β -Ketoacyl acyl carrier protein reductase (FabG) activity of the fatty acid biosynthetic pathway is a determining factor of 3-oxo-homoserine lactone acyl chain lengths

Tung T. Hoang,[†] Sarah A. Sullivan,[‡] John K. Cusick[§]
and Herbert P. Schweizer

Author for correspondence: Herbert P. Schweizer. Tel: +1 970 491 3536. Fax: +1 970 491 1815.
e-mail: herbert.schweizer@colostate.edu

Department of
Microbiology, Immunology
and Pathology, Colorado
State University, Fort Collins,
CO 80523-1682A, USA

The two acyl-homoserine lactones (AHLs) *N*-(butyryl)-L-homoserine lactone and *N*-[3-oxododecanoyl]-L-homoserine lactone (3-oxo-C₁₂-HSL) are required for quorum sensing in *Pseudomonas aeruginosa*. These AHLs derive their invariant lactone rings from *S*-adenosylmethionine and their variable acyl chains from the cellular acyl-acyl carrier protein (ACP) pool. This reaction is catalysed by specific AHL synthases, which exhibit acyl chain specificity. Culture supernatants of *P. aeruginosa* contain multiple 3-oxo-AHLs that differ in their acyl chain lengths but their physiological role, if any, remains unknown. An *in vitro* fatty acid-3-oxo-AHL synthesis system was established utilizing purified *P. aeruginosa* Fab proteins, ACP and the LasI 3-oxo-AHL synthase. In the presence of excess protein, substrates and cofactors, this system produced almost exclusively 3-oxo-C₁₂-HSL. When the β -ketoacyl-ACP reductase (FabG) catalysed step was made rate-limiting by switching from the preferred NADPH cofactor to NADH, increased levels of short chain 3-oxo-AHLs were produced, presumably because shorter-chain ketoacyl-ACPs accumulated and thus became LasI substrates. Consistent with these *in vitro* observations, a *fabG*(Ts) mutant produced increased amounts of 3-oxo-AHLs *in vivo*. Thus, *in vitro* and *in vivo* evidence indicated that modulation of FabG activity of the fatty acid biosynthetic pathway may determine the acyl chain lengths of these 3-oxo-AHLs and that the LasI 3-oxo-AHL synthase is sufficient for their synthesis.

Keywords: *Pseudomonas*, homoserine lactone, fatty acid synthesis, synthase

INTRODUCTION

The expression of many extracellular *Pseudomonas aeruginosa* virulence factors (Passador *et al.*, 1993; Van Delden & Iglewski, 1998) and other cellular processes, such as biofilm maturation *in vitro* (Davies *et al.*, 1998) and biofilm formation in the lungs of cystic fibrosis

patients (Singh *et al.*, 2000) are regulated in a cell-density-dependent manner by a process called cell-to-cell communication or quorum sensing. Cell-to-cell communication in *P. aeruginosa* involves the two acyl homoserine lactones (AHLs) *N*-(butyryl)-L-homoserine lactone (C₄-HSL) and *N*-[3-oxododecanoyl]-L-HSL (3-oxo-C₁₂-HSL). Although these two AHLs seem to be the main players involved in quorum sensing, *P. aeruginosa* produces other AHLs which differ by their acyl chain lengths but their physiological roles, if any, remain unclear. A quinolone signal (Pesci *et al.*, 1999) and perhaps cyclic peptides (Holden *et al.*, 1999) also seem to participate in some of these regulatory networks.

Several previous studies revealed that bacterial AHLs derive their invariant homoserine lactone rings from *S*-adenosyl methionine (SAM) and their variable acyl

[†] Present address: Department of Microbiology, University of Hawaii at Manoa, Honolulu, HI 96822, USA.

[‡] Present address: VWR International, 106 Gray Road, Suite D, Indianapolis, IN 46237, USA.

[§] Present address: National Jewish Hospital, 1400 Jackson Street, Denver, CO 80206, USA.

Abbreviations: ACP, acyl carrier protein; AHL, acyl homoserine lactone; C₄-HSL, *N*-(butyryl)-L-homoserine lactone; Fab, fatty acid biosynthesis; HSL, homoserine lactone; SAM, *S*-adenosyl methionine.

Fig. 1. Fatty acid biosynthesis in *P. aeruginosa*, and acyl-ACPs as acyl donors in cellular metabolism and AHL synthesis. There are several potential pathways to generate acetoacetyl-ACP and initiate fatty acid synthesis (Cronan & Rock, 1996) but not all are shown for the sake of clarity. In the reaction shown, which explains the *in vitro* system established in this study, malonyl-ACP is decarboxylated to acetyl-ACP by FabB, which then condenses these two molecules to acetoacetyl-ACP to initiate the cycle (Cronan & Rock, 1996). The malonyl-ACP is derived from malonyl-CoA by malonyl-CoA:ACP acyltransferase (FabD). Subsequent cycles are initiated by condensation of malonyl-ACP with acyl-ACP, catalysed by FabB (β -ketoacyl ACP synthase I). The β -ketoacyl-ACP from the FabB reaction is reduced to a β -hydroxyacyl-ACP by FabG, a NADPH-dependent β -ketoacyl-ACP reductase. The subsequent dehydration step is catalysed by either FabA or FabZ, depending on the lengths of the acyl groups in the β -hydroxyacyl-ACP substrates. The final step involves reduction of the dehydratase product to an acyl-ACP via FabI, a NADH-dependent enoyl-ACP reductase. Subsequent cycles are initiated by a FabB-catalysed condensation of malonyl-ACP with acyl-ACP. For synthesis of 3-oxo-C₁₂-HSL, LasI utilizes the 3-oxo-dodecanoyl-ACP from the Fab pathway. Similarly, RhII uses crotonyl-ACP for synthesis of C₄-HSL. Enzymes involved in 3-OH-AHL synthesis probably use D-3-hydroxy-ketoacyl-ACP substrates from the Fab cycle. Other biosynthetic pathways, including the phospholipid, lipopolysaccharide, haemolysin and other pathways, also use acyl-ACP intermediates. Other abbreviations: ACC, acetyl-CoA carboxylase; ACP, acyl carrier protein; FabH, β -ketoacyl ACP synthase III.

chains from the cellular acyl-ACP (acyl carrier protein) pool (Hoang & Schweizer, 1999; Moré *et al.*, 1996; Parsek *et al.*, 1999; Val & Cronan, 1998) (Fig. 1). Acyl chain specificity resides in critical amino acid residues within the AHL synthase sequences (Watson *et al.*, 2002). The AHL synthases (LasI for 3-oxo-C₁₂-HSL and RhII for C₄-HSL) are sufficient for catalysis of the acyl transfer and lactonization reactions (Moré *et al.*, 1996; Parsek *et al.*, 1999; Hoang & Schweizer, 1999; Hoang *et al.*, 1999). *P. aeruginosa* culture supernatants contain 3-oxo-AHLs with various acyl chain lengths but their metabolic origins have not been elucidated. In this study, we attempted to elucidate the molecular basis for the synthesis of these 3-oxo-AHLs. Since LasI competes

with NADPH-dependent β -ketoacyl-ACP reductase, FabG, for the 3-oxo-acyl-ACP precursors for synthesis of these 3-oxo-AHLs (Fig. 1), we reasoned that FabG activity may be a modulating factor determining acyl chain lengths in 3-oxo-AHLs. Because most Fab (fatty acid biosynthesis) enzymes, including FabG, are essential, conventional mutant analysis cannot be used to address their roles in cellular metabolism. To circumvent these problems, a complete *in vitro* Fab system using purified *Escherichia coli* Fab proteins and ACP was previously described and was shown to produce the types and distribution of acyl-ACP species found *in vivo* (Heath & Rock, 1996a, b). Since the *E. coli* and *P. aeruginosa* Fab systems are quite similar, we reasoned

that an *in vitro* Fab-3-oxo-AHL synthesis system could be used to explore FabG activity as a factor determining acyl chain lengths of 3-oxo-AHLs. To this end, we purified the *P. aeruginosa* Fab proteins as hexahistidine (H_6) fusion proteins and developed an *in vitro* Fab-AHL synthesis system by coupling them to purified LasI. Some of the observations made with the *in vitro* system were supported by preliminary *in vivo* data obtained with a conditional, temperature-sensitive *fabG*(Ts) mutant.

METHODS

Strains and growth media. *Escherichia coli* strains used in this study were DH5 α (Liss, 1987), BL21(DE3) (Novagen), SA1503(DE3) (Hoang *et al.*, 1999) and the 3-oxo- C_{12} -HSL reporter strain MG4/pKDT17 (*lasR*⁺ *lasB*-*lacZ*) (Schaefer *et al.*, 2000). The wild-type *P. aeruginosa* strain PAO1 was previously described (Watson & Holloway, 1978). *P. aeruginosa* strain 4 is a clinical wound isolate from the Glaxo SmithKline collection and is similar to PAO1 in terms of extracellular protein profiles, exoenzyme S production and nucleotide sequences. The *fabG*(Ts) mutant ts-67 was derived from strain 4 by Dr J. Huang (Collegeville, PA, USA) at Glaxo SmithKline and strain ts-67R1 is a revertant of strain ts-67. The *Agrobacterium tumefaciens* strains NTL4/pZLR4 (containing *traR* and *traG*::*lacZ*) and NT1/pTiC58 Δ *accR* were from S. Farrand (University of Illinois, Urbana, USA). The *Erwinia carotovora* strain EC14 was previously described (Schweizer, 1994). Unless otherwise indicated, bacterial strains were grown in LB medium (Difco), which for plasmid maintenance in *E. coli* was supplemented with 100 μ g ampicillin ml⁻¹ and/or 25 μ g chloramphenicol ml⁻¹.

Construction of expression vectors and affinity purification of proteins. The coding sequences for the individual enzymes were PCR amplified from PAO1 genomic DNA utilizing *Taq* polymerase and previously described conditions (Hoang & Schweizer, 1999; Hoang *et al.*, 1998). The general strategy involved the use of a forward primer that incorporated an *NdeI* restriction site at the start codon of the respective gene and a reverse primer that incorporated a *BamHI* restriction site after the stop codon of the same gene (Table 1). The gel-

purified (QIAquick gel extraction kit; Qiagen) PCR fragments were digested with *NdeI*/*BamHI* and then ligated between the same sites of pET-15b (Novagen). Since *fabG* contained a *BamHI* site, the reverse primer incorporated a *BglII* site, which allowed subcloning into the *BamHI* site of pET-15b. Standard molecular biological techniques were used (Sambrook & Russell, 2001). Subcloning into pET-15b yielded the expression vectors pPS837 (FabB), pPS980 (FabG), pPS998 (FabH) and pPS937 (FabZ). For FabA, the PCR fragment was first cloned into the TA cloning vector pGEM-T (Promega) to yield pPS847. An *NdeI*-*BamHI* fragment derived from this plasmid was then subcloned between the same sites of pET-15b (Novagen) to yield the FabA expression vector pPS848. For expression of the resulting proteins with NH_2 -terminal hexahistidine (H_6) tags, the plasmids were transformed into BL21(DE3) (Novagen). Screening of H_6 -Fab protein-expressing transformants, cell lysis and purification of the soluble fusion proteins on Ni^{2+} agarose affinity columns (Qiagen) was performed as previously described (Hoang *et al.*, 1999), except for FabD. Since FabD eluted from the columns with 40 mM imidazole, washing of the column was done with 30 bed vols buffer with 20 mM imidazole.

ACP was purified via an intein chitin-binding domain fusion protein as previously described (Kutchma *et al.*, 1999), except that it was coexpressed with acyl-ACP synthase (AcpS) to maximize holo-ACP formation. To this end, the AcpS expressing pPS1118 was constructed by subcloning the *acpS* gene from *E. coli* on a 470 bp *AseI*-*HindIII* fragment from pDPJ (Lambalot & Walsh, 1995) between the same sites of pACYC184 (Chang & Cohen, 1978). For coexpression of ACP and AcpS, the expression strain was grown in LB + ampicillin + chloramphenicol medium to maintain the *acpP*- and *acpS*-containing plasmids. H_6 -LasI was purified using a published procedure (Hoang *et al.*, 1999).

Protein concentrations were determined using the Bradford dye-binding assay (Bio-Rad) and BSA as the standard. Proteins were analysed by electrophoresis on 0.1% SDS-10% polyacrylamide gels (SDS-PAGE) (Makowski & Ramsby, 1993) and visualized by staining with Coomassie Brilliant Blue R-250 (Chen *et al.*, 1993).

Complementation assays. The coding sequences for the H_6 -tagged FabA, FabB and FabD proteins were subcloned into the

Table 1. List of PCR primers

The forward primers incorporated an *NdeI* restriction site (underlined) at the start codon of the respective genes. In most cases, the reverse primers incorporated a *BamHI* restriction site (underlined) after the stop codon with the exception of FabG (reverse), which incorporated a *BglII* site after the *fabG* stop codon.

Name	Sequence (5'-3')
FabA (forward)	TCATATGACCAAACAACACGCCTTCAC
FabA (reverse)	GGATCCCCCTTAGAAGCTGTCAGTGGAG
FabB (forward)	TCCATATGCGTCGCGTCGTTATCACCGGTC
FabB (reverse)	ATGGATCCAATCAACCCTGCCAGCGCTTGAGGA
FabG (forward)	TGACATATGCCGCGCGCCGCCGTGGTCT
FabG (reverse)	GACAGATCTTATGACAGACCCGAGAAAGGTAAC
FabH (forward)	TGACATATGCCGCGCGCCGCCGTGGTCT
FabH (reverse)	GTGGATCCCTCTTCAGTCCATTGTCCG
FabZ (forward)	CCTCATATGATGGACATCAACGAGATTTCG
FabZ (reverse)	GAGGATCCATCAAACATAGTTTGCGT

broad-host-range vector pUCP21T (Schweizer *et al.*, 1996) on *Bam*HI–*Xba*I fragments. Subcloning between the *Bam*HI and *Xba*I sites of pUCP21T placed the H_6 -Fab coding sequences in the correct transcriptional orientation with respect to the *lac* promoter contained on this cloning vector and yielded pPS1013 (H_6 -FabA), pPS1025 (H_6 -FabB) and pPS1019 (H_6 -FabD). To test for expression of functional H_6 -FabA and H_6 -FabB proteins, pPS1013 and pPS1025 were transformed (Hoang *et al.*, 1998) into strain PAO191 (*fabA*) and PAO192 (*fabB*) (Hoang & Schweizer, 1997), respectively. Since FabA and FabB are required for unsaturated fatty acid synthesis, PAO191 and PAO192 will not grow at 42 °C unless supplemented with oleic acid or complemented with either a FabA- or FabB-expressing plasmid. Complementation was therefore scored as the ability to grow at 42 °C on RB medium without oleate supplementation (Hoang & Schweizer, 1997). To test for expression of a functional H_6 -FabD protein, pPS1019 was transformed into the *fabD*(Ts) mutant PAO204 (Kutchma *et al.*, 1999). Successful complementation was scored as the ability of the transformants to grow on LB plates at 42 °C. In all instances, strains were transformed with pUCP21T as a negative control.

Reconstitution of the Fab-AHL pathway and extraction of 3-oxo-acyl-HSLs. Complete reactions (total volume 500 μ l) contained buffer [10 mM Tris/HCl (pH 7.4), 330 mM NaCl, 15%, w/v, glycerol, 0.7 mM DTT, 2 mM EDTA, 25 mM $MgSO_4$, 0.1 mM $FeSO_4$] (Moré *et al.*, 1996), 2 μ g ACP, 1 μ g each FabA, FabB, FabD, FabH, FabI and FabZ, 0.5 μ g FabG, 5 μ g LasI, 0.25 mM SAM, 0.08 mM acetyl-CoA, 0.8 mM malonyl-CoA and 0.6 mM each NADH and NADPH (substrates and cofactors were obtained from Sigma). Reactions were incubated at 37 °C for 1 h and extracted three times with 250 μ l ethyl acetate. Extracted AHLs were dried by rotary vacuum evaporation and resuspended in 20 μ l acetonitrile. For detection of fractions containing AHLs, 5–10 μ l each fraction was spotted on a C_{18} reverse-phase TLC plate (Whatman) and the plates were dried at 37 °C for 15 min before being overlaid with the detection strain. For TLC analysis of AHL fractions, the plates were developed in 60% methanol in water (v/v) and then dried for 20 min at 37 °C prior to being overlaid with the detection strain.

Detection, identification and quantification of AHLs. *A. tumefaciens* reporter strain NTL4/pZLR4 was grown at 30 °C for 48 h in M9 medium (Miller, 1992) with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.6% glucose and 30 μ g gentamicin ml^{-1} (Shaw *et al.*, 1997). Cells were harvested and resuspended in warm (~ 45 °C) fresh M9 medium with 0.4% agar, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.6% glucose and 40 μ g X-Gal ml^{-1} . This suspension was used immediately to overlay the TLC plates. The presence of AHLs was usually evident by the appearance of blue spots after incubation at room temperature for 36–48 h. Synthetic 3-oxo- C_{12} -HSL, and bacterial-derived 3-oxo- C_8 -HSL and 3-oxo- C_6 -HSL were included as standards. The latter two were extracted from 10 ml stationary-phase clarified culture supernatants of *A. tumefaciens* strain NT1/pTiC58 $\Delta accR$ or *Erw. carotovora* strain EC14, respectively, using a previously described method (Shaw *et al.*, 1997). The concentrations of 3-oxo- C_{12} -HSL were estimated utilizing the *Esc. coli* reporter strain MG4/pKDT17 (*lasR*⁺ *lasB*–*lacZ*) as previously described (Schaefer *et al.*, 2000) and by using a dilution series of synthetic 3-oxo- C_{12} -HSL to establish a standard curve. For determination of HSL levels in the supernatants of the *fabG*(Ts) mutant ts-67, its parental strain 4 and the ts-67R1 revertant of strain ts-67, the strains were grown in LB medium until the cultures reached an optical density of ~ 1.6 (600 nm). The pH in the cultures was

monitored to avoid excess alkalization of the medium since AHLs are very unstable at alkaline pH values (Schaefer *et al.*, 2000). Aliquots (1 ml) were harvested by centrifugation. The supernatants were extracted three times with 1 ml acidified ethyl acetate (ethyl acetate containing 0.1 ml glacial acetic acid per litre), dried and suspended in 200 μ l acidified ethyl acetate. For detection of fractions containing 3-oxo-HSLs, 10 μ l each fraction was spotted on a C_{18} reverse-phase TLC plate. The plates were processed as described above and then overlaid with the *A. tumefaciens* detection strain.

RESULTS AND DISCUSSION

Purification and *in vivo* activity of Fab proteins

Our initial goal was to set up a complete *in vitro* Fab-AHL synthesis system using only *P. aeruginosa* proteins by coupling purified Fab enzymes to LasI 3-oxo-AHL synthase. Since we previously described the purification and activity of ACP (Kutchma *et al.*, 1999), FabD (Kutchma *et al.*, 1999), FabI (Hoang & Schweizer, 1999) and LasI (Hoang *et al.*, 1999), we still needed to purify FabA, FabB, FabG, FabH and FabZ, assuming that all of these proteins are needed to synthesize acyl-ACPs from acetyl-CoA and malonyl-CoA (Fig. 1). Using non-denaturing conditions and metal chelation affinity chromatography, all Fab proteins were purified to near homogeneity after overexpression in *E. coli* (Fig. 2). When expressed *in vivo* from the *lac* promoter, the genes encoding the H_6 -tagged FabA, FabB and FabD proteins complemented the corresponding *P. aeruginosa* mutations, indicating that the constructs expressed enzymically active H_6 -Fab proteins. We previously showed that

Fig. 2. Gel electrophoretic analysis of purified proteins. Samples of purified ACP and the various Fab proteins were analysed by electrophoresis on a 0.1% SDS-13% PAGE. The gel was stained with Coomassie blue. All proteins, except ACP, were purified with NH_2 -terminal H_6 -tag containing extensions. ACP was purified in its native form via an ACP-intein chitin-binding domain fusion protein. The sizes of protein markers (M) from Bio-Rad are indicated in kDa and were (top to bottom): myosin, β -galactosidase, BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme.

Fig. 3. Enzymic synthesis of 3-oxo-AHLs in a reconstituted enzyme system. Ethyl acetate extracts of reactions were analysed for the presence of 3-oxo-AHLs by spotting samples on a C_{18} -reverse-phase TLC plate and overlaying it with the *A. tumefaciens* NTL4(pZLR4) detection strain in the presence of X-Gal. Spots indicate the presence of 3-oxo-acyl-HSLs and a 3-oxo- C_{12} -HSL standard (Std). The complete reaction (All) contained ACP, FabA, FabB, FabD, FabG, FabH, FabI, FabZ, LasI, SAM, acetyl-CoA, malonyl-CoA, and NADH and NADPH. The other reactions lacked the indicated enzymes, substrates or co-factors. Some reactions contained 50 μ M of the Fab inhibitors thiolactomycin (TLM), cerulenin (Cer), diazaborine (Dia) or triclosan (Tri). Enzyme abbreviations are explained in Fig. 1. Other abbreviations: AcCoA, acetyl-CoA; MaCoA, malonyl-CoA.

expressed H_6 -FabI complemented an *E. coli fabI*(Ts) mutant and was enzymically active (Hoang & Schweizer, 1999). Complementation experiments were not possible for FabH and FabZ since no mutants were available.

Establishment of an *in vitro* Fab-3-oxo-AHL synthesis system

The Fab-3-oxo-AHL pathway was reconstituted *in vitro* and biologically active 3-oxo-AHLs were detected using an *A. tumefaciens* indicator strain (Fig. 3). The results showed that the minimal Fab-3-oxo-AHL biosynthetic pathway consists of ACP, FabB, FabD, FabG, FabI, FabZ and LasI. Essential metabolites included malonyl-CoA and SAM. Lesser amounts of 3-oxo-AHLs were produced when acetyl-CoA and NADH were omitted. While our experiments confirmed the previously established importance of some components of the Fab system in AHL synthesis, i.e. the dependency on ACP, metabolites and cofactors (Moré *et al.*, 1996; Parsek *et al.*, 1999; Val & Cronan, 1998), the minimal pathway was to date unknown and could not have been determined without establishing the experimental system described in this study. The *in vitro* system also allowed

an assessment of the relative contribution of the seemingly redundant components of the Fab system.

Synthases. Since FabB is the major condensing enzyme, it was essential for AHL formation from malonyl-CoA. In contrast, FabH was not required presumably since FabB can decarboxylate malonyl-ACP to acetyl-ACP and then condenses these two molecules to initiate the cycle without FabH (Fig. 1), as has been suggested for *E. coli* FabB (Cronan & Rock, 1996). This would also explain the formation of 3-oxo-AHLs in the reactions containing no acetyl-CoA.

Dehydratases. Of the two dehydratases, only FabZ was essential for AHL formation but not FabA. This is probably due to the fact that FabZ is mostly required in the initial cycles since its *E. coli* counterpart has greatest affinity for C_4 - C_8 β -hydroxyacyl-ACP intermediates, but can use substrates with longer acyl chains (Heath & Rock, 1996a). In contrast, *E. coli* FabA acts preferably on C_{10} - C_{14} β -hydroxyacyl-ACP intermediates.

Reductants. Exclusion of NADH led to detectable AHL production but at much reduced levels. Since NADH is the reductant preferred by FabI (Hoang & Schweizer, 1999), this result indicates that FabI can utilize NADPH but that this step becomes rate-limiting in the absence of NADH.

When the known Fab inhibitors cerulenin, triclosan, diazaborine and thiolactomycin were added to the reaction mixture, only cerulenin and triclosan efficiently inhibited 3-oxo-AHL formation at the concentration tested (50 μ M). For unknown reasons, at the same concentrations, thiolactomycin and diazaborine had little effect but from other experiments we suspected that these two antimicrobials, which are not available commercially, had lost much of their activities during storage (data not shown).

Nature of AHL molecules synthesized *in vitro*

TLC analysis (Fig. 4) was used to identify AHL species contained in representative positive reactions shown in Fig. 3. The analysis showed that reactions containing all essential components of the Fab-3-oxo-AHL synthesis system almost exclusively yielded 3-oxo- C_{12} -HSL, and only minute amounts of shorter chain 3-oxo-AHLs were discernible. Conversely, in the absence of NADPH but presence of NADH, LasI synthesized hardly any 3-oxo- C_{12} -HSL but larger amounts of 3-oxo- C_{10} -HSL and 3-oxo- C_8 -HSL, and lesser amounts of 3-oxo- C_6 -HSL (lane labelled -NADPH). Since 3-oxo- C_8 -HSL is the cognate *A. tumefaciens* AHL, its spot size is not indicative of a higher quantity of 3-oxo- C_8 -HSL relative to the other 3-oxo-AHLs, but rather indicates a better response to its native AHL. According to the pathway model (Fig. 1), LasI and FabG compete for 3-oxo-acyl-ACP substrates from the Fab system. Although FabG can utilize NADH, NADPH is its preferred cofactor and in its absence the FabG-catalysed reduction step becomes rate limiting, leading to accumulation of shorter chain 3-oxo-acyl-

Fig. 4. Identification of AHLs produced in *in vitro* synthesis reactions. Extracted and concentrated products from selected reactions shown in Fig. 3 were spotted onto a C_{18} -reverse phase TLC plate. The plate was developed with 60% (v/v) methanol in water and overlaid with the *A. tumefaciens* NTL4(pZLR4) detection strain in the presence of X-Gal. All, complete reaction mixtures contained ACP, FabA, FabB, FabD, FabG, FabH, FabI, FabZ, LasI, SAM, acetyl-CoA, malonyl-CoA, and NADH and NADPH; other reactions lacked the indicated enzymes, substrates or co-factors. The relative mobility of known 3-oxo-acyl-HSLs, analysed on the same TLC plate but in a portion that is not shown, and the sample origin are marked on the right.

ACPs. This now enables LasI to compete for the shorter-chain 3-oxo-acyl-ACP substrates and use them for synthesis of the corresponding shorter chain 3-oxo-AHLs. These results also proved that LasI alone is sufficient for synthesis of the shorter chain 3-oxo-AHLs found in *P. aeruginosa* culture supernatants.

A *fabG*(Ts) mutant is altered in 3-oxo-AHL production

A *fabG*(Ts) mutant was used to obtain preliminary *in vivo* experimental evidence for some of the *in vitro* observations. To examine whether altered FabG activity influenced 3-oxo-AHL production *in vivo*, AHL formation was analysed in a *fabG*(Ts) mutant grown in LB medium at permissive temperature (30 °C) and 37 °C, a temperature that is close to non-permissive (38 °C or higher). The *fabG*(Ts) mutant produced elevated levels of all 3-oxo-AHLs at both temperatures, most notably 3-oxo- C_6 -HSL which under these experimental conditions was undetectable in supernatants obtained from wild-type and revertant strains, respectively (Fig. 5). Whereas the parental wild-type and the revertant strain produced levels of 3-oxo- C_{12} -HSL that remained nearly constant over the temperature range examined, the *fabG*(Ts) strain produced elevated levels of this 3-oxo-AHL, which increased with increasing temperatures (Table 2). These increasing 3-oxo- C_{12} -HSL levels were paralleled with a slight decrease in growth rates of the *fabG*(Ts) mutant as the temperature increased. The doubling

Fig. 5. Identification of 3-oxo-AHLs produced by a *fabG*(Ts) strain, its parent and a revertant. AHLs were extracted from cells grown at the indicated temperatures and samples of the concentrated reaction products were spotted onto a C_{18} -reverse phase TLC plate. The plate was developed with 60% (v/v) methanol in water and overlaid with the *A. tumefaciens* NTL4(pZLR4) detection strain in the presence of X-Gal. Samples analysed were from wild-type strain 4 (WT), its *fabG*(Ts) derivative (TS) and a revertant that contained a restored wild-type *fabG* sequence (RE). Standards included 3-oxo- C_6 -HSL (C_6), 3-oxo- C_8 -HSL (C_8) and 3-oxo- C_{12} -HSL (C_{12}). The relative mobility of 3-oxo- C_{10} -HSL (C_{10}), for which no standard was available, and the origin (O) are marked on the right.

times at 37 °C were 24 min for wild-type and revertant, and 36 min for the *fabG*(Ts) mutant. Similar observations to those presented in Fig. 5 and Table 2 were made when AHLs were extracted from cultures grown to lesser cell densities (data not shown). The most plausible explanation for these observations is that even at permissive temperatures the *fabG*(Ts) strain produces a FabG protein whose reductase activity is decreased when compared to wild-type or revertant FabG. Decreased FabG activity would lead to an increase in the intracellular 3-oxo-acyl-ACP pools, enabling LasI to compete for these substrates, ultimately resulting in increased 3-oxo-acyl-HSL levels.

Conclusions

The 3-oxo-AHLs normally found in *P. aeruginosa* culture supernatants contain acyl chains of 6–12 carbons (Shaw *et al.*, 1997) and the relative abundances of different 3-oxo-AHLs change during growth. The results obtained with our *in vitro* system gave the first clues that modulation of FabG activity by substrate and/or cofactor availability may at least partially explain these observations. In the presence of LasI, this AHL synthase

Table 2. Estimation of 3-oxo-C₁₂-HSL production by a *fabG*(Ts) mutant, its parent and a revertant

The strains were grown in LB medium to OD₆₀₀ ~1.6 at the indicated temperatures. One millilitre aliquots were harvested by centrifugation. The supernatants were extracted three times with 1 ml acidified ethyl acetate, dried and suspended in 200 µl acidified ethyl acetate. The concentrations of 3-oxo-C₁₂-HSL were estimated using the *E. coli* reporter strain MG4/pKDT17 (*lasR*⁺ *lasB*-*lacZ*) and a dilution series of synthetic 3-oxo-C₁₂-HSL to establish a standard curve. The values shown are the means ± standard deviations of triplicate measurements.

Strain	Extracellular 3-oxo-C ₁₂ -HSL (nM)		
	22 °C	30 °C	37 °C
4 (wild-type)	48.1 ± 8.8	39.3 ± 15.0	42.6 ± 15.4
ts-67 [<i>fabG</i> (Ts)]	252 ± 88.7	301 ± 79.8	487 ± 133
ts-67R1 (revertant to wild-type)	55.6 ± 10.7	47.1 ± 25.4	26.4 ± 7.39

and FabG compete for 3-oxo-acyl-ACP substrates from the fatty acid biosynthetic pathway. When FabG activity is high, turnover of the short chain 3-oxo-acyl-ACP substrates is rapid and LasI cannot compete for them, presumably because its affinity for them is lower than that of FabG. Once the acyl chain length reaches 12 carbons, LasI efficiently competes for the 3-oxo-C₁₂-ACP, resulting in synthesis of 3-oxo-C₁₂-HSL. When the FabG catalysed step becomes rate limiting, as mimicked in our experimental system by switching cofactors from the preferred NADPH to NADH, accumulation of shorter chain 3-oxo-acyl-ACPs results. This enables LasI to compete for these shorter-chain 3-oxo-acyl-ACP substrates and use them for synthesis of the corresponding shorter chain 3-oxo-AHLs. This explains why in the absence of NADPH only minute amounts of 3-oxo-C₁₂-HSL were synthesized in the *in vitro* reactions, while the levels of 3-oxo-C₈-HSL and 3-oxo-C₁₀-HSL were greatly elevated (Fig. 4). Consistent with these observations and conclusions, a *fabG*(Ts) mutant produced overall elevated levels of 3-oxo-AHLs, especially when it was grown at increasing temperatures (Fig. 5; Table 2), presumably since the respective 3-oxo-acyl-ACPs become available for LasI as the growth rate and therefore the demand for fatty acids for other biosynthetic processes decreases. The potential physiological relevance of 3-oxo-AHLs in *P. aeruginosa* other than 3-oxo-C₁₂-HSL, and the regulation of their relative abundances during cellular growth by modulation of FabG activity is currently unclear and awaits further investigation. FabG activity may be controlled at the genetic level (e.g. via transcriptional regulation of *fabG*) or at the protein level (via substrate allosteric effects).

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