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Acyl-Acyl Carrier Protein Specificity of UDP-GlcNAc Acyltransferases from Gram-Negative Bacteria: Relationship to Lipid A Structure

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Lipid A, the component of lipopolysaccharide that provides the membrane anchor of the core and O-antigen sugars, is known to contain characteristic *R*-3-hydroxy fatty acids bound to the 2,2′ (N-linked) and 3,3′ (O-linked) positions of the glucosamine disaccharide in different gram-negative bacteria. The studies reported here show that it is the acyl-acyl carrier protein specificities of the enzymes UDP-GlcNAc-*O*-acyltransferase and UDP-3-*O*-[(*R*)-3-hydroxyacyl]-GlcN-*N*-acyltransferase that determine the nature of these fatty acids.

Lipid A, the endotoxic component of lipopolysaccharide of gram-negative bacteria, anchors the core and O-antigen sugars to the outer membrane. The structure of this unusual lipid has been determined for a variety of species. In members of the family *Enterobacteriaceae*, such as *Escherichia coli* (16, 25), *Salmonella typhimurium* (24), *Proteus mirabilis* (20), and *Serratia marcescens* (1), *R*-3-hydroxymyristoyl moieties are found in the 2,2′ (N-linked) and 3,3′ (O-linked) positions of the glucosamine disaccharide. In other gram-negative organisms, such as *Pseudomonas aeruginosa* (9), *Rhodobacter* (*Rhodopseudomonas*) *sphaeroides* (15, 19), and *Neisseria gonorrhoeae* (23), the O- and N-linked fatty acids have been found to differ. Another member of the *Neisseria-Moraxella* family, *Acinetobacter calcoaceticus*, has been reported to contain O- and N-linked 3-hydroxylaurate (27). The acyl groups attached directly to the glucosamine disaccharide of these types of lipid A are summarized in Table 1.

UDP-*N*-acetylglucosamine-*O*-acyltransferase has been shown to catalyze the first committed step of lipid A biosynthesis (3). The enzyme from *E. coli* has been purified to homogeneity and extensively characterized (5b). Its structural gene, *lpxA*, has been cloned and sequenced (6, 7). Of particular interest is the fact that the enzyme is very specific for *R*-3-hydroxymyristoyl-acyl carrier protein (ACP) as the acyl donor. The product of *O*-acyltransferase activity, UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc, is then deacylated, and the second *R*-3-hydroxymyristoyl residue is added to the nitrogen of the glucosamine (Fig. 1). The enzymes responsible for these transformations have been demonstrated in extracts of *E. coli* but have not yet been purified (4). This pathway has not been studied in organisms other than *E. coli* and *S. typhimurium* (16). We wanted to determine whether similar enzymes are present in organisms of clinical interest and whether the acyl-ACP specificity of the *O*- and *N*-acyltransferases in vitro correlates with the characteristic fatty acids found in the lipid A of a particular gram-negative bacterium in vivo.

Cultures. All cultures except *E. coli* were obtained from the American Type Culture Collection (Rockville, Md.). *E. coli* SM105 and JB1104 were obtained as described previously (5, 8).

Preparation of extracts. Cultures (with the exception of *A. calcoaceticus* and *R. sphaeroides*) were grown with aeration in 1 liter of LB medium containing 10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract per liter to an optical density at 600 nm of 1.0 to 1.3. The strains of *A. calcoaceticus* were grown in brain heart infusion broth; *R. sphaeroides* was grown anaerobically in R8AH medium under a tungsten light source for 48 h at 25°C (26). *E. coli*, *Enterobacter aerogenes*, *P. aeruginosa*, *Klebsiella oxytoca*, *Citrobacter freundii*, *P. mirabilis*, and *A. calcoaceticus* were grown at 37°C. The strains of *S. marcescens* were grown at 30°C. The cells were harvested by centrifugation, washed once in 0.9% NaCl, and suspended in 3.0 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) sodium buffer (pH 7.5) containing DNase II (700 U) and a mixture of protease inhibitors (17 μg each of leupeptin, antipain, chymostatin, and pepstatin A per ml). Preparations of cell extracts were carried out at 2 to 4°C. The cells were ruptured by one passage in a French press at 18,000 lb/in^2. Insoluble material was removed by centrifugation at 100,000 × g_{av} for 90 min. The supernatant solutions were stored at −80°C until assayed. Protein concentration was determined by using the bicinchoninic acid method with bovine serum albumin as the standard (21).

Preparation of acyl-ACPs. ACP was prepared from *E. coli* K-12 (1 kg; Grain Processing, Muscatine, Iowa) as described previously (18). Acyl-ACP synthetase was prepared from *E. coli* K-12 as described previously (17) and used immobilized on Blue Sepharose to prepare the acyl-ACPs (3). The 10- and 12-carbon hydroxy acids are much poorer substrates for acyl-ACP synthetase than is the 14-carbon hydroxy acid. Quantitative acylation of ACP was obtained with *R*-3-hydroxymyristate. Its concentration was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) as described previously (3). About 20 to 30% acylation of ACP with *RS*-3-hydroxylaurate or *RS*-3-hydroxydecanoate was obtained. The concentrations of these acyl-ACPs were estimated by comparison with standards of known concentration after polyacrylamide gel electrophoresis as described previously (11).

Preparation of 3-hydroxy fatty acids. 3-Ketodecanoate, 3-ketolaurate, and 3-ketomyristate methyl esters were pre-

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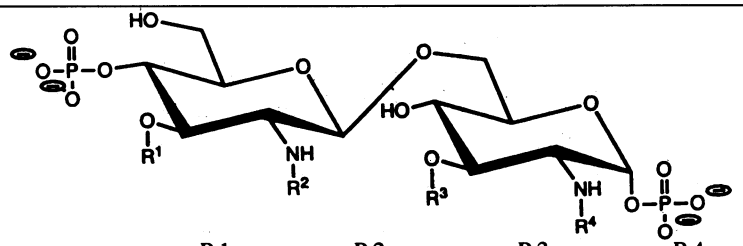
TABLE 1. Nature of the acyl groups bound directly to the glucosamine-disaccharide of lipid A in various gram-negative bacteria[a]



Culture	R1	R2	R3	R4	References
Escherichia, Salmonella, Proteus, Serratia	3-OHC14	3-OHC14	3-OHC14	3-OHC14	(24,25,20,1)
Pseudomonas	3-OHC10	3-OHC12	3-OHC10	3-OHC12	(9)
Rhodobacter	3-OHC10	3-OHC14	3-OHC10	3-ketoC14	(15,19)
Neisseria	3-OHC12	3-OHC14	3-OHC12	3-OHC14	(23)
Acinetobacter	3-OHC12	3-OHC12	3-OHC12	3-OHC12	(27)

[a] Normal fatty acids in acyloxyacyl linkage are not indicated.

pared from the appropriate acid chlorides and Meldrum's acid (14). *RS*-3-Hydroxy-decanoate, -laurate, and -myristate were prepared from the corresponding keto compounds by reduction with sodium borohydride as described previously (3). *R*-3-Hydroxymyristate was prepared from the racemic mixture by cocrystallization with (−)-ephedrine as described previously (11a). The ephedrine salt of *R*-3-hydroxymyristate is water soluble and is efficiently utilized by acyl-ACP synthetase.

Preparation of UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc. [α-^{32}P]UDP-GlcNAc was prepared as described previously (2, 12) from [α-^{32}P]UTP. UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc, [α-^{32}P]UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc, and UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-[6-^{3}H]GlcNAc were prepared enzymatically from the corresponding UDP-GlcNAc preparations and *R*-3-hydroxymyristoyl-ACP with UDP-GlcNAc-*O*-acyltransferase purified from an overproducing strain of *E. coli* (3, 4). At least a 10-fold excess of UDP-GlcNAc over *R*-3-hydroxymyristoyl-ACP was used to obtain complete conversion of acyl-ACP to the product. A typical reaction mixture contained the following components (in a total volume of 0.3 ml): 0.83 mM UDP-GlcNAc, UDP-GlcNAc labeled with ^{3}H (3×10^5 to 4×10^5 dpm/nmol) or ^{32}P (5×10^4 to 10×10^4 dpm/nmol), 80 μM *R*-3-hydroxymyristoyl-ACP, 140 mM sodium HEPES (pH 7.5), 17 mg of bovine serum albumin (fatty acid free) per ml and purified *O*-acyltransferase (0.01 U). The mixture was incubated at 30°C for 2 h. In some experiments, this reaction mixture was used without purification as the source of UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc. In other experiments, the UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc was first purified of contaminating ACP, UDP-GlcNAc-*O*-acyltransferase, and residual UDP-GlcNAc by a new, rapid purification procedure as follows. The reaction mixture was loaded onto DEAE-Sepharose (10 ml; previously equilibrated with 10 mM bis-Tris-HCl [pH 6.0]) packed in a plastic, disposable column (Econocolumn, 20-ml total volume; Bio-Rad) to whose outlet was directly attached a C_{18} SepPak cartridge (Waters Associates; prepared for use by

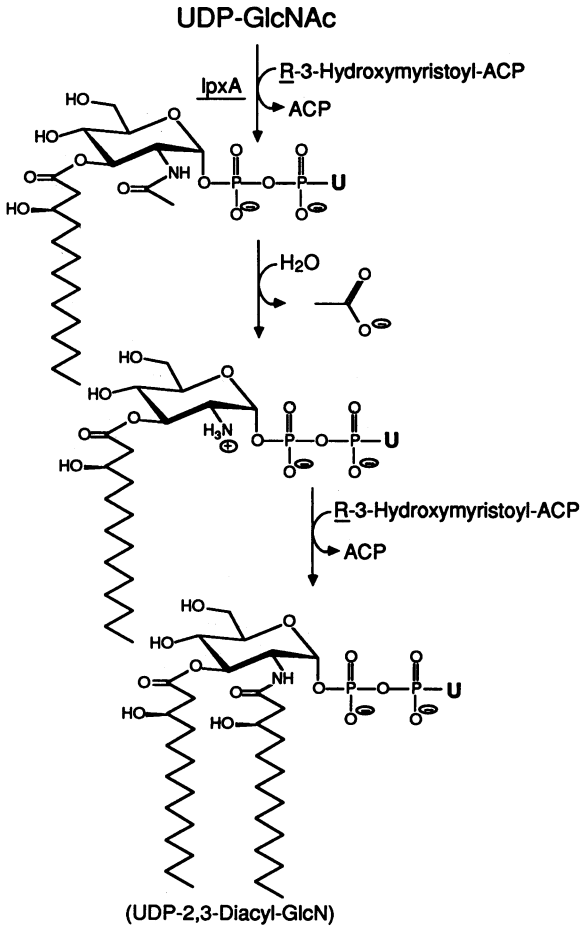


FIG. 1. Fatty acylation of UDP-GlcNAc. Evidence for this scheme has been presented previously (16). U, uridine.

TABLE 2. Acyl-ACP donor specificity of UDP-GlcNAc-*O*-acyltransferase in various strains of bacteria[a]

Culture	nmol of product/5 min/mg of protein (% maximum activity) with:		
	R-3-OHC14	*RS*-3-OHC12	*RS*-3-OHC10
E. coli SM105[b]	6.80 (100)	0.17 (2)	0 (0)
E. coli JB1104[c]	2.28 (100)	0.04 (2)	0.01 (0.4)
C. freundii 6750[b]	3.44 (100)	0.08 (2)	0.01 (0.3)
K. oxytoca 8724[b]	5.05 (100)	0.32 (6)	0.03 (0.6)
E. aerogenes 13048[c]	6.52 (100)	0.08 (1)	0.02 (0.3)
S. marcescens 264[c]	7.06 (100)	0.19 (3)	0.02 (0.3)
S. marcescens 29632[c]	8.64 (100)	0.16 (2)	0.04 (0.5)
P. mirabilis 27035[b]	0.54 (100)	0.28 (52)	0.03 (6)
P. mirabilis 29906[b]	0.64 (100)	0.07 (11)	0.01 (2)
A. calcoaceticus 33304[b]	0.08 (15)	0.54 (100)	0.08 (15)
A. calcoaceticus 33305[b]	0.03 (7)	0.42 (100)	0.07 (16)
P. aeruginosa 12055[c]	0.11 (7)	0.16 (10)	1.52 (100)
P. aeruginosa 15692[b]	0 (0)	0.10 (14)	0.71 (100)
P. aeruginosa 21036[c]	0.14 (5)	0.21 (8)	2.78 (100)
P. aeruginosa 35151[c]	0.09 (5)	0.28 (16)	1.76 (100)
R. sphaeroides 17023[b]	0 (0)	0.02 (5)	0.37 (100)

[a] The reaction mixtures contained the following (in a total volume of 20 μl): 0.2 mM UDP-GlcNAc, UDP-GlcNAc labeled with ^3H (3.5×10^5 dpm/nmol) or α-^{32}P (1.0×10^5 dpm/nmol), 10 mg of bovine serum albumin per ml, 85 mM sodium HEPES (pH 8.0), membrane-free cell extract (1.0 to 2.6 mg of protein per ml), and either 50 μM *R*-3-hydroxymyristoyl-ACP, 60 μM *RS*-3-hydroxylauroyl-ACP, or 60 μM *RS*-3-hydroxydecanoyl-ACP. Reaction mixtures were incubated at 30°C for 5 min. Reactions containing ^3H were stopped by the addition of 1.0 ml of 10 mM sodium HEPES (pH 8.0). The diluted reaction mixtures were loaded onto individual C_{18} SepPak cartridges (Waters Associates), and the products were determined as described previously (8). Reactions containing ^{32}P were stopped by spotting a portion (5 μl) of each mixture on a 20- by 20-cm silica gel plate, and the products were determined as described previously (3). *R*-3-OHC14, *R*-3-hydroxymyristoyl-ACP; *RS*-3-OHC12, *RS*-3-hydroxylauroyl-ACP; *RS*-3-OHC10, *RS*-3-hydroxydecanoyl-ACP.

[b] Determined with the SepPak assay.

[c] Determined by using thin-layer chromatography.

washing with 10 ml of methanol, 10 ml of water, and 10 ml of 10 mM bis-Tris buffer). The column and cartridge were washed with 30 ml of 200 mM NaCl. The SepPak cartridge (to which the desired product was bound) was removed from the column and attached to an empty syringe. The cartridge was washed with 30 ml of distilled water. Methanol was then used to elute the monoacylated product. All fractions containing radioactivity were combined and taken to dryness under a stream of nitrogen. The residue was dissolved in 0.1 ml of 10 mM sodium HEPES (pH 8.0) and used immediately. The average yield of purified product obtained (in seven trials; calculated from the amount of acyl-ACP added) was 62% ± 12%.

Acyl-ACP specificity of UDP-GlcNAc-*O*-acyltransferase in various bacteria. Table 2 shows the results of an experiment in which the supernatant fraction derived from late-log-phase cells was incubated with either ^3H- or ^{32}P-labeled UDP-GlcNAc in the presence of different 3-hydroxyacyl derivatives of ACP. No formation of product was observed with any of the extracts in the absence of added acyl-ACP (data not shown). All of the *Enterobacteriaceae* tested (*E. coli*, *P. mirabilis* [two strains], *K. oxytoca*, *C. freundii*, *S. marcescens* [two strains], and *E. aerogenes*) utilized *R*-3-hydroxymyristoyl-ACP most efficiently as the acyl donor. Most had some activity with *RS*-3-hydroxylauroyl-ACP but much less with *RS*-3-hydroxydecanoyl-ACP. In the absence of octyl glucoside (an inhibitor of the deacetylase) and with *R*-3-hydroxymyristoyl-ACP as acyl donor, conditions that in *E. coli* are known to permit the action of the deacetylase and *N*-acyltransferase (4), the time-dependent formation of UDP-2,3-diacyl-GlcN and the monoacylated product was found with all of these extracts (data not shown). In contrast, all strains of *P. aeruginosa* and *R. sphaeroides* utilized *RS*-3-hydroxydecanoyl-ACP most efficiently, but only the synthesis of the monoacylated material was observed. Finally, the two *Acinetobacter* strains utilized *RS*-3-hydroxylauroyl-ACP most efficiently.

Specificity of UDP-3-*O*-(3-hydroxyacyl)-GlcN-*N*-acyltransferase in various bacteria. Next, we wanted to determine the acyl-ACP specificities of the *N*-acyl transferases. This was more complicated than the previous work with the *O*-acyltransferase, since the substrate required, UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc, is somewhat unstable (4) and the overall rate of reaction depends on the activity of both the deacetylase and *N*-acyltransferase. To minimize stability problems with the substrate, initial experiments were done with UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc, generated by incubation of a 10-fold molar excess of UDP-GlcNAc over that of *R*-3-hydroxymyristoyl-ACP with purified *E. coli* UDP-GlcNAc-*O*-acyltransferase and used without further purification. Under these conditions, all of the *R*-3-hydroxymyristoyl-ACP is utilized to form UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc. This mixture was incubated with the supernatant fraction of late-log-phase cells in the presence of different acyl donors. With extracts of all of the *Enterobacteriaceae* and *A. calcoaceticus*, there was substantial synthesis of UDP-2,3-diacyl-GlcN in the absence of any added acyl-ACP. The addition of *R*-3-hydroxymyristoyl-ACP gave, at best, a twofold stimulation of the activity (data not shown).

Purification of the UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc away from ACP and UDP-GlcNAc-*O*-acyltransferase was found to remove most of the apparent N-acylation activity in the absence of acyl donor (data not shown). Therefore, the experiments on acyl-ACP specificities of these *N*-acyltransferases were repeated with purified substrate (Table 3). In *E. coli*, *K. oytoca*, *C. freundii*, *P. mirabilis*, *S. marcescens*, and *E. aerogenes*, *R*-3-hydroxymyristoyl-ACP was the preferred acyl donor. However, substantial activity was still observed with *RS*-3-hydroxylauroyl-ACP or even *RS*-3-hydroxydecanoyl-ACP.

In contrast to the results with the *Enterobacteriaceae*, extracts of *P. aeruginosa* (Table 4) showed only a low level of UDP-2,3-diacyl-GlcN synthesis when provided with unpurified UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc in the absence of added acyl-ACP. The most efficient acyl donor was *RS*-3-hydroxylauroyl-ACP. When these experiments were repeated with purified substrate, the same relative results were obtained (data not shown). When a similar experiment was done with extracts of *R. sphaeroides*, no synthesis of UDP-2,3-diacyl-GlcN was observed under any condition, although monoacyl-UDP-GlcNAc formation was obtained when *RS*-3-hydroxydecanoyl-ACP was added to the reaction mixture (data not shown).

Since no *O*-acyltransferase activity had been seen in the absence of an added acyl donor, it seemed unlikely that the N-acylation activity observed without acyl donor addition could be due to acyl-ACP contamination of the extracts. Therefore, an experiment was performed with an *Enterobacter* extract to determine which of the components of the O-acylation reaction used to generate the substrate were required to obtain N acylation in the absence of added acyl-ACP (Fig. 2). It can be seen that the best activity was

TABLE 3. Acyl-ACP specificity of N-acylation in members of the *Enterobacteriaceae* and *A. calcoaceticus*[a]

Culture	Acyl-ACP donor	UDP-2,3-diacyl-GlcN formed (nmol/min/mg)	% of maximum activity
E. coli JB1104	None	0.013	4
	R-3-OHC14	0.356	100
	RS-3-OHC12	0.104	29
	RS-3-OHC10	0.017	5
S. marcescens 29632	None	0.058	4
	R-3-OHC14	1.302	100
	RS-3-OHC12	0.360	28
	RS-3-OHC10	0.107	8
C. freundii 6750	None	0.027	3
	R-3-OHC14	0.908	100
	RS-3-OHC12	0.169	19
	RS-3-OHC10	0.065	7
K. oxytoca 8724	None	0.076	11
	R-3-OHC14	0.692	100
	RS-3-OHC12	0.367	53
	RS-3-OHC10	0.078	11
A. calcoaceticus 33304	None	0.000	0
	R-3-OHC14	0.715	100
	RS-3-OHC12	0.483	68
	RS-3-OHC10	0.017	2
P. mirabilis 27035	None	0.040	9
	R-3-OHC14	0.425	100
	RS-3-OHC12	0.155	36
	RS-3-OHC10	0.015	4

[a] Each reaction mixture contained the following (in a total volume of 25 μl): 50 μM UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc (prepared with [α-^{32}P] UDP-GlcNAc [1 × 10^5 dpm/nmol] and purified), 8 mg of bovine serum albumin per ml, 35 mM sodium HEPES (pH 8.0), membrane-free cell extract (0.8 to 2.1 mg/ml), and either 50 μM *R*-3-hydroxymyristoyl-ACP (*R*-3-OHC14), 60 μM *RS*-3-hydroxylauroyl-ACP (*RS*-3-OCH12), or 40 μM *RS*-3-hydroxydecanoyl-ACP (*RS*-3-OCH10). After 5, 10, 15, and 30 min of incubation at 30°C, portions (5 μl) were removed from each and spotted on a 20- by 20-cm silica gel thin-layer chromatography plate. The amount of UDP-2,3-diacyl-GlcN formed was determined as described previously (3). The reaction rate was linear for at least 10 min under all conditions.

obtained when ACP and the purified *O*-acyltransferase were added together.

These data and the fact that purified and unpurified substrates gave the same results in *Pseudomonas* extracts suggested that UDP-GlcNAc-*O*-acyltransferase catalyzes the back reaction, the conversion of monoacyl-UDP-GlcNAc and ACP to UDP-GlcNAc and acyl-ACP. Since the monoacyl-UDP-GlcNAc added to the *Pseudomonas* extract contained *R*-3-hydroxymyristate, the back reaction could only generate *R*-3-hydroxymyristoyl-ACP, which is not an effective acyl-donor for either the *O*- or *N*-acyltransferases of this organism. To test this question directly, purified *E. coli* UDP-GlcNAc-*O*-acyltransferase was incubated with [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc and ACP, and the synthesis of UDP-GlcNAc was monitored as a function of time (Table 5). Substantial reversal of the reaction was found.

All of the results obtained on the acyl-ACP specificities of the UDP-GlcNAc-*O*-acyltransferases are consistent with the conclusion that it is the substrate specificity of this enzyme that determines the characteristic acyl group found in the 3,3′ positions of the organism's lipid A. The results on the acyl-ACP specificity of the *N*-acyltransferase are also generally consistent with the conclusion that its specificity determines the nature of the acyl group found in the 2,2′ position of lipid A. The only exception found thus far is *A. calcoaceticus*, which has been reported to contain 2,2′ 3-hydroxylaurate (27) but whose specificity in vitro is for *R*-3-hydroxymyristoyl-ACP. The reason for this discrepancy is unknown. However, in light of these results and the fact that another member of the same family, *N. gonorrhoeae*, has been found to contain N-linked *R*-3-hydroxymyristate (23), it may be that the nature of the N-linked fatty acid in *A. calcoaceticus* warrants reinvestigation.

Acyl-ACP dependence of the *N*-acyltransferase reaction in extracts of the species of *Enterobacteriaceae* and *A. calcoaceticus* could not be demonstrated when UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc was added to the extracts along with ACP and purified *E. coli* UDP-GlcNAc-*O*-acyltransferase. This result suggested that the *O*-acyltransferase can catalyze the reverse reaction, the formation of *R*-3-hydroxymyristoyl-ACP and UDP-GlcNAc, in the presence of UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc and ACP (Fig. 1). The results of the reconstruction experiment (Fig. 2) are consistent with this hypothesis; the addition of both ACP and *O*-acyltransferase is required for maximum UDP-2,3-diacyl-GlcN formation in the absence of preformed *R*-3-hydroxymyristoyl-ACP. The reversibility of the reaction catalyzed by UDP-GlcNAc-*O*-acyl-transferase was then demonstrated directly with the purified enzyme (Table 5). Taken together, these results imply that *R*-3-hydroxymyristoyl-ACP (a thioester) is more stable than the product, UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc (an oxygen ester). This anomaly might be explained by the relatively low pKa of sugar hydroxyls and possible steric constraints at the 3 position (5c). Prolonged incubation of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc in water at room temperature has been shown to result in migration of the 3-*O*-acyl moiety to the 6 position (4).

The fact that UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc serves as a substrate for the deacetylases and *N*-acyltransferases of *P. aeruginosa* and *A. calcoaceticus*, whose lipid A-s contain O-linked fatty acids other than *R*-3-hydroxymyristate, implies that the deacetylase and *N*-acyltransferase from these organisms are relatively nonspecific with respect to the nature of the acyl group at the 3 position. It is

TABLE 4. Acyl-ACP specificity of N-acylation in *P. aeruginosa*[a]

Strain	Acyl-ACP	UDP-2,3-diacyl-GlcN formed (nmol/min/mg)	% of maximum activity
12055	None	0.029	4%
	R-3-OHC14	0.040	6%
	RS-3-OHC12	0.717	100%
	RS-3-OHC10	0.018	2%
21036	None	0.005	0.4%
	R-3-OHC14	0.033	10%
	RS-3-OHC12	0.343	100%
	RS-3-OHC10	0.005	0.4%
15692	None	0.010	5%
	R-3-OHC14	0.012	6%
	RS-3-OHC12	0.193	100%
	RS-3-OHC10	0.033	17%

[a] Each reaction mixture contained the following (in a total volume of 25 μl): 60 μM UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-GlcNAc (prepared from [α-^{32}P] UDP-GlcNAc [5 × 10^4 dpm/nmol] and used without further purification), 10 mg of bovine serum albumin per ml, 50 mM sodium HEPES (pH 8.0), membrane-free cell extract (0.8 to 1.4 mg/ml), and either 50 μM *R*-3-hydroxymyristoyl-ACP (*R*-3-OHC14), 50 μM *RS*-3-hydroxylaurate (*RS*-3-OHCl 12), or 50 μM *RS*-3-hydroxydecanoate (*RS*-3-OHC10). After 5 min of incubation at 30°C, a portion (5 μl) of each reaction mixture was spotted on a 20- by 20-cm silica gel thin-layer chromotography plate. The amount of UDP-2,3-diacyl-GlcN formed was determed as described previously (3).

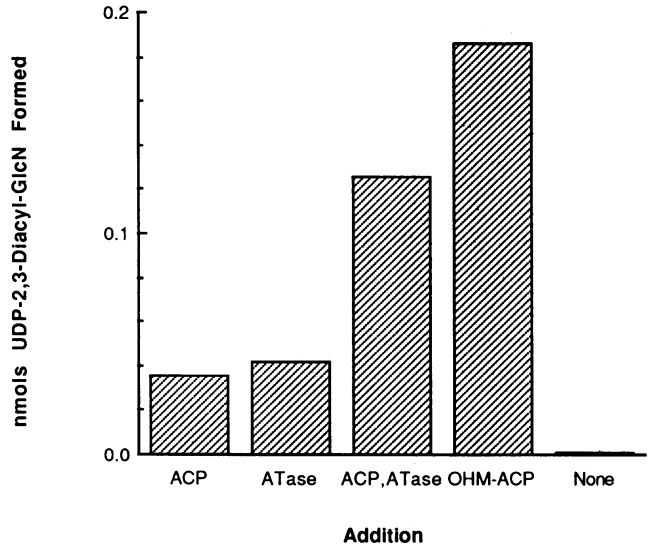


FIG. 2. Effect of components of the UDP-GlcNAc-*O*-acyltransferase reaction on formation of UDP-2,3-diacyl-GlcN in extracts of *E. aerogenes* ATCC 13048. Each reaction mixture contained the following (in a total volume of 25 μl): 50 μM UDP-3-*O*-[(*R*)-3-hydroxymyristoyl-GlcNAc (prepared from [^{3}H]UDP-GlcNAc [4 × 10^5 dpm/nmol] and purified), 8 mg of bovine serum albumin per ml, 35 mM sodium HEPES (pH 8.0), membrane-free cell extract (2.0 mg of protein per ml), and, as indicated, either 32 μM ACP, purified *E. coli* UDP-GlcNAc-*O*-acyltransferase (0.001 U) (ATase), 53 μM *R*-3-hydroxymyristoyl-ACP (OHM-ACP), or no addition. After 5, 10, 20, and 30 min of incubation at 30°C, a portion (5 μl) was removed from each sample and spotted on a 20- by 20-cm silica gel thin-layer chromatography plate. The amount of UDP-2,3-diacyl-GlcN formed was determined as described previously (3), except that the plates were sprayed with En^{3}Hance before autoradiography. The data presented in the figure were obtained after 10 min of incubation and are representative.

not clear why we have been unable to demonstrate *N*-acyltransferase activity in extracts of *R. sphaeroides* when the *O*-acyltransferase activity is easily seen. It is possible that 3-ketomyristoyl-ACP is required or that the extracts are deficient in deacetylase activity.

TABLE 5. Formation of UDP-GlcNAc from [α-^{32}P]UDP-3-*O*-(*R*-3 hydroxymyristoyl)-GlcNAc and ACP catalyzed by purified *E. coli* UDP-GlcNAc-*O*-acyltransferase[a]

Time (min)	% of substrate converted to UDP-GlcNAc	
	Expt 1	Expt 2
5	7	7
60	23	24
240	31	31

[a] Reaction mixtures contained the following (in a total volume of 20 μl): 1 μM [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc (6.25 × 10^6 cpm/nmol), 1 μM ACP (reduced with dithiothreitol before the reaction), 1 mg of bovine serum albumin (fatty acid free) per ml, 100 mM sodium HEPES (pH 7.5), and 24 nM UDP-GlcNAc-*O*-acyltransferase (treated with 1 mM dithiothreitol in the presence of 1 mg of bovine serum albumin per ml and 100 mM sodium HEPES [pH 7.5] immediately before use to insure maximum activity [5a]). Incubation was at 30°C. At the indicated times, the reaction was stopped by spotting a portion (4 μl) of the reaction mixture directly onto a silica gel thin-layer plate. The plate was developed in chloroform-methanol-acetic acid-water (25/15/2/4, vol/vol), and the products were determined as previously described (3).

Lipid A molecules from a number of species of bacteria have been reported to contain branched, odd-chain-length, or extremely long-chain fatty acids (10, 13, 22, 27). Because of the difficulty of preparing the acyl-ACP derivatives of these acids, we have not attempted to study the acyl transferases of these organisms. However, based on the results presented here, we would anticipate that the addition of the appropriate acyl-ACP would be required to detect *O*- and *N*-acyltransferase activity. In organisms known to contain only *R*-3-hydroxymyristate, -laurate, or -decanoate, however, it should be possible to determine the positions of acyl substitution in lipid A by studying the acyl-ACP specificity of the *O*- and *N*-acyltransferases in cell extracts. Finally, these results with clinically important gram-negative bacteria establish the generality of the lipid A biosynthetic pathway and its potential as a target for the development of novel antibacterials.

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REFERENCES

1. **Adams, G. A., and P. P. Singh.** 1970. The chemical constitution of lipid A from *Serratia marcescens*. Can. J. Biochem. **48**:55–62.
2. **Anderson, M. S., C. E. Bulawa, and C. R. H. Raetz.** 1985. The biosynthesis of gram-negative endotoxin: formation of lipid A precursors from UDP-GlcNAc in extracts of *Escherichia coli*. J. Biol. Chem. **260**:15536–15541.
3. **Anderson, M. S., and C. R. H. Raetz.** 1987. Biosynthesis of lipid A precursors in *Escherichia coli*. A cytoplasmic acyltransferase that converts UDP-N-acetylglucosamine to UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine. J. Biol. Chem. **262**: 5159–5169.
4. **Anderson, M. S., A. D. Robertson, I. Macher, and C. R. H. Raetz.** 1988. Biosynthesis of lipid A in *Escherichia coli*: identification of UDP-3-O-[(*R*)-3-hydroxymyristoyl]-α-D-glucosamine as a precursor of UDP-N^2,O^3-bis-[(*R*)-3-hydroxymyristoyl]-α-D-glucosamine. Biochemistry **27**:1908–1917.
5. **Bulawa, C. E., and C. R. H. Raetz.** 1984. Isolation and characterization of *Escherichia coli* strains defective in CDP-diglyceride hydrolase. J. Biol. Chem. **259**:11257–11264.
5a. **Bull, H.** Unpublished observation.
5b. **Bull, H., S. Galloway, K. Radika, and C. R. H. Raetz.** Unpublished data.
5c. **Bull, H., and C. R. H. Raetz.** Unpublished observations.
6. **Coleman, J., and C. R. H. Raetz.** 1988. First committed step of lipid A biosynthesis in *Escherichia coli*: sequence of the *lpxA* gene. J. Bacteriol. **170**:1268–1274.
7. **Crowell, D. N., M. S. Anderson, and C. R. H. Raetz.** 1986. Molecular cloning of the genes for lipid A disaccharide synthase and UDP-*N*-acetylglucosamine acyltransferase in *Escherichia coli*. J. Bacteriol. **168**:152–159.
8. **Galloway, S., and C. R. H. Raetz.** 1990. A mutant of *Escherichia coli* defective in the first step of endotoxin biosynthesis. J. Biol. Chem. **265**:6394–6402.
9. **Goldman, R. C., C. C. Doran, S. K. Kadam, and J. O. Capobianco.** 1988. Lipid A precursor from *Pseudomonas aeruginosa* is completely acylated prior to addition of 3-deoxy-D-manno-octulosonate. J. Biol. Chem. **263**:5217–5223.
10. **Hollingsworth, R. I., and R. W. Carlson.** 1989. 27-Hydroxyoctacosanoic acid is a major structural fatty acyl component of the lipopolysaccharide of *Rhizobium trifolii* ANU843. J. Biol. Chem. **264**:9300–9303.
11. **Jackowski, S., and C. O. Rock.** 1983. Ratio of active to inactive forms of acyl carrier protein in *Escherichia coli*. J. Biol. Chem. **258**:15186–15191.
11a. **Kogyo, F. Y.** Japanese patent 86-281640/43, 1986.
12. **Lang, L., and S. Kornfeld.** 1984. A simplified procedure for synthesizing large quantities of highly purified uridine [β-^{32}P]

diphospho-N-acetyl-glucosamine. Anal. Biochem. **140**:264–269.
13. **Moule, A. L., and S. G. Wilkinson.** 1989. Composition of lipopolysaccharides from *Altermonas putreficans*. J. Gen. Microbiol. **135**:163–173.
14. **Oikawa, Y., K. Sugano, and O. Yonemitsu.** 1978. Meldrum's acid in organic synthesis. II. A general and versatile synthesis of β-keto esters. J. Org. Chem. **43**:2087–2088.
15. **Qureshi, N., J. P. Honovich, H. Hara, R. J. Cotter, and K. Takayama.** 1988. Location of fatty acids in lipid A obtained from lipopolysaccharide of *Rhodopseudomonas sphaeroides* ATCC 17023. J. Biol. Chem. **263**:5502–5504.
16. **Raetz, C. R. H.** 1990. Biochemistry of endotoxin. Annu. Rev. Biochem. **59**:129–170.
17. **Rock, C. O., and J. E. Cronan.** 1979. Solubilization, purification, and salt activation of acyl-acyl carrier protein synthetase from *Escherichia coli*. J. Biol. Chem. **254**:7116–7122.
18. **Rock, C. O., and J. E. Cronan.** 1980. Improved purification of acyl carrier protein. Anal. Biochem. **102**:362–364.
19. **Salimath, P. V., J. Weckesser, W. Strittmatter, and H. Mayer.** 1983. Structural studies on the non-toxic lipid A from *Rhodopseudomonas sphaeroides* ATCC 17023. Eur. J. Biochem. **136**: 195–200.
20. **Sidorczyk, Z., U. Zahringer, and E. T. Rietschel.** 1983. Chemical structure of the lipid A component of the lipopolysaccharide from a *Proteus mirabilis* Re-mutant. Eur. J. Biochem. **137**:15–22.
21. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**:76–85.
22. **Sonesson, A., E. Jantzen, K. Bryn, L. Larsson, and J. Eng.** 1989. Chemical composition of a lipopolysaccharide from *Legionella pneumophila*. Arch. Microbiol. **153**:72–78.
23. **Takayama, K., N. Qureshi, K. Hyver, J. Honovich, R. J. Cotter, P. Mascagni, and H. Schneider.** 1986. Characterization of a structural series of lipid A obtained from lipopolysaccharides of *Neisseria gonorrhoeae*. J. Biol. Chem. **261**:10624–10631.
24. **Takayama, K., N. Qureshi, and P. Mascagni.** 1983. Complete structure of lipid A obtained from the lipopolysaccharides of the heptoseless mutant of *Salmonella typhimurium*. J. Biol. Chem. **258**:12801–12803.
25. **Takayama, K., N. Qureshi, P. Mascagni, M. A. Nashed, L. Anderson, and C. R. H. Raetz.** 1983. Fatty acyl derivatives of glucosamine-1-phosphate in *Escherichia coli* and their relation to lipid A. Complete structure of a diacyl GlcN-1-P found in a phosphatidylglycerol-deficient mutant. J. Biol. Chem. **258**:7379–7385.
26. **Weckesser, J., G. Drews, and I. Fromme.** 1972. Chemical analysis of and degradation studies on the cell wall lipopolysaccharide of *Rhodopseudomonas capsulata*. J. Bacteriol. **109**: 1106–1113.
27. **Wollenweber, H. W., U. Seydel, B. Lindner, O. Luderitz, and E. T. Rietschel.** 1984. Nature and location of amide-bound (R)-3-acyloxyacylgroups in lipid A of lipopolysaccharides from various gram-negative bacteria. Eur. J. Biochem. **145**:265–272.