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Exogenous Myristic Acid Can Be Partially Degraded prior to Activation To Form Acyl-Acyl Carrier Protein Intermediates and Lipid A in *Vibrio harveyi*

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To study the involvement of acyl carrier protein (ACP) in the metabolism of exogenous fatty acids in *Vibrio harveyi*, cultures were incubated in minimal medium with [9,10-^{3}H]myristic acid, and labeled proteins were analyzed by gel electrophoresis. Labeled acyl-ACP was positively identified by immunoprecipitation with anti-*V. harveyi* ACP serum and comigration with acyl-ACP standards and [^{3}H]β-alanine-labeled bands on both sodium dodecyl sulfate- and urea-polyacrylamide gels. Surprisingly, most of the acyl-ACP label corresponded to fatty acid chain lengths of less than 14 carbons: C_{14}, C_{12}, C_{10}, and C_8 represented 33, 40, 14, and 8% of total [^{3}H]14:0-derived acyl-ACPs, respectively, in a dark mutant (M17)^r of *V. harveyi* which lacks myristoyl-ACP esterase activity; however, labeled 14:0-ACP was absent in the wild-type strain. 14:0- and 12:0-ACP were also the predominant species labeled in complex medium. In contrast, short-chain acyl-ACPs ($\leq C_6$) were the major labeled derivatives when *V. harveyi* was incubated with [^{3}H]acetate, indicating that acyl-ACP labeling with [^{3}H]14:0 in vivo is not due to the total degradation of [^{3}H]14:0 to [^{3}H]acetyl coenzyme A followed by resynthesis. Cerulenin increased the mass of medium- to long-chain acyl-ACPs ($\geq C_8$) labeled with [^{3}H]β-alanine fivefold, while total incorporation of [^{3}H]14:0 was not affected, although a shift to shorter chain lengths was noted. Additional bands which comigrated with acyl-ACP on sodium dodecyl sulfate gels were identified as lipopolysaccharide by acid hydrolysis and thin-layer chromatography. The levels of incorporation of [^{3}H]14:0 into acyl-ACP and lipopolysaccharide were 2 and 15%, respectively, of that into phospholipid by 10 min. Our results indicate that, in contrast to the situation in *Escherichia coli*, exogenous fatty acids can be activated to acyl-ACP intermediates after partial degradation in *V. harveyi* and can effectively label products (i.e., lipid A) that require ACP as an acyl donor.

The mechanism of uptake and degradation of exogenous fatty acids in *Escherichia coli* is well established; for reviews, see references 19, 24, and 35. The uptake of long-chain fatty acids involves protein-mediated transport across the outer membrane (2) and then activation to acyl coenzyme A (acyl-CoA) by acyl-CoA synthetase on the cytoplasmic side of the inner membrane (20). These fatty acids can then be incorporated into membrane phospholipids by glycerol 3-phosphate acylation (14) or degraded to acetyl coenzyme A (acetyl-CoA) via β-oxidation (24). In contrast, de novo fatty acid synthesis involves elongation of acyl-acyl carrier protein (acyl-ACP) intermediates; these ultimately serve as donors for the acylation of both phospholipids and the lipid A moiety of outer membrane lipopolysaccharides (27). Exogenous fatty acids are not elongated in *E. coli*, indicating that they do not have access to biosynthetic pathways involving acyl carrier protein (ACP) (33). Although exogenous fatty acids can be transferred to phospholipids by 2-acylglycerophosphatidylethanolamine acyltransferase/acyl-ACP synthetase (10, 17), the transient acyl-ACP intermediates bound to this inner membrane enzyme are not available to enzymes of fatty acid biosynthesis or glycerol 3-phosphate acylation. Thus, there is no acyl group exchange between acyl-CoA and acyl-ACP, and the biosynthesis and degradation of fatty acids are chemically segregated in *E. coli*.

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We previously showed that the metabolism of exogenous fatty acids in the bioluminescent bacterium *Vibrio harveyi* exhibits a number of differences from that in *E. coli*, despite overall similarities in the fatty acid and phospholipid compositions of these gram-negative organisms (5). First, exogenous labeled fatty acids can be elongated prior to incorporation into phospholipids in *V. harveyi* (6), indicating that these fatty acids have access to biosynthetic pathways. Second, there is a small transient pool of unesterified fatty acids (5), arising from the action of a luminescence-specific esterase which specifically cleaves 14:0-ACP to supply 14:0 for reduction to form the myristyl aldehyde substrate of luciferase (8, 11); free 14:0 is regenerated by light-emitting luciferase oxidation. Myristoyl-ACP esterase is defective in a dark mutant strain of *V. harveyi* (mutant M17) which requires exogenous 14:0 for light emission (23, 34). Finally, we recently isolated from *V. harveyi* a soluble acyl-ACP synthetase which is capable of activating a broad range of fatty acids to acyl-ACP in vitro (7, 12, 32).

Elongation of exogenous fatty acids in *V. harveyi* could in principle occur by a number of possible mechanisms, including: (i) activation and elongation as acyl-CoA derivatives, as occurs in eucaryotes (9), or (ii) activation to form biosynthetic acyl-ACP intermediates, either directly upon entry into the cell or by transfer of the acyl group from coenzyme A (CoA) to ACP (without complete degradation to acetyl-CoA). To date, labeled acyl-ACP formation from exogenous fatty acids has not been reported in any bacterium. In the present study, we demonstrate that acyl-ACP is indeed labeled with exogenous [^{3}H]14:0, although the appearance of shorter-chain acyl-ACPs

suggests partial degradation, as acyl-CoA intermediates, can occur prior to acyl-ACP activation. Moreover, we show that lipid A, a cellular component that requires acyl-ACP for its synthesis in *E. coli*, is efficiently labeled with [^{3}H]14:0 in *V. harveyi*.

MATERIALS AND METHODS

Materials. [1-^{14}C]octanoic acid (8:0; 53.5 Ci/mol) and [1-^{14}C]palmitic acid (16:0; 58 Ci/mol) were obtained from Du Pont-NEN (Du Pont Canada Inc., Mississauga, Ontario). [1-^{14}C]lauric acid (12:0; 57 Ci/mol) and [1-^{14}C]myristic acid (14:0; 54 Ci/mol) were obtained from Amersham Canada Ltd. (Oakville, Ontario). [1-^{14}C]hexanoic acid (6:0; 8 Ci/mol) and [1-^{14}C]decanoic acid (10:0; 10.6 Ci/mol) were purchased from Sigma Chemical Co. (St. Louis, Mo.). [9,10-^{3}H]myristic acid (20 Ci/mmol) was obtained by thin-layer chromatographic purification of the products of tritiation of myristoleic acid prepared by Amersham. β-[^{3}H]alanine (87 Ci/mmol) was obtained from Du Pont-NEN, while sodium [^{3}H]acetate (22 Ci/mmol) was obtained from ICN Biomedicals Canada Ltd. (Mississauga, Ontario). Fixed *Staphylococcus aureus* Cowan 1 was purchased from Terochem Laboratories Ltd. (Markham, Ontario). All equipment for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was obtained from BioRad (Canada) Ltd. (Mississauga, Ontario), and low-molecular-weight standards for gel electrophoresis were purchased from Pharmacia. En^{3}Hance was obtained from Du Pont-NEN.

***V. harveyi* ACP and acyl-ACP standards.** *V. harveyi* ACP was purified by a modification of the procedure described by Rock and Cronan (28) for *E. coli* ACP (31a). *V. harveyi* acyl-ACP standards with acyl chain lengths of C_6 to C_{16} were prepared by use of ^{14}C-labeled fatty acids and partially purified *V. harveyi* acyl-ACP synthetase as previously described (32). A rabbit anti-ACP serum was prepared against purified *V. harveyi* ACP by subcutaneous injection of 250 μg of ACP in 1 ml of Freund's complete adjuvant (15). Three booster injections, each with 200 μg of ACP in incomplete adjuvant, were given at 2- to 3-week intervals. Serum was collected 2 weeks after the last injection.

In vivo radiolabeling and immunoprecipitation. For β-[^{3}H]alanine labeling, *V. harveyi* cells in exponential growth were subcultured 1:250 in 1 ml of minimal medium [10 g of NaCl, 3.7 g of Na_2HPO_4, 1 g of KH_2PO_4, 0.5 g of $(NH_4)_2HPO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 2 ml of glycerol per liter] containing 0.3 μM β-[^{3}H]alanine (87 Ci/mmol) and grown to an optical density at 660 nm (OD_{660}) of 2 to 3. Complex medium contained, in addition, 5 g of Bacto-Tryptone (Difco) and 0.5 g of yeast extract (Difco) per liter (5). For [^{3}H]myristic acid labeling, 1 ml of cells in minimal or complex medium was incubated with 20 μl of 0.2 mM [^{3}H]14:0 (20 Ci/mmol) at 27°C. Cell pellets were collected in a microcentrifuge (7,000 × *g* for 1 min), washed once with 1 ml of minimal medium, and resuspended in 50 μl of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl [pH 7.5] (15). After sonication (two 20-s bursts), the lysate was centrifuged at 13,000 × *g* for 5 min to remove unbroken cells, heated at 80°C for 5 min, and centrifuged again to remove denatured proteins. The supernatant was frozen in liquid nitrogen or used directly for electrophoresis or for immunoprecipitation. The heating step was added to avoid possible metabolism or degradation of acyl-ACPs in routine analysis (no effect on acyl-ACPs was noted). Repeated freezing and thawing or prolonged incubation during immunoprecipitation was found to alter the acyl-ACP profile to a small extent.

Immunoprecipitation was carried out by incubating 30 μl of labeled cell lysate with 20 μl of antiserum or preimmune serum and 5 μl of 5× RIPA buffer at 0°C for 90 min (15). One hundred microliters of 20% (vol/vol) fixed *S. aureus* cells, which had been washed with and resuspended in RIPA buffer, was added and incubated for a further 2 h at 0°C. The pellets were washed three times with RIPA buffer, collected by centrifugation, resuspended in 200 μl of SDS sample buffer (2% SDS, 10% glycerol, 60 mM Tris-HCl [pH 6.8], 0.001% bromphenol blue), and heated to 85°C for 5 min to release antigen.

Electrophoresis and fluorography. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (21) with resolving and stacking gels of 15 and 4% (wt/vol) acrylamide, respectively. Polyacrylamide gels containing 2 M urea were prepared as described by Post-Beittenmiller et al. (26) with 15 and 5% (wt/vol) acrylamide resolving and stacking gels, respectively. No urea was added to the running buffer (3.1 g of Tris-bis and 14.4 g of glycine per liter). Lysate samples or acyl-ACP standards were diluted with equal volumes of 2×-concentrated SDS sample buffer before being loaded onto either SDS- or urea-polyacrylamide gels. After electrophoresis, protein bands in SDS gels were visualized by staining with Coomassie blue, and the labeled proteins were visualized by fluorography with Kodak X-Omat AR film without flashing as described previously (37). Urea gels were soaked in at least two changes of fixing solution (10% acetic acid–30% methanol [vol/vol]) overnight to remove urea from the gels before fluorography. The relative incorporation of label into acyl-ACP intermediates was determined by densitometric scanning of fluorograms with an Apple OneScanner and National Institutes of Health Image 1.45 software; absolute quantitation was established by counting tritium label in [^{3}H]14:0-labeled bands excised from the corresponding dried polyacrylamide gel (36). Variation in the labeling of individual acyl-ACP species from samples incubated with [^{3}H]14:0 in duplicate was less than ±2% of the total acyl-ACP label.

Analysis of phospholipids and lipid A. Extraction and analysis of phospholipid and lipid A were carried out essentially as described by Galloway and Raetz (13). *V. harveyi* M17 cells labeled with [^{3}H]14:0 in complex medium were centrifuged and washed once with 1 ml of complex medium containing 0.1% bovine serum albumin. The cell pellet was resuspended in complex medium and mixed with 3.75 volumes of methanol-chloroform (2:1 [vol/vol]) (3), and the insoluble residue containing lipopolysaccharide was separated from the organic phospholipid extract by centrifugation. To prepare monophosphoryl lipid A degradation products from lipopolysaccharide, the insoluble residue was resuspended in 0.2 N HCl by sonication and heated at 100°C for 90 min (13). After neutralization of the hydrolysate with 2 N NaOH, thin-layer chromatographic (TLC) analysis was performed with silica gel G plates developed in chloroform-methanol-water-ammonia (40:25:4:2 [vol/vol]) and sprayed with En^{3}Hance prior to fluorography.

RESULTS

Identification and analysis of acyl-ACPs labeled in vivo. To establish whether the previously observed elongation of exogenous saturated ^{14}C-fatty acids in *V. harveyi* (6) might involve activation to form acyl-ACP, cultures in minimal medium were incubated with labeled precursors, and cell extracts were subjected to PAGE in the presence of 2 M urea (26). As shown in Fig. 1, incubation of the M17 mutant of *V. harveyi* (chosen for most studies to avoid potential interference of the luminescence-specific myristoyl-ACP esterase in the detection of

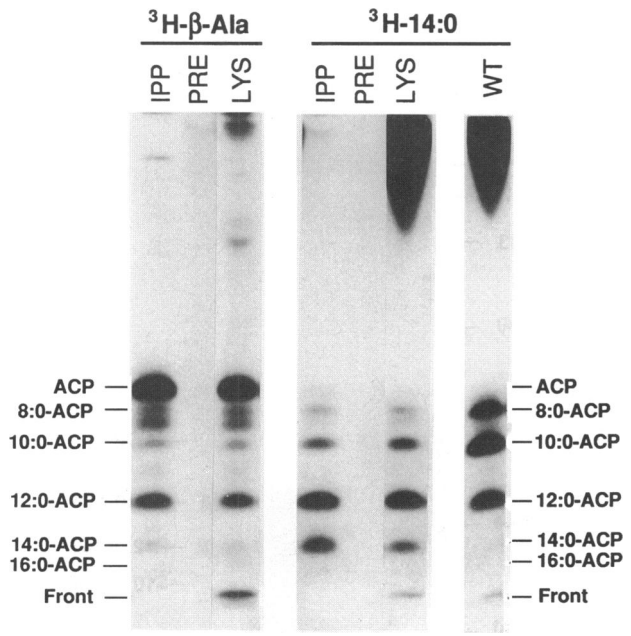

FIG. 1. Urea-PAGE of *V. harveyi* proteins labeled with [^{3}H]β-alanine or [^{3}H]14:0 in minimal medium. *V. harveyi* M17 cells (1 ml) were labeled with 0.3 μM [^{3}H]β-alanine (87 Ci/mmol) during growth (40 h to an OD_{660} of 2.5) in minimal medium at 27°C. For [^{3}H]myristic acid labeling, M17 or wild-type (WT) cells were grown similarly, and 1 ml was incubated with 20 μl of 0.2 mM [^{3}H]14:0 (20 Ci/mmol) for 10 min. Cells were lysed and treated with either anti-ACP serum (IPP) or preimmune serum (PRE) as described in Materials and Methods. Four microliters of cell lysate (LYS and WT) or immunoprecipitates from 15 μl of lysate (IPP and PRE) were fractionated by 2 M urea-PAGE. A fluorogram of a 30-day exposure is shown.

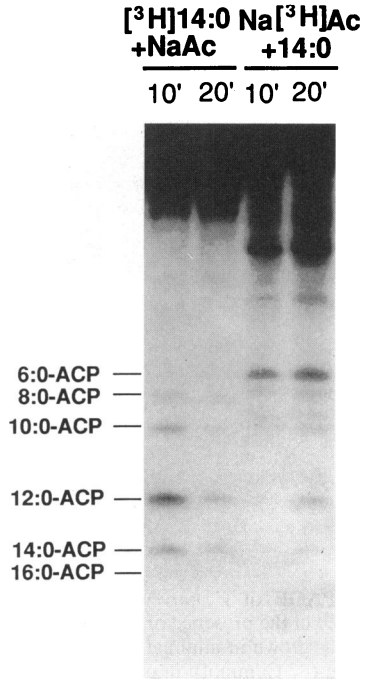

FIG. 2. Urea-PAGE of *V. harveyi* M17 proteins labeled with [^{3}H]14:0 or sodium [^{3}H]acetate (Na[^{3}H]Ac) in minimal medium. *V. harveyi* M17 cells were incubated for 10 or 20 min in minimal medium (1 ml) containing either 5 μM unlabeled 14:0 and 66 μM [^{3}H]acetate (22 Ci/mmol) or 5 μM [^{3}H]14:0 (20 Ci/mmol) and 66 μM unlabeled acetate (NaAc) as indicated. Radiolabeled cells were lysed as described in Materials and Methods, and 5 μl of cell lysate was analyzed by urea-PAGE. A fluorogram of an 80-day exposure is shown.

acyl-ACP intermediates) with [^{3}H]14:0 resulted in the labeling of proteins which were immunoprecipitated with anti-*V. harveyi* ACP serum but not preimmune serum. These bands comigrated with authentic ^{14}C-acyl-ACP standards (C_8 to C_{14}), which were well resolved in this gel system, with migration increasing as a function of fatty acid chain length (Fig. 1). Incubation with [^{3}H]β-alanine (a specific precursor of the pantotheine prosthetic group of ACP) also resulted in the labeling of medium- to long-chain acyl-ACPs, as well as higher-molecular-weight bands corresponding to free ACP and short-chain ($\leq C_8$) acyl-ACPs. These results provide conclusive evidence that acyl-ACPs can be labeled with exogenous [^{3}H]14:0 in *V. harveyi*.

Surprisingly, only about one-third of the [^{3}H]14:0-derived label associated with acyl-ACP was found in 14:0-ACP in M17 cells under these conditions. Densitometric scanning of fluorograms following urea-PAGE of cell lysates revealed 95% of the acyl-ACP label in four species: 14:0-ACP (33.0% ± 7.9%), 12:0-ACP (39.8% ± 4.4%), 10:0-ACP (14.4% ± 5.4%), and 8:0-ACP (8.5% ± 2.4%) (mean ± standard deviation for eight samples). While the incorporation of [^{3}H]14:0 into the phospholipid fraction of M17 cells in minimal medium increased with time to 20 min, the amount of label in acyl-ACPs was relatively constant between 2 and 10 min and decreased slightly thereafter: label in total acyl-ACPs decreased from 5% of that in phospholipid at 5 min to about 1% at 20 min (data not shown). There was no significant change in the relative labeling of individual acyl-ACP species over this period. Acyl-ACP bands labeled with [^{3}H]14:0 disappeared upon chasing for 10 min with unlabeled 14:0, indicating that they were metabolically active intermediates.

The appearance of [^{3}H]14:0-labeled acyl-ACP chain lengths shorter than C_{14} was even more pronounced in wild-type *V. harveyi* cells (Fig. 1, WT). Indeed, no labeled 14:0-ACP was detected at all in wild-type cells labeled in minimal medium, while increases in the amounts of labeled 8:0-ACP (10-fold) and 10:0-ACP (6-fold) relative to those in M17 cells were observed. These differences indicate that the activity of the luminescence-specific 14:0-ACP esterase in the wild-type strain depletes 14:0-ACP and also influences the labeling of other acyl-ACP intermediates. To ensure that acyl-ACP formation from exogenous fatty acids was not an artifact of restricted growth in minimal medium, cells were incubated with [^{3}H]14:0 in complex medium. Although extremely long exposure times were necessary under these conditions, 12:0-ACP and 14:0-ACP were observed in both wild-type and M17 cells (data not shown). Little or no 16:0-ACP was observed under any conditions in the present study and, as expected, *E. coli* exhibited no acylation of ACP.

Labeled acyl-ACP intermediates do not arise from the total degradation of 14:0 to acetyl-CoA. The observed labeling of acyl-ACPs with [^{3}H]14:0 could in theory arise from β-oxidation of [9,10-^{3}H]14:0 to form [^{3}H]acetyl-CoA (which would be released during degradation of the parent chain at the [^{3}H]6:0-CoA and [^{3}H]4:0-CoA stages), followed by de novo resynthesis. This possibility was investigated by incubating *V. harveyi* M17 cells with either [^{3}H]14:0 (and unlabeled acetate) or [^{3}H]acetate (and unlabeled 14:0) in minimal medium (Fig. 2). The labeling pattern of acyl-ACPs as a function of chain length

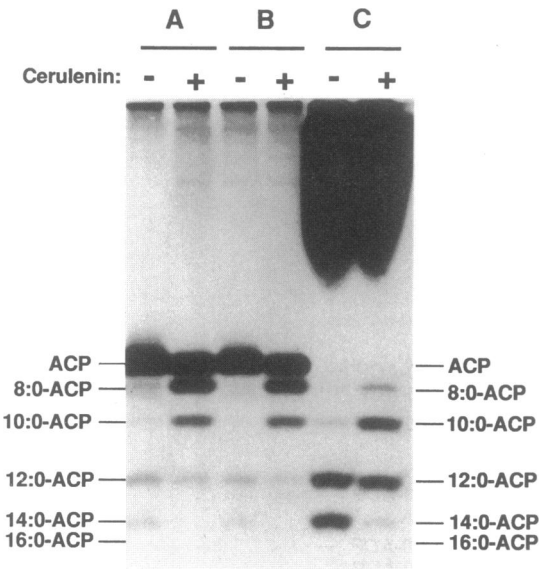

FIG. 3. Urea-PAGE of *V. harveyi* proteins labeled with [^{3}H]β-alanine or [^{3}H]14:0 in the presence or absence of cerulenin. (A and B) *V. harveyi* M17 was grown in minimal medium (1.2 ml) containing 0.3 μM [^{3}H]β-alanine (87 Ci/mmol); in panel B, unlabeled 14:0 (3.3 μM) and Na acetate (55 μM) were also added for 10 min prior to harvest. (C) Cells in minimal medium were incubated with [^{3}H]14:0 (4.2 μM; 20 Ci/mmol) and 55 μM unlabeled acetate for 10 min prior to harvest. As indicated, cerulenin (10 μg/ml) was also added for 1 h prior to harvest and lysis at an OD_{660} of 2.8 as described in Materials and Methods. Cell lysates (10 μl) were fractionated by urea-PAGE; a fluorogram of a 10-day exposure is shown.

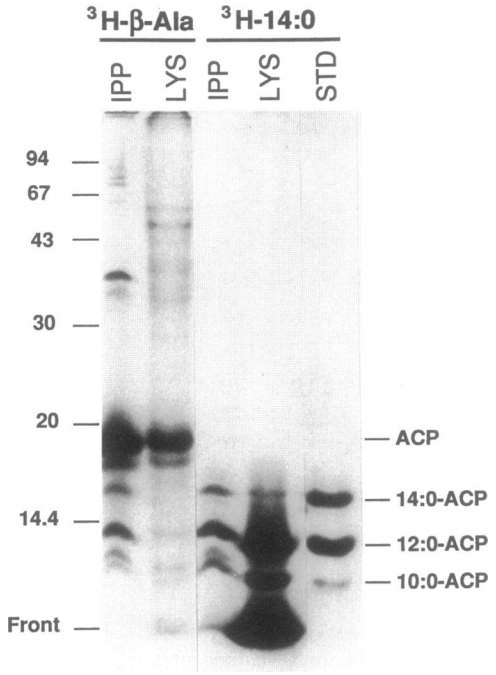

FIG. 4. SDS-PAGE of *V. harveyi* M17 proteins labeled with [^{3}H]β-alanine or [^{3}H]14:0 in minimal medium. *V. harveyi* M17 cells (1 ml) were labeled with [^{3}H]β-alanine or [^{3}H]14:0 as described in the legend to Fig. 1. Cell lysates (LYS, 2 μl), immunoprecipitates (IPP) from 10 μl of lysate, and ^{14}C-acyl-ACP standards (STD) were fractionated by SDS-PAGE as described in Materials and Methods. The migration of ACP and standard molecular mass proteins (in kilodaltons) are also indicated. A fluorogram of a 20-day exposure is shown.

would be expected to be identical under these chemically equivalent conditions were exogenous [^{3}H]14:0 totally degraded to [^{3}H]acetyl-CoA and then resynthesized to form ^{3}H-acyl-ACP. In contrast, the labeling pattern observed with [^{3}H]14:0 was significantly different from that observed with [^{3}H]acetate: with [^{3}H]14:0 as a precursor, 12:0-ACP was the major acyl-ACP band, representing 46% of the total label associated with acyl-ACPs, and short-chain acyl-ACPs ($\leq C_6$) were not detected; however, short-chain acyl-ACPs represented 52% of the total acyl-ACP label from [^{3}H]acetate, and 12:0-ACP represented <10% (Fig. 2). Moreover, the temporal pattern of incorporation was different, with [^{3}H]14:0 labeling of long-chain acyl-ACP decreasing with longer incubation times (as noted above), while the opposite was observed with [^{3}H]acetate. This experiment indicates that acyl-ACPs are labeled following partial, but not total, degradation of the parent [9,10-^{3}H]14:0 chain.

Effect of cerulenin on acyl-ACP intermediates. To determine whether acyl-ACP intermediates derived from (partially degraded) exogenous labeled fatty acids can undergo chain elongation, *V. harveyi* M17 cells were incubated with labeled precursors in the presence or absence of cerulenin, which blocks fatty acid synthesis by irreversibly inhibiting 3-ketoacyl-ACP synthase (25). Cerulenin had a dramatic effect on the relative mass composition of acyl-ACPs labeled with [^{3}H]β-alanine, with large increases in the amounts of 8:0-ACP (25-fold) and 10:0-ACP (16-fold) (Fig. 3A). On the other hand, 14:0-ACP was essentially undetectable in the presence of cerulenin, even upon longer fluorographic exposure. Comparable effects of cerulenin were noted with [^{3}H]acetate as a precursor (data not shown). Inclusion of unlabeled 14:0 alone or with acetate (Fig. 3B) did not affect the pattern of labeling of ACP derivatives with [^{3}H]β-alanine (with or without cerulenin), indicating that these precursors do not significantly perturb the mass composition of the acyl-ACP pool. In marked contrast to the results obtained with [^{3}H]β-alanine, there was no change (<2% decrease) in the total incorporation of [^{3}H]14:0 label into acyl-ACPs in the presence of cerulenin; however, the labeling of 14:0-ACP was substantially decreased, while the labeling of both 8:0-ACP and 10:0-ACP was increased sixfold (Fig. 3C). These results suggest that long-chain acyl-ACPs labeled with [^{3}H]14:0 have not completely equilibrated with the mass pool (labeled with [^{3}H]β-alanine) under these conditions.

Labeling of lipid A with exogenous myristic acid. As shown in Fig. 4, immunoprecipitated acyl-ACPs labeled in vivo with either [^{3}H]β-alanine or [^{3}H]14:0 could also be resolved by SDS-PAGE (note that the migration of long-chain acyl-ACPs decreases as a function of chain length in this gel system). However, in contrast to the situation with urea gels in which acyl-ACP composition could be directly analyzed without immunoprecipitation, we observed that most of the [^{3}H]14:0-labeled material which migrated in the acyl-ACP region of SDS gels was not immunoprecipitated with anti-ACP serum (Fig. 4).

We investigated the possibility that these labeled bands might correspond to lipopolysaccharide, which is known to migrate as defined bands or "ladders" in SDS gels (38). In the experiment shown in Fig. 5, lysates from M17 cells labeled with [^{3}H]14:0 in complex medium were analyzed in parallel by SDS-PAGE and TLC. The labeled bands appeared as a doublet on SDS-PAGE of cell lysates (lane A); acyl-ACPs

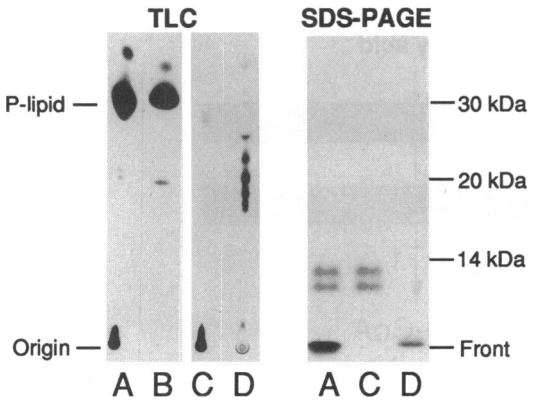

FIG. 5. Comparison of [^{3}H]14:0-labeled products in *V. harveyi* cells by SDS-PAGE and TLC. *V. harveyi* M17 (OD_{660} = 1.2) in 1 ml of complex medium was incubated with 20 μl of 0.2 mM [^{3}H]14:0 (20 Ci/mmol) for 10 min. One half of the washed cell pellet was used to prepare a cell lysate (A) in 125 μl of RIPA buffer; 0.5- and 2.0-μl portions were analyzed by TLC and SDS-PAGE, respectively, as described in Materials and Methods. The other half of the cell pellet was used to prepare a lipid extract (total volume, 2.2 ml); 6 μl of this lipid extract (B) was used for TLC analysis. One half of the insoluble residue containing lipopolysaccharide (C) was resuspended in 68 μl of RIPA buffer by sonication, diluted with an equal volume of 2× SDS sample buffer, and analyzed by TLC (1 μl) or SDS-PAGE (4 μl). The other half of the insoluble residue (D) was heated in 68 μl of 0.2 N HCl, neutralized with NaOH, diluted with SDS sample buffer, and analyzed by TLC (2 μl) or SDS-PAGE (4 μl). Fluorography exposures were 2 days for TLC and 1 day for SDS-PAGE. P-lipid, phospholipid.

were not visualized under these conditions and short exposure times. These bands could not be extracted with chloroform-methanol and corresponded to the label remaining at the origin on TLC (lane C), while the labeled material at the SDS gel front could be extracted with organic solvent and was identified as glycerophospholipid by TLC (lane B). The labeled bands in SDS-PAGE were resistant to proteinase K treatment but were destroyed by acid hydrolysis of the chloroform-methanol-insoluble residue, resulting in the appearance of a series of labeled spots on TLC (lane D). This pattern is characteristic of the 4′-monophosphoryl lipid A degradation products released by acid hydrolysis of *E. coli* lipopolysaccharide, as reported by Galloway and Raetz (13). The migration of these [^{3}H]14:0-labeled products in this TLC system also corresponded to the migration of those from samples prepared from both *E. coli* RR1 and *V. harveyi* labeled with $^{32}P_i$, for which virtually identical patterns were observed (data not shown). These observations confirm that the lipid A component of outer membrane lipopolysaccharide can be labeled with exogenous myristic acid in *V. harveyi*. The incorporation of [^{3}H]14:0 into lipopolysaccharide was approximately 15% of that into phospholipid in *V. harveyi* but was not detected in *E. coli*, as reported previously (4).

DISCUSSION

The present study has clearly shown that acyl chains derived from exogenous myristic acid can be activated to form acyl-ACP and lipid A in *V. harveyi*. Acyl-ACPs labeled by incubation with [9,10-^{3}H]14:0 were positively identified by (i) comigration with authentic acyl-ACP standards in two different gel systems, (ii) comigration with acyl-ACP labeled specifically with [^{3}H]β-alanine, and (iii) immunoprecipitation with anti-*V. harveyi* ACP serum. To our knowledge, this is the first direct demonstration of the activation of exogenous fatty acids to biosynthetic acyl-ACP intermediates in bacteria and provides strong evidence that the elongation of these fatty acids observed previously for *V. harveyi* involves ACP (5, 6). The present results also provide a further contrast to fatty acid metabolism in *E. coli*, in which exogenous fatty acids can be activated to both acyl-CoA and acyl-ACP at the inner cell membrane (30), but neither intermediate has access to pathways of fatty acid synthesis. In *E. coli*, acyl-CoA is oxidized or incorporated directly into phospholipids by glycerol-3-phosphate acyltransferase (14), while acyl-ACP derived from exogenous fatty acids is a transient, tightly bound intermediate in the reacylation of the 1 position of phosphatidylethanolamine and is not elongated (10, 17). *V. harveyi* also differs from *E. coli* in that fatty acids do not appear to induce enzymes responsible for their metabolism (i.e., a *fad*-like operon), nor can they support growth in the absence of other carbon sources (6).

An unexpected result of this study, and one that would not be predicted from earlier metabolic labeling studies, is that exogenous 14:0 can be partially degraded to shorter chain lengths prior to acyl-ACP formation. An alternative explanation, that labeling of acyl-ACPs with [^{3}H]14:0 is due to the total degradation of fatty acids to form acetyl-CoA, followed by de novo resynthesis, is unlikely for several reasons. First, labeling with [^{3}H]14:0 and [^{3}H]acetate under identical conditions resulted in completely different acyl-ACP profiles (Fig. 2): fatty acid label appeared exclusively in medium- to long-chain (C_8 to C_{14}) acyl-ACPs, while acetate label appeared mostly in shorter-chain derivatives ($\leq C_6$). These differences occurred without a substantial influence of either 14:0 or acetate on acyl-ACP pool sizes (Fig. 3B). Moreover, only one-sixth of the label in [9,10-^{3}H]14:0 would be expected to appear in acyl chains via the recycling of acetate; the label in the 9 position of the fatty acid would be lost as ^3H_2O upon degradation, and two-thirds of the remaining label in [^{3}H]acetyl-CoA (i.e., from the 10 position of [^{3}H]14:0) would be lost during resynthesis. This situation is clearly at odds with the rapid appearance of labeled acyl-ACP (5% of lipid label by 5 min) from [^{3}H]14:0 but not from [^{3}H]acetate itself (Fig. 2). Also, we found no evidence of general protein labeling from precursors derived from [^{3}H]14:0 via [^{3}H]acetate, whereas SDS-PAGE bands other than acyl-ACP were labeled with [^{3}H]acetate in a time-dependent manner. Finally, we showed previously that all of the fatty acid label from [1-^{14}C]12:0 and [1-^{14}C]14:0 appears in saturated acyl chains (6), with none in 16:1 and 18:1, which account for over 60% of the fatty acids in *V. harveyi* (5). Both saturated and unsaturated fatty acids would be expected to be labeled with [1-^{14}C]acetate.

A model of exogenous fatty acid metabolism in *V. harveyi* that is consistent with the findings of this and earlier studies is presented in Fig. 6. On the basis of the appearance of labeled acyl-ACPs with chain lengths shorter than 14:0, as well as the observed formation of $^{14}CO_2$ from [1-^{14}C]14:0 (6), we suggest that exogenous 14:0 may be initially activated to acyl-CoA (as in *E. coli*) and partially oxidized as CoA derivatives. These partially degraded acyl moieties can be transferred from acyl-CoA to ACP and can also be reelongated by fatty acid synthase. The latter step would be blocked by cerulenin, resulting in the observed shift of labeled acyl-ACPs to shorter chain lengths (Fig. 3). The dramatic decrease in the amount of labeled 14:0-ACP in the presence of cerulenin likely reflects its efficient use as an intermediate in phospholipid acylation (14:0 is about 10% of total *V. harveyi* fatty acids [5]) in the absence of competing endogenous 14:0-ACP. Note that our results do not rule out direct activation or transfer of at least some of the intact 14:0 chain to ACP (i.e., without degradation). Indeed,

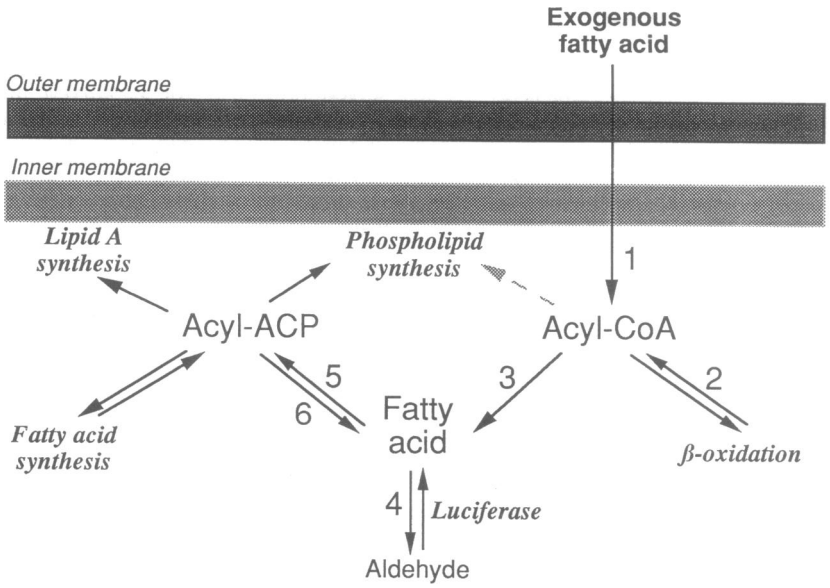

FIG. 6. Proposed model for the metabolism of exogenous fatty acids in *V. harveyi*. Exogenous fatty acids are transported and activated to form acyl-CoA (step 1), which can be oxidized to shorter chain lengths (step 2). Long-chain acyl-CoA intermediates may also be hydrolyzed to free fatty acids (step 3) as substrates for aldehyde synthesis by fatty acid reductase (step 4; 14:0 only) or activation by acyl-ACP synthetase to form acyl-ACP (step 5). In wild-type bacteria (but not mutant M17), endogenous 14:0 for bioluminescence is provided by cleavage of 14:0-ACP by a specific esterase (step 6).

30% of the labeled fatty acid derived from [1-^{14}C]14:0 appears in *V. harveyi* phospholipids as 16:0 in complex medium (6): this labeled 16:0 must arise from direct elongation of the 14:0 precursor, as the label would be lost after a single round of β-oxidation. Thus, degradation of exogenous fatty acids does not appear to be a prerequisite for transfer to ACP.

The mechanism of acyl chain transfer from acyl-CoA to acyl-ACP in *V. harveyi* has not been established. One possibility is direct transacylation by a fatty acid condensing enzyme synthase (β-ketoacyl-ACP synthase). This mechanism has been suggested for the in vivo acylation of ACP by exogenously derived fatty acids in the angiosperm *Spirodela oligorrhiza*, on the basis of inhibition by cerulenin and in vitro transfer of labeled acyl-CoA to ACP (22). However, we favor the involvement of a free fatty acid intermediate (as shown in Fig. 6) for several reasons. First, acyl-ACP is labeled when *V. harveyi* cell extracts are incubated with [^{3}H]14:0 (plus ATP) but not with [^{3}H]14:0-CoA (37). Second, cerulenin might be expected to decrease the labeling of all acyl-ACPs by similar extents, rather than to produce a shift in their chain length profile, if a single condensing enzyme were involved in acyl-CoA transfer. Third, a transient free fatty acid pool (product of the luciferase reaction) does exist and is detectable under certain conditions in *V. harveyi* (5). Finally, we have isolated a soluble *V. harveyi* acyl-ACP synthetase which activates a broad range of fatty acids (C_6 to C_{16}) to acyl-ACP in vitro (7, 12, 32) and could in theory be involved in the activation of free fatty acids to acyl-ACP in vivo.

The relative levels of acyl-ACP intermediates should reflect the balance of enzyme activities involved in their synthesis and utilization. In bioluminescent bacteria, 14:0-ACP is the specific substrate of a luminescence-related esterase which provides endogenous 14:0 for aldehyde synthesis (8, 11). This study indicates that the presence of this enzyme in wild-type *V. harveyi* dramatically affects the species of acyl-ACP labeled with exogenous [^{3}H]14:0, as labeled 14:0-ACP was virtually absent in wild-type cells compared with M17 cells incubated in minimal medium (Fig. 1). The amount of labeled 14:0-ACP was also decreased in the presence of cerulenin, while 8:0-ACP and 10:0-ACP accumulated (Fig. 3). These results suggest that elongation of longer-chain acyl-ACPs might be particularly sensitive to cerulenin and that 14:0-ACP (a suitable substrate for phospholipid acylation) is quickly removed under these conditions. The composition of the growth medium also affected the labeling of acyl-ACP with [^{3}H]14:0 in minimal medium; the balance appeared to be shifted towards oxidation of 14:0 prior to ACP acylation, with acyl-ACPs as short as C_8 being detected, while less degradation of 14:0 was noted in complex medium, in which 12:0-ACP was the only shortened product observed. The absence of labeling of 16:0-ACP with [^{3}H]14:0, even in complex medium, likely reflects the fact that 16:0-ACP is a preferred substrate for phospholipid acylation and does not accumulate as an intermediate to any appreciable extent.

Previous investigations examining the mass composition of acyl-ACP pools in both plants (26, 31) and *E. coli* (18, 29) have been valuable in providing insight into the rate-determining steps in fatty acid biosynthesis. The incorporation of exogenous fatty acid precursors in *V. harveyi* should provide another dimension to this approach, particularly in studies of the control of the synthesis of less abundant cell components that require acyl-ACP as acyl donors, such as secreted proteins (16) and lipid A. In *E. coli*, the transfer of fatty acids from 3-hydroxymyristoyl-ACP to UDP-GlcNAc is the first committed step in the synthesis of lipid A (1, 27). While lipid A cannot be directly labeled with exogenous fatty acids in *E. coli* (4), it is effectively labeled with [^{3}H]14:0 in *V. harveyi* (Fig. 5). As 12:0-ACP and 14:0-ACP are the major labeled acyl-ACP intermediates, we would expect that all the major fatty acids of lipid A (12:0, β-hydroxy-14:0, and 14:0) would also be labeled to some extent; further characterization of *V. harveyi* lipid A is presently under way. In any case, *V. harveyi* should provide a

good model for studying competition for acyl-ACP intermediates between different pathways and products, shedding light on, for example, the regulatory process which diverts about 20% of acyl-ACP to the formation of lipid A rather than phospholipid (19).

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