynthetic pathway. *Appl Microbiol Biotechnol* 54, 665–670.
- Hoffmann, N., Steinbüchel, A. & Rehm, B. H. A. (2000b). The *Pseudomonas aeruginosa phaG* gene product is involved in the synthesis of polyhydroxyalkanoic acid consisting of medium-chain-length constituents from non-related carbon sources. *FEMS Microbiol Lett* 184, 253–259.
- Hoffmann, N., Amara, A. A., Beermann, B. B., Qi, Q., Hinz, H. J. & Rehm, B. H. A. (2002). Biochemical characterization of the *Pseudomonas putida* 3-hydroxyacyl ACP:CoA transacylase, which diverts intermediates of fatty acid *de novo* biosynthesis. *J Biol Chem* 277, 42926–42936.
- Huisman, G. W., de Leeuw, O., Eggink, G. & Witholt, B. (1989). Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. *Appl Environ Microbiol* 55, 1949–1954.
- Kessler, B., Kraak, M. N., Ren, Q., Klinke, S., Prieto, M. & Witholt, B. (1998). Enzymology and molecular genetics of PHA₁ biosynthesis. In *Biochemical Principles and Mechanisms of Biosynthesis and Degradation of Polymers*, pp. 48–56. Edited by A. Steinbüchel. Weinheim, Germany: Wiley-VCH.
- Maier, R. M. & Soberon-Chavez, G. (2000). *Pseudomonas aeruginosa* rhamnolipids, biosynthesis and potential applications. *Appl Microbiol Biotechnol* 54, 625–633.
- Moller, S., Sternberg, C., Andersen, J. B., Christensen, B. B., Ramos, J. L., Givskov, M. & Molin, S. (1998). *In situ* gene expression in mixed-culture biofilms, evidence of metabolic interactions between community members. *Appl Environ Microbiol* 64, 721–732.
- Nivens, D. E., Ohman, D. E., Williams, J. & Franklin, M. J. (2001). Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. *J Bacteriol* 183, 1047–1057.
- Ochsner, U. A. & Reiser, J. (1995). Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 92, 6424–6428.
- Ohman, D. E. & Chakrabarty, A. M. (1981). Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. *Infect Immun* 33, 142–148.
- Qi, Q., Steinbüchel, A. & Rehm, B. H. A. (2000). *In vitro* synthesis of poly(3-hydroxydecanoate), purification and enzymatic characterization of type II polyhydroxyalkanoate synthases PhaC1 and PhaC2 from *Pseudomonas aeruginosa*. *Appl Microbiol Biotechnol* 54, 37–43.
- Rahim, R., Ochsner, U. A., Olvera, C., Graninger, M., Messner, P., Lam, J. S. & Soberon-Chavez, G. (2001). Cloning and functional characterization of the *Pseudomonas aeruginosa rhIC* gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. *Mol Microbiol* 40, 708–718.
- Rehm, B. H. A. (2003). Polyester synthases, catalysts for plastics. *Biochem J* 376, 15–33.
- Rehm, B. H. A. & Steinbüchel, A. (1999). Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. *Int J Biol Macromol* 25, 3–19.
- Rehm, B. H. A., Krüger, N. & Steinbüchel, A. (1998). A new metabolic link between fatty acid *de novo* synthesis and polyhydroxyalkanoic acid synthesis – the *phaG* gene from *Pseudomonas putida* KT2440 encodes a 3-hydroxyacyl-acyl carrier protein coenzyme A transferase. *J Biol Chem* 273, 24044–24051.
- Rehm, B. H. A., Mitsky, T. A. & Steinbüchel, A. (2001). Role of fatty acid *de novo* biosynthesis in polyhydroxyalkanoic acid (PHA) and rhamnolipid synthesis by pseudomonads, establishment of the transacylase (PhaG)-mediated pathway for PHA biosynthesis in *Escherichia coli*. *Appl Environ Microbiol* 67, 3102–3109.
- Ren, Q., de Roo, G., Kessler, B. & Witholt, B. (2000). Recovery of active medium-chain-length-poly-3-hydroxyalkanoate polymerase from inactive inclusion bodies using ion-exchange resin. *Biochem J* 349, 599–604.
- Ruiz, J. A., Lopez, N. I., Fernandez, R. O. & Mendez, B. S. (2001). Polyhydroxyalkanoate degradation is associated with nucleotide accumulation and enhances stress resistance and survival of *Pseudomonas oleovorans* in natural water microcosms. *Appl Environ Microbiol* 67, 225–230.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schweizer, H. P. & Hoang, T. T. (1995). An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. *Gene* 158, 15–22.
- Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering, transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* 1, 784–791.
- Webb, J. S., Givskov, M. & Kjelleberg, S. (2003). Bacterial biofilms, prokaryotic adventures in multicellularity. *Curr Opin Microbiol* 6, 578–585.
- Zhang, Y. & Miller, R. M. (1992). Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). *Appl Environ Microbiol* 58, 3276–3282.