System-wide Studies of N-Lysine Acetylation in Rhodopseudomonas palustris Reveal Substrate Specificity of Protein Acetyltransferases

Received for publication, February 10, 2012, and in revised form, March 2, 2012. Published, JBC Papers in Press, March 13, 2012. DOI 10.1074/jbc.M112.352104

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N-Lysine acetylation is a posttranslational modification that has been well studied in eukaryotes and is likely widespread in prokaryotes as well. The central metabolic enzyme acetyl-CoA synthetase is regulated in both bacteria and eukaryotes by acetylation of a conserved lysine residue in the active site. In the purple photosynthetic α-proteobacterium Rhodopseudomonas palustris, two protein acetyltransferases (RPPat and the newly identified RPKatA) and two deacetylases (RPLdaA and RPSrtN) regulate the activities of AMP-forming acyl-CoA synthetases. In this work, we used LC/MS/MS to identify other proteins regulated by the N-lysine acetylation/deacetylation system of this bacterium. Of the 24 putative acetylated proteins identified, 14 were identified more often in a strain lacking both deacetylases. Nine of these proteins were members of the AMP-forming acyl-CoA synthetase family. RPPat acetylated all nine of the acyl-CoA synthetases identified by this work, and RPLdaA deacetylated eight of them. In all cases, acetylation occurred at the conserved lysine residue in the active site, and acetylation decreased activity of the enzymes by >70%. Our results show that many different AMP-forming acyl-CoA synthetases are regulated by N-lysine acetylation. Five non-acyl-CoA synthetases were identified as possibly acetylated, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Rpa1177, a putative 4-oxalocrotonate tautomerase. Neither RPKatA nor RPKatA acetylated either of these proteins in vitro. It has been reported that Salmonella enterica Pat (SePat) can acetylate a number of metabolic enzymes, including GAPDH, but we were unable to confirm this claim, suggesting that the substrate range of SePat is not as broad as suggested previously.

N-Lysine acetylation is a posttranslational modification of the ε amino group of lysine residues; such modification has been found in a wide variety of proteins, ranging from histones to tubulin and metabolic enzymes (for recent reviews, see Refs. 1 and 2). The acyl group most frequently transferred onto proteins is the acetyl moiety of acetyl-CoA, although the transfer of propionyl, butyryl, succinyl, and malonyl groups has also been reported (3–7). N-Lys-ε acylation/deacylation is a system for the rapid control of the biological activity of proteins. Acetylation of a protein can have a positive (8) or negative effect on its activity (9); deacetylation has the opposite effect. In most of the examples currently in the literature, acetylation inactivates a protein, and deacetylation restores its function. It is clear that N-Lys acylation is widespread among prokaryotes (for a review, see Ref. 10).

Acetyl-CoA synthetase (AcS) was the first enzyme reported to be under N-Lys acylation/deacylation control (9). AcS is a member of the AMP-forming acyl-CoA synthetase family of enzymes that activate organic acids to the corresponding acyl-CoA thioesters. These reactions occur in two steps via an enzyme-bound acyl-AMP intermediate (11). AMP-forming acyl-CoA synthetases participate in the degradation of short-, medium-, and long-chain fatty acids as well as aromatic acids and dicarboxylic acids. These enzymes are also required for the biosynthesis of a number of natural products, including some antibiotics (12) and signaling molecules (13).

In Salmonella enterica, AcS is regulated by acetylation of a conserved lysine residue (Lys-609). Acetylation of Lys-609 abolishes activity of the enzyme by preventing formation of the acyl-AMP intermediate (9). After the discovery that AcS in S. enterica was regulated by acetylation, similar modes of regulation were identified in Bacillus subtilis (14), Rhodopseudomonas palustris (15), Mycobacterium smegmatis (16), yeast (17), and mammals (18, 19).

This work was supported, in whole or in part, by National Institutes of Health Grants R01 GM62203, a USPHS grant from the NIGMS (to J. C. E.-S.), and T32 GM08349, a USPHS biotechnology training grant (to H. A. C.). This work was also supported by the Genomic Science Program, Office of Biological and Environmental Research, United States Department of Energy under Contract DE-AC05-00OR22725 with Oak Ridge National Laboratory, managed and operated by UT-Battelle, LLC (for G. B. H. and D. A. P.). This article contains supplemental Fig. S1 and Tables S1–S6.

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‡The abbreviations used are: N-Lys, ε-amino-Lys; AcS, acetyl-CoA synthetase; TCEP, tris(2-carboxyethyl)phosphine; Pat, protein acetyltransferase; KatA, lysine acetyltransferase Ac ϵ Lys, monocarboxylic fatty acid; DC ϵ Lys, dicarboxylic fatty acid; rTEV, recombinant tobacco etch virus; Se, S. enterica; Rp, R. palustris; MEME, Multiple Em for Motif Elicitation.

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In addition to Acs, two acyl-CoA synthetases (BadA, which activates benzoate, and HbaA, which activates 4-hydroxybenzoate) and the aliphatic acyl-CoA synthetase AliA are regulated by N-Lys acetylation in R. palustris (15), and propionyl-CoA synthetase (PrpE) is regulated by N-Lys propionylation in S. enterica (3).

In all of the above mentioned bacterial systems, yGcn5p-related N-acetyltransferases (pfam00583) catalyze N-Lys acetylation. In R. palustris and S. enterica, this acetyltransferase is called Pat (for protein acetyltransferase). Here, we report work performed with RrPat (907 amino acids) and SePat (886 amino acids). Each of these proteins consists of a large N-terminal domain of unknown function with homology to nucleoside diphosphate-forming acyl-CoA synthetases and a smaller C-terminal yGcn5p-like acetyltransferase domain (15, 20).

There are different types of protein deacetylases in prokaryotes. S. enterica has one known protein deacetylase, CobB, which is a Sir2p (sirtuin)-type deacetylase that requires NAD⁺ as a substrate and transfers the acetyl group to ADP-ribose, producing nicotineamide and 2'-O-acetyl-ADP-ribose as products (9, 21–23). In contrast, R. palustris has two deacetylases, both of which appear to regulate the activities of acyl-CoA synthetases in this bacterium (15). One deacetylase is a sirtuin-type enzyme (RpSrtN), and the other, lysine deacetylase A (RpLdaA), is a zinc-dependent member of the class IIa histone deacetylases (24).

In R. palustris strains lacking ldaA (formerly rpa0954) and srtN (formerly rpa2524) functions, the acyl-CoA synthetases have elevated levels of acetylation. Consequently, cultures grow poorly on carbon sources such as benzoate, the metabolism of which requires the activity of the acyl-CoA synthetase BadA, HbaA, or AliA (25). Deletion of the pat gene in a deacetylase-deficient R. palustris (lidaA srtN) strain restores growth on benzoate, but when BadA is isolated from this strain, it is still partially acetylated, suggesting the existence of a second acetyltransferase whose identity was unknown (15) but is revealed in this work.

Recently, several groups used proteomics approaches as a less biased way to identify proteins that may be acetylated in vivo. Three of these studies used high performance liquid chromatography and tandem mass spectrometry (LC/MS/MS) to identify acetylated proteins in Escherichia coli (26, 27) and S. enterica (28). These studies identified between 85 and 191 different proteins that were putatively acetylated with some overlap between each set of proteins.

In the two E. coli studies, limited validation was done to confirm that the targets were actually acetylated, although one group did find Acs to be acetylated at the expected lysine residue (26). In contrast, the S. enterica study suggested that three proteins could be acetylated by SePat and deacetylated by SeCobB and that there was an effect on enzyme activity. These three proteins were the central metabolic enzymes glyceraldehyde-3-phosphate dehydrogenase (GapA) and isocitrate lyase (AceA) and the glyoxylate shunt regulator isocitrate dehydrogenase kinase/phosphatase (AceK).

A fourth system-wide approach to identify acetylated proteins in bacteria used an E. coli proteome microarray that was probed with SePat and radiolabeled acetyl-CoA (29). The authors identified seven putative substrates of SePat, including the response regulator RcsB. Although SePat acetylated RcsB in vitro, the authors did not conclude that RcsB was a substrate of SePat in vivo.

In this work, we used an LC/MS/MS-based proteomics approach to identify acetylated proteins in R. palustris. We show in vitro evidence that nine of these proteins are AMP-forming acyl-CoA synthetases that are substrates of RrPat, and some are also substrates of a newly identified acetyltransferase that we refer to as RpKatA (encode by kata, formerly rpa3031). We also show that N-Lys acetylation inactivates the above mentioned acyl-CoA synthetases and that their acetylated forms can be deacetylated by RpLdaA. This increased knowledge of substrate specificity of RpPat prompted a re-evaluation of the reported substrate specificity of SePat. Our results obtained with SePat were inconsistent with those reported by others.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—All strains and plasmids used in this study are listed in supplemental Tables S1 and S2. E. coli strains were grown at 37 °C in lysogenic broth (LB; Difco) (30, 31). All R. palustris strains are derivatives of R. palustris CGA009 (32) and were cultured at 30 °C in photosynthetic medium (33) supplemented with succinate (10 mM) or with NaHCO₃ (10 mM) and benzoate (3 mM). When used, ampicillin was at 100 µg ml⁻¹, chloramphenicol was at 20 µg ml⁻¹, and kanamycin was at 50 (E. coli) or 75 µg ml⁻¹ (R. palustris). Radiolabeled [1⁻¹⁴C]acetyl-CoA (54 mCi/mmol) was purchased from Moravek, and all other chemicals were from Sigma. Monocarboxylic acids are abbreviated as Cₓ and dicarboxylic acids are abbreviated as DCₓ where X is carbon chain length. Acids tested in this work were acetate (C₂), propionate (C₃), butyrate (C₄), valerate (C₅), hexanoate (C₆), heptanoate (C₇), octanoate (C₈), nonanoate (C₉), decanoate (C₁₀), undecanoate (C₁₁), laurate (C₁₂), tridecanoate (C₁₃), myristate (C₁₄), malonate (DC₂), glutarate (DC₃), adipate (DC₄), pimelate (DC₅), suberate (DC₆), azelaic (DC₇), sebacate (DC₁₄), dodecanedioic (DC₁₂), tetradecanedioic (DC₁₄), isobutyrate, isovalerate, β-hydroxybutyrate, crotonate, acetoacetate, and benzoate. Acids longer than 10 carbon atoms were solubilized with Triton X-100 (1% (v/v) in a 2 mM carboxylic acid stock solution).

**Molecular Biology Techniques**—DNA manipulations were performed using standard techniques (34). Restriction endonucleases were purchased from Fermentas. DNA was amplified using PfuUltra II Fusion DNA polymerase (Stratagene), and site-directed mutagenesis was performed using the QuickChange® kit (Stratagene). Plasmid DNA was purified using the Wizard® Plus SV Miniprep kit (Promega), and PCR products were purified using the Wizard SV Gel and PCR Clean-Up kit (Promega). DNA sequencing was performed using Applied Biosystems BigDye® terminator cycle methodology, and samples were resolved and analyzed at the University of Wisconsin-Madison Biotechnology Center. Oligonucleotide primer sequences are listed in supplemental Table S3. In-frame deletion of RpKatA (formerly rpa3031) was generated using the method of Schäfer et al. (35) as described elsewhere (15).
**Mass Spectrometry**—Strains *R. palustris* CGA009, JE11616, JE11937, and JE12800 were grown on benzoate as described above in 800-ml volumes to an optical density (OD) of ~0.5 at 600 nm. Pellets with masses of ~1 g were collected by centrifugation and stored at –80 °C. Each pellet was resuspended in Tris-HCl buffer (50 mM, pH 7.6) containing EDTA (10 mM); cells were lysed by sonication. Cell debris was removed by centrifugation at 5000 × g at 4 °C. The resulting supernatant was denatured in 6 M guanidine hydrochloride, 10 mM dithiothreitol (DTT) for 1 h at 60 °C and diluted to 1 M with Tris-HCl buffer (50 mM, pH 7.6) containing CaCl2 (10 mM); proteins were digested overnight at 37 °C using trypsin (10 μg) followed by addition of an additional 10 μg of trypsin and an extended incubation (4–5 h) at 37 °C. Digests were concentrated and desalted using Sep-Pak Lite C18 solid-phase extraction cartridges (Waters, Milford, MA).

Each strain was analyzed in triplicate using the multidimensional protein identification technology approach (36). As described previously (37), eluent from a two-dimensional (strong cation exchange followed by reverse phase) HPLC separation (Accela quaternary HPLC pump, ThermoScientific) was introduced to the quadrupole ion trap mass spectrometer (LTQ XL, ThermoScientific) via a nanoelectrospray interface (Proxeon, Odense, Denmark). Tandem mass spectra were acquired in the data-dependent mode (37).

Two independent software tools were used to identify peptides from the tandem mass spectra. For both tools, the list of candidate amino acid sequences was obtained from a database containing amino acid sequences from 4833 *R. palustris* proteins (32) as well as 44 common contaminant and standard proteins. A sequence-reversed version of each protein provided a “decoy” for estimation of the false discovery rate for peptide identification (38, 39), yielding a total of 9754 protein sequences in the database. SEQUEST® (version 27) searches (40) were performed with a differential modification of 42,037 Da on lysines to permit identification of both acetylated and non-acetylated peptides. Searches were specific for peptides resulting from trypsin digestion with four allowed missed cleavages. Peptide identifications were filtered and collated using DTASelect (version 1.9) (41). Peptide-level filters required an XCorr of ≥2.1 for z = 1, ≥2.8 for z = 2, and ≥3.5 for z = 3 where z is the charge on the ion. DeltaCN was required to be ≥0.08. These filters provided false discovery rates comparable with those obtained using InSpecT (see below), generally <0.1% at the peptide level. At the protein level, identification of a minimum of two different peptides or two charge states of the same peptide was required for protein identification; the false discovery rate at the protein level was 0.7% or less. InSpecT (42) searches were performed with optional modifications of +42 (acetylation) and +104 Da (benzoylation) allowed on lysines and arginines. Other parameters were as follows: instrument, ESLION TRAP; protease, trypsin; number of modifications per peptide, 3; parent mass tolerance, 3 m/z units; fragment ion mass tolerance, 0.5 m/z units. The InSpecT PValue module filtered peptide identifications, retaining those with p values of 0.01 or less, whereas the Summary module retained proteins with two or more identified peptides. Only unmodified peptides or peptides containing singly acetylated lysines were included in the reported results. Peptide and protein identification results from SEQUEST and InSpecT were imported into a Microsoft Access database for further analysis.

**Plasmids Used for Protein Purification**—*R. palustris* acetylation targets encoded by prpE (formerly rpa4504), hcsA (formerly rpa1003), fca (formerly rpa1702), ibuA (formerly rpa2302), fadD (formerly rpa4267), lcsA (formerly rpa4421), rpa1177, and cbbG were amplified from *R. palustris* genomic DNA using the primers listed in supplemental Tables S2 and S3. The PCR products were cut with the specified restriction enzymes and ligated into the overexpression vector pTEV5 (43). The resulting plasmids expressed each gene with an N-terminal hexahistidine (His6) tag, which was removed using recombinant tobacco etch virus (rTEV) protease (44). RpKatA (rpa3031) was cloned into plasmid pET-16b. The genes encoding *S. enterica* proteins AceA, GapA, PckA, Pgk, and SodB were amplified from strain TR6583 and cloned into pTEV5 for overexpression. Site-directed mutagenesis of acyl-CoA synthetases was performed using the QuikChange protocol (Stratagene).

**Protein Overproduction**—Plasmids encoding all potential acetyltransferase protein substrates were transformed into strain JE9314, a Pka (formerly YfiQ)-deficient derivative of *E. coli* C41(DE3) (45), to ensure that proteins were purified in their unacetylated state. The resulting strains were grown until early stationary phase and subcultured 1:100 in 2 liters of lysogenic broth supplemented with ampicillin (100 μg ml−1) unless otherwise noted. Cultures were grown with shaking to an OD600 ~0.6, and protein synthesis was induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside. Cultures were grown overnight at 30 °C, cells were harvested by centrifugation at 8000 × g for 12 min in a Beckman Coulter Avanti J-20 XOI refrigerated centrifuge with a JLA-8.1000 rotor, and cell pellets were frozen at –80 °C until used.

**Protein Purification**—*S. enterica* AcAs was purified using a chitin affinity tag as described (9). *S. enterica* Pat was purified as a His10-maltose-binding protein fusion, and both tags were removed using rTEV protease as described (29). The purification of *R. palustris* Pat, AcA, BdaA, HbaA, and AliA was performed as described (15). rTEV protease was purified as described (44). Protein concentrations were determined using a NanoDrop 1000 spectrophotometer (Fisher) and A280 molar extinction coefficients for each protein, which were obtained from the Integrated Microbial Genomes (IMG) database (46). *R. palustris* RpKatA was synthesized as a His10 N-terminal fusion protein in strain JE9314. Cells were grown in 6 liters of lysogenic broth and after induction with isopropyl 1-thio-β-D-galactopyranoside were allowed to grow overnight at 37 °C. After harvesting the cells by centrifugation, the pellet was resuspended in 30 ml of binding buffer (sodium phosphate buffer (50 mM, pH 8.0), NaCl (300 mM), imidazole (10 mM)) containing tris(2-carboxyethyl)phosphine (TCEP; 0.5 mM) and lysozyme (1 mg ml−1). Cells were lysed using a Sonic Dismembrator (Fisher) at power level 9 with 2-s pulses separated by 5-s breaks for 1.5 min. Debris was removed by centrifugation at 39,000 × g for 30 min at 4 °C, and the soluble fraction was passed through a 0.45-μm filter (Thermo Scientific). His10-RpKatA was purified by nickel affinity purification using a 1.5-ml bed volume of nickel-nitrilotriacetic acid Superflow resin (Qia-
Acetyl-CoA synthetases were acetylated with [1-14C]Ac-CoA and RpPat as described above, and the acetylation reaction was stopped by buffer exchange into HEPES buffer (50 mM, pH 7.5) containing TCEP (1 mM) and KCl (20 mM) using Microcon YM-30 centrifugal filter units (Millipore). A 2-μL sample of clarified cell-free extracts containing RpLdaA or no-enzyme control was added to 18 μL (54 pmol) of 14C-acetylated acetyl-CoA synthetase, and the reaction was incubated at 30 °C for 30 min. Proteins were resolved using SDS-PAGE, and radioactivity was quantified using phosphorimaging as described above. Each experiment was performed three times, and the amount of acetylation of each enzyme was normalized to the acetylation measured before the addition of RpLdaA.

**In Vitro Acyl-CoA Synthetase Activity Assay**—Acetyl-CoA synthetases (1.5 μM each) were individually incubated with RpPat or RpKatA (0.5 μM) plus or minus 50 μM acetyl-CoA for 1 h at 30 °C using the same buffer system described above for 14C-acetylation assays. Acetyl-CoA synthetase activity was quantified using an NAHAD consumption assay (48). Reactions contained HEPES buffer (50 mM, pH 7.5), TCEP (1 mM), ATP (2.5 mM), coenzyme A (CoASH) (0.5 mM), MgCl2 (5 mM), phosphoenolpyruvate (3 mM), NADH (0.1 mM), pyruvate kinase (1 unit), myokinase (5 units), lactate dehydrogenase (1.5 units), and organic acid substrate (0.2 mM). All reactions were started by adding acetyl-CoA synthetase (30 nm), and changes in the absorbance at 340 nm were monitored for 8 min in a 96-well plate format using a Spectramax Plus UV-visible spectrophotometer (Molecular Devices). Enzyme activities were calculated as described (3).

**RESULTS**

**RpKatA, a New Acetyltransferase Encoded by rpa3031, Acetylates Acyl-CoA Synthetases**

We previously showed that the RpPat enzyme acetylates acetyl-CoA synthetases in R. palustris and predicted the existence of a redundant acetyltransferase based on the detection of acetylated benzoyl-CoA synthetase (BadA) in a ∆apat strain (15). R. palustris has 26 genes encoding putative Gcn5p-type N-acetyltransferases, most of which are ~100–200 amino acids in length (pfam00583; Ref. 49). The protein encoded by rpa3031 (predicted size, 207 amino acids; hereafter referred to as RpKatA) is 31% identical to the Sulfolobus solfataricus Pat enzyme encoded by locus sso2813 (supplemental Fig. S1). The rpa3031 gene is located immediately 3′ to the gene encoding a putative phenylacetyl-CoA thioesterase, suggesting that RpKatA may be involved in the metabolism of aromatic compounds. Such homology to a known protein acetyltransferase and the proximity to a putative thioesterase led us to investigate whether RpKatA was the redundant acetyltransferase that modified acyl-CoA synthetases.

As shown in Fig. 1A, RpKatA acetylated cyclohexanecarboxyl-CoA synthetase (AliA) once at residue Lys-532, and as reported elsewhere, acetylation of AliA at Lys-532 inactivated the enzyme (15). These results suggested that, in R. palustris, the activity of acyl-CoA synthetases might be regulated by an N-Lys acylation/deacetylation system consisting of at least two
Acetylated Proteins Present in R. palustris Grown under Photoheterotrophic Conditions

In this work, we sought to identify new protein substrates of the N-Lys acylation/deacetylation system of R. palustris using a system-wide approach. For this purpose, we constructed several strains whose acetylome were compared with that of the wild-type strain. To enrich for acetylated proteins, we constructed strain JE11616 (supplemental Table S1), which lacked the two known deacetylases (ΔldaA ΔsrtN). To block protein acetylation, we constructed two strains, one of which (JE11937) lacked both deacetylases and the acetyltransferase RpPat (ΔldaA ΔsrtN Δpat) and a second strain (JE12800) that lacked both deacetylases and both acetyltransferases (ΔldaA ΔsrtN Δpat ΔkatA). We hypothesized that comparisons of results obtained from strains JE11937 and JE12800 would identify subsets of protein substrates for each acetyltransferase. For these experiments, all strains were grown photosynthetically using benzoate as a carbon source, and acetylation of total cell proteins was assessed for three technical replicates of each strain using LC/MS/MS.

The raw mass spectrometry data were analyzed using two independent software packages, SEQUEST (40) and InSpecT (42), which identified peptides from tandem mass spectra. Across all strains and replicates, SEQUEST identified a total of 2252 proteins for which evidence of acetylated lysines could be found in 230. InSpecT identified 2373 total proteins with evidence of acetylated lysines in 243 of these (supplemental Table S5). The number of proteins identified per single LC/MS/MS analysis by SEQUEST averaged around 1400, whereas InSpecT identified an average of ~1440 proteins (supplemental Table S4). SEQUEST provided evidence of acetylated lysines in 23–54 proteins per LC/MS/MS analysis, whereas InSpecT identified acetylated lysines in 17–40 proteins per run (supplemental Table S5). Because SEQUEST and InSpecT use different approaches (42), we expected (50) that the most robust identifications would be those found by both search tools within a single LC/MS/MS analysis. Surprisingly, the overlap between these two analyses was only 32 peptides, corresponding to 24 different proteins. Of these 24 proteins, 14 were identified more frequently in the deacetylase-deficient JE11616 (ΔldaA ΔsrtN) strain than in the other strains used in this work (Table 1), suggesting that they were more likely to be substrates of RpPat or RpKatA. Notably, of the 14 proteins mentioned above, nine were putative members of the acyl-CoA (AMP-forming) synthetase family, including the previously identified substrates AliA, BadA, and HbaA (15). Acetylation of another previously identified substrate, AcS, was detected with InSpecT but not SEQUEST and therefore was eliminated by our stringent analysis parameters; the acetylation was furthermore identified in only one of the three LC/MS/MS analyses of strain JE11616.

Although AcS was robustly identified from unmodified peptides with 21–45% sequence coverage by identified peptides (supplemental Table S5), the region of the protein containing the predicted acetylation site (Lys-606) was not among the identified tryptic peptides either acetylated or unmodified; the single exception was the case mentioned above when InSpecT identified a peptide containing acetylated Lys-606 (TRSGKAcIMR). In contrast to the present approach in which only endogenously occurring AcS protein was analyzed along with all other expressed proteins, previous studies confirming acetylation at the predicted lysine residue were performed using larger amounts of the AcS protein that had been expressed with an affinity tag and purified (9), leading to increased sensitivity.

Consistent with previous results, AliA, BadA, and HbaA showed some acetylation in strain JE11937 (ΔldaA ΔsrtN Δpat), but no acetylation of these proteins was detected in strain JE12800 (ΔldaA ΔsrtN Δpat ΔkatA) (Table 1). These results suggested that RpKatA acetylated acyl-CoA synthetases in vivo and that RpPat and RpKatA may be the only acetyltransferases that modify AMP-forming acyl-CoA synthetases in R. palustris.

In Vitro Assessment of Enzymatic Activity and Substrate Preference for Putative Acyl-CoA Synthetases

Of the nine acyl-CoA synthetases identified by LC/MS/MS, six were of unknown function. We isolated to homogeneity putative acyl-CoA synthetases encoded by rpa4504, rpa1003, rpa1702, rpa2302, rpa4267, and rpa4421 and assessed their activities using a coupled spectrophotometric assay with ATP, CoA, and a variety of organic acids as substrates.
TABLE 1
Selected proteins identified by LC/MS/MS and putative location of acetyl moieties
Selection was based on more frequent identification of acetylated peptide in the deacetylase-deficient strain (ldaA srtN).

| Locus tag | Protein name | Acyl-CoA synthetase | Peptidea | Position modified | WT | ldaA srtN | ldaA srtN pat | ldaA srtN pat katA |
|-----------|--------------|---------------------|----------|-------------------|-----|-----------|-------------|------------------|
| RPA0071   | AcclD        | No                  | ETPENLWJK/CPTDGQL/VFYYKDVEQNFQVFGNHYMR | 27   | 2          | 2           |             |
| RPA0651   | AliA         | Yes                 | DAMPATSQGK/QFK | 532  | 3          | 3           |             |
| RPA0651   | AliA         | Yes                 | LVRDAMPATSQGK/QFK | 532  | 3          | 2           |             |
| RPA0661   | BadA         | Yes                 | TATGK/QFQFK | 512  | 2          | 1           |             |
| RPA0661   | BadA         | Yes                 | STVIEALPKTATGK/QIR | 512  | 3          | 3           |             |
| RPA0669   | HbaA         | Yes                 | WIQIMDLPKTSQGK/LQR | 503  | 2          | 2           |             |
| RPA0817   | No           | FaaA56                                                                        75   | 1          |
| RPA0944   | CbbG         | No                  | DNXAATTAGKRR | 107  | 3          | 2           | 1             |
| RPA0944   | CbbG         | Yes                 | AAIAMMSPTGTAAGK/AIGLVPHELK | 215  | 2          | 1           |             |
| RPA0944   | CbbG         | No                  | AIGLVPHELK/GKGLDGVAIR | 225  | 2          | 1           |             |
| RPA1003   | HcsA         | Yes                 | NANGK/QKTVLR | 524  | 2          |             |             |
| RPA1177   | No           | TPVERKAAGK*         | 67       | 2          |             |             |             |
| RPA1535   | CycA         | No                  | ADK*NMMVGPAALGVGVR | 46   | 1          | 3           |             |
| RPA1702   | FcsA         | Yes                 | SYVEAEEPLRTFGK*LIVK | 496  | 1          |             |             |
| RPA2302   | IbuA         | Yes                 | TPSGK/QIR | 539  | 1          |             |             |
| RPA2302   | IbuA         | Yes                 | EIEFLEQLPRTSGK/QIR | 539  | 2          |             |             |
| RPA2467   | FadD         | Yes                 | TNVGK/QLR | 546  | 1          |             |             |
| RPA2467   | FadD         | Yes                 | TTTLPKTNVGK/QLR | 546  | 1          |             |             |
| RPA4421   | LcsA         | Yes                 | SAIGK*VLKR | 499  | 2          |             |             |
| RPA4504   | PrpE         | Yes                 | SGK*ILGTIK | 598  | 3          | 3           | 1             |
| RPA4504   | PrpE         | Yes                 | K*IADGDTWTMPATIEDPTALDDISSALK | 606  | 1          |             |             |

*a* Acetylated lysine residue indicated by *. In some cases, more than one peptide was identified from the same protein.

*b* Three technical replicates of each strain were analyzed, and the number represents how many of these replicates contained the indicated acetylated peptide identified by both SEQUEST and InSpecT.

Short- and Medium-chain Fatty Acyl-CoA Synthetases—Bioinformatics analysis identified Rpa4504 as a homologue of propionyl-CoA synthetase (PrpE) from other organisms. Homogeneous Rpa4504 activated propionate, acetate, and isobutyrate, although propionate was the preferred substrate (Fig. 2). Rpa2302 activated short- to medium-chain monocarboxylic acids but had highest activity with isobutyrate (Fig. 2), thus we suggest naming it isobutyryl-CoA synthetase (IbuA). The highest activity of Rpa1003 was measured when the medium-chain monocarboxylic acid hexanoate (C6) was used as substrate (Fig. 2); thus, we named this enzyme hexanoyl-CoA synthetase A (HcsA).

Medium-long Chain Fatty Acyl-CoA Synthetases—Rpa1702 had the broadest range and preferred medium- to long-chain mono- and dicarboxylic acids as substrates. It activated C8, C9, C10, C12, and C14 monocarboxylic acids and C12 and C14 dicarboxylic acids with similar efficiencies (Fig. 2). We named Rpa1702 FcsA for fatty acyl-CoA synthetase A.

Rpa4267 and Rpa4421 preferred long-chain dicarboxylic acids. Rpa4267 was most active with the C12 dicarboxylic acid dodecanedioate, and Rpa4421 showed the highest activity with the C14 dicarboxylic acid tetradecanedioate; we did not test any longer dicarboxylic acids to see whether they were also substrates of this enzyme (Fig. 2). We named Rpa4267 RpFadD on the basis of its identity level (57%) with the long-chain fatty acyl-CoA synthetase FadD of *E. coli* and named Rpa4421 LcsA for long-chain acyl-CoA synthetase A.

*R. palustris* Acyl-CoA Synthetases Are Acetylated in Vitro by RpPat and RpKatA with Different Specificities

After confirming that the six newly identified acyl-CoA synthetases had enzymatic activity in *vitro*, we tested whether RpPat or RpKatA could acetylate them. RpPat acetylated all of the wild-type proteins but not variant proteins in which the conserved active site lysine residue (equivalent to Lys-512 in BadA) was changed to alanine (Fig. 3). These data showed that all proteins were acetylated only once and that acetylation occurred at the above mentioned conserved lysine residue. This information was consistent with the location of acetylated lysine residues identified by LC/MS/MS with the exception of PrpE in which residues Lys-598 and Lys-606 were acetylated (Table 1). Notably, RpPat and RpKatA acetylated only residue Lys-598, and the peptide of PrpE containing AcLys-606 was detected only in the strain lacking both acetyltransferases. RpKatA did not have as broad a substrate range as RpPat. The best substrates of RpKatA were AliA, BadA, HbaA, and PrpE, whereas FcsA, IbuA, and FadD were poor substrates, and neither HcsA nor LcsA was a substrate (Fig. 3).

Acetylation of Newly Identified Acyl-CoA Synthetases by RpPat or RpKatA Inactivates Enzymes

We investigated whether acetylation affected the activities of the newly identified acyl-CoA synthetases. For this purpose, each acyl-CoA synthetase was preincubated with either RpPat or RpKatA and acetyl-CoA followed by specific activity measurements (Table 2). The resulting activity level was compared with the activity of enzymes measured in a reaction mixture devoid of acetyl-CoA.
Table 1, suggested that RpKatA acetylated AliA, PrpE, BadA, HbaA, and possibly Acs in vivo and that as low as \( \frac{1}{3} \) enzyme inactivation under the conditions of this assay was biologically relevant.

Acetylated Acyl-CoA Synthetases Are Deacetylated by Lysine Deacetylase A (RpLdaA) Enzyme

We investigated whether acetylated acyl-CoA synthetases could be enzymically deacetylated. To do this, we used cell-free extracts of a deacetylase-deficient E. coli K12 strain expressing RpLdaA. After incubation of \(^{14}\)C-acetylated acyl-CoA synthetases with RpLdaA-enriched cell-free extracts, proteins were resolved by SDS-PAGE, and the removal of radiolabeled acetyl moieties from acetylated proteins was monitored using phosphorimaging. In each case, the extent of protein acetylation was compared with the level of acetylation observed before the addition of RpLdaA and with the level of acetylation observed after addition of cell-free extracts of a strain harboring the empty cloning vector used to express RpLdaA. As shown in Fig. 4, RpLdaA deacetylated all acyl-CoA synthetases tested (\( >75\% \) deacetylation). RpFadD was not tested because it was unstable.

Acetylation of Some Proteins Identified by LC/MS/MS Does Not Depend on RpPat or RpKatA

Five non-acyl-CoA synthetase proteins were identified by LC/MS/MS to be putatively acetylated more often in the deacetylase-deficient JE11616 strain (Table 1). These proteins were AccD (\( \beta \)-subunit of acetyl-CoA carboxylase), Rpa0817 (a putative acetyl-CoA acetyltransferase/thiolase), CbbG (glycer-aldehyde-3-phosphate dehydrogenase (GAPDH)), RPA1177 (a putative 4-oxalocrotonate tautomerase), and CycA (cytochrome \( c_5 \)). CbbG (GAPDH) was of interest because it has been identified in all three previous mass spectrometry-based proteomics studies in bacteria (26–28).

In our study, multiple acetylation sites were identified in CbbG (Lys-107, Lys-215, and Lys-225), but none of these
lysines were identified in any of the other studies. In total, 10 different lysine residues have been identified as putatively acetylated in the four studies, and only one of those, Lys-194 (Lys-192 in E. coli numbering), was identified in more than one study (26, 27). In our work, only Lys-107 was found more frequently in the deacetylase-deficient strain (Table 1), and this lysine is not conserved in E. coli (28). As shown in Fig. 5, KatA in the presence of 14C-labeled acetyl-CoA did not detect any acetylation (Fig. 5A). RpBadA (benzoyl-CoA synthetase), a substrate of RpPat and RpKatA, was used as positive control for RpPat and RpKatA activities. On the basis of the above results, we concluded that CbbG and Rpa1177 proteins were not substrates of RpPat or RpKatA.

**Inability to Replicate Results Reported by Others Raises Important Questions Regarding Identity of True Substrates of Pat Enzymes**

Failure of RpPat to acetylate RpCbbG was an unexpected result because its homologue in S. enterica (GapA) was recently reported to be a substrate of S. enterica Pat (28). Because SePat and RpPat are homologues (38% identical over 886 amino acids) and both acylate Acss, we investigated whether SePat could acetylate SeGapA. Strikingly, we saw very limited acetylation of SeGapA, and more importantly, it was not dependent on SePat activity (Fig. 5B); control experiments showed that the SePat used in these experiments was active. These results prompted us to test whether SePat could acetylate other reported substrates of SePat, such as isocitrate lyase (AceA), isocitrate dehydrogenase phosphatase/kinase (AceK), phosphoenolpyruvate carboxykinase (PckA), phosphoglycerate kinase (Pgtk), and superoxide dismutase (SodB) (28). As shown in Fig. 5B, SePat did not acetylate any of the above mentioned proteins.
**DISCUSSION**

Evidence reported here provides insights into several important aspects of N-Lys protein acylation in prokaryotes in general. First, the data support the involvement of putative prokaryotic γGcn5p-like acetyltransferases in the posttranslational control of the biological activity of prokaryotic proteins (Table 2). Second, the data expand the scope of protein acetylation in the α-proteobacterium *R. palustris* (Fig. 3). Third, the data support the assignment of function and substrate preference for six newly identified acyl-CoA synthetases (Fig. 2). Fourth, the data highlight differences in the protein substrate specificity of *RpPat* and *RpKatA* (Fig. 3).

Sixth, the data indicate that the previously identified lysine deacetylase A (*RpLdaA*) enzyme has broad substrate specificity in vitro, suggesting that it may play a role in the in vivo modulation of activity of all newly identified acyl-CoA synthetases.

*RpPat* and *RpKatA* May Have Evolved to Specifically Modify AMP-forming Acyl-CoA Synthetases—It is important to note that all known *RpPat* substrates are AMP-forming acyl-CoA synthetases and that all are acetylated at a conserved lysine residue (Fig. 6). *SePat* and its *E. coli* homologue, *Pka* (formerly *YfIQ*) (52), also acylate the acyl-CoA synthetases *Acs* and *PrpE*, which contain the same conserved lysine residue and surrounding motif. Although it has been reported that *SePat* acetylates *GapA*, *AceA*, and *AceK* (28), we could not confirm those results here, and we did not observe acetylation of the *R. palustris* *GapA* homologue, *CbbG*, by *RpPat* either. It has also been suggested that *Pka* (the *E. coli* homologue of *SePat*) acetylates the α subunit of RNA polymerase (53), but direct evidence for this acetylation was not provided. It has recently been shown that *E. coli* *Pka* can acetylate RNase *R* both in vitro and in vivo and that acetylation affects the stability of the protein (52, 54). The RNase *R* homologue in *R. palustris* (*Rpa3125*) contains an alanine residue at the reported acetylation site, suggesting that it is unlikely that RNase *R* is acetylated in this bacterium. Taken together, this information suggests that *RpPat* may specifically acetylate AMP-forming acyl-CoA synthetases as we have no evidence of other substrates in this organism. Given the strong conservation of the acetylation site that we see among known substrates of *Pat* homologues from *R. palustris*, *S. enterica*, and *E. coli*, it seems likely that the primary role for all three *Pat* homologues may be in regulating AMP-forming acyl-CoA synthetases. It is possible that *E. coli* *Pka* and *SePat* have broader substrate ranges than *RpPat*, which may explain how they are able to acetylate such diverse proteins as *Acs*, *RcsB*, and RNase R.

### FIGURE 4. Deacetylation of newly identified acyl-CoA synthetases using cell-free extracts enriched for *RpLdaA*. 14C-Acetylated acyl-CoA synthetases were incubated with cell lysates of *E. coli* harboring either plasmid pRpLDA1 or cloning vector. Deacetylated reactions were resolved using SDS-PAGE, and the specificity of bacterial protein acetyltransferases was quantified by phosphorimaging and normalized to the reaction mixture devoid of cell-free extract. Data represent averages and S.D. of three independent experiments.

### FIGURE 5. Probing acetylation of other proteins reported to be substrates of *Pat*. A. *R. palustris* proteins BadA (positive control), CbbG, and Rpa1177 were incubated with [1-14C]acyl-CoA and with or without *RpPat* or *RpKatA*. B. *S. enterica* proteins Acs (positive control), AceA, AceK, GapA, PckA, Pga, and SodB were incubated with [1-14C]acyl-CoA and with or without *SePat*. Molecular mass standards in kDa are shown on the left side of the gel. Top panels show SDS-PAGE of acetylation reactions, and the bottom panels show the phosphorimage of the same gels.
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“Acetylation Motif” Recognized by Pat Enzymes—Salient features within the proposed acetylation motif found in AMP-forming acyl-CoA synthetases are glycines immediately preceding the acetylation site and a proline six positions upstream of the lysine (PKTRSGK) (Fig. 6A). At present, it is not clear whether these or other residues in specific positions direct the recognition of the protein substrate by the acetyltransferase and/or whether conservation of the above residues is required for synthesis of the acyl-CoA product.

Newly Discovered RpKatA Acetyltransferase Is Likely Needed under Physiological Conditions Different from Those where RpPat Function Is Required—Clearly, RpKatA has a different substrate specificity than that of RpPat (Fig. 3), but it is not evident why RpKatA specifically targets PrpE, AliA, Acs, BadA, and HbaA but not other acyl-CoA synthetases. It is also unclear why RpPat contains a large (~700-amino acid) N-terminal domain because RpKatA appears to function similarly but lacks this large domain. We speculate that such a domain is involved in regulation, oligomerization, or substrate recognition.

Many AMP-forming Acyl-CoA Synthetases Are Controlled by Acetylation—The number of reports of AMP-forming acyl-CoA synthetases (from bacteria to humans) whose activities are posttranslationally controlled by N-Lys acylation/deacylation systems is on the rise (3, 9, 14–16, 18, 19). This work alone expands the number of acyl-CoA synthetases that are regulated by N-Lys acylation from four to 10 in R. palustris, indicating that in this bacterium this type of posttranslational control is commonly used to modulate the activity of this important class of enzymes.

Why Are So Many AMP-forming Fatty Acyl-CoA Synthetases Posttranslationally Controlled?—We posit that acetylation is a feedback mechanism that helps maintain energy and carbon homeostasis. Many acyl-CoA synthetases activate acids that are eventually degraded to acetyl-CoA. If the intracellular concentration of acetyl-CoA rose for any reason, it would be important for the cell to slow down fatty acid activation. In addition, fatty acid activation is an energy-intensive reaction, and it may be preferable to use other carbon sources if they are available. Consistent with the idea of controlling energy consumption, work by Escalante-Semerena and co-workers (55) recently established a link between N-Lys acylation/deacylation and energy homoeostasis in S. enterica. Whether acylation/deacylation control of this class of enzymes in R. palustris is needed to respond to the same physiological stress remains to be determined.

It is interesting to note that during log phase photosynthetic growth of R. palustris using benzoate as a carbon source we did not detect acetylation of acyl-CoA synthetases in the wild-type strain (Table 1). It is clear that the acetylation/deacetylation machinery was expressed and active, however, because elimination of the deacetylases ldaA and srtN resulted in readily detectable acetylation of acyl-CoA synthetases (Table 1) and a concomitant growth defect (15). Further work is required to determine when acetylation of acyl-CoA synthetases occurs, although it could become important during different growth phases or when multiple carbon sources are present.

Proteomics Approaches Allow for Identification of New Acetylation Targets—System-wide approaches like the one used in this work provide a powerful approach for identifying potentially acetylated proteins. However, we cannot emphasize enough the importance of rigorous validation.

In our work, we were able to reduce some of the “noise” in the mass spectrometry data set by combining the analyses obtained from using the SEQUEST and InSpecT software packages and by considering only acetylated proteins that were identified using both programs. We also focused on substrates of the known protein acetylation/deacetylation system in R. palustris by selecting proteins that were more frequently acetylated in a strain lacking both deacetylases. Although some acetylated proteins were certainly overlooked in our work using these stringent parameters, including one known acetylated protein, RpAcs, the fraction of putative substrates that could be verified in vitro was high. Using these techniques, we reduced the list of potentially acetylated proteins from several hundred to 14 of which nine were bona fide substrates of RpPat.

There are many possible explanations for why some proteins appear to be acetylated but cannot be verified in vitro. For example, our interpretation presumes that the modified peptides and proteins identified from the LC/MS/MS data are acetylated. However, it is not possible under the experimental conditions used here to distinguish between acetylation and trimethylation of lysine residues (56). This isobaric interference may have contributed to observations that fall outside the predictions based on our model of the N-Lys acetylation/deacetylation system of R. palustris. In addition, we ignore the extent and impact of non-enzymic acetylation and the role of many putative, uncharacterized acetyltransferases.

The failure of RpPat and RpKatA to acetylate RpCbbG or Rpa1177 in vitro (Fig. 5A) illustrates that even this reduced group of putative substrates identified by LC/MS/MS con-
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tained proteins that were not acetylated by this acetylation/deacetylation system. At present, we cannot rule out the possibility that RpPat and RpKpKatA require ancillary proteins or factors to acetylate RpCbbG or Rpa1177 and that, because our reaction mixtures contained homogeneous RpPat or RpKpKatA proteins, it would not be surprising that neither acetyltransferase modified RpCbbG or Rpa1177. The argument for ancillary proteins or factors is not very strong in light of the recent report by others who claim that homogeneous SePat modifies ScGapA (a homologue of RpCbbG) and that acetylated GapA has higher enzymatic activity than unacetylated GapA (28).

We could not replicate the results by Wang et al. (28) using either RpPat/RpCbbG or SePat/ScGapA (Fig. 6). In fact, SePat failed to acetylate S. enterica GapA, AceA, and AceK, the three proteins that were used by these authors to verify their proteomics results (28). Our negative results were not due to the use of inactive SePat because the enzyme strongly acetylated its well documented substrate Acs (Fig. 6).

Conclusions—The work reported here augments the data available in the literature in strong support of the idea that Pat homologues have broad substrate specificity for AMP-forming acyl-CoA synthetases.

Acknowledgments—We thank Caroline S. Harwood (University of Washington) for helpful discussion, Alison L. Russell (Oak Ridge National Laboratory) for advice on the use of InSpecT, Manesh B. Shah (Oak Ridge National Laboratory) for assistance in the performance of SEQUEST and InSpecT analyses, and Patricia Lankford (Oak Ridge National Laboratory) for assistance with protein extraction and mass spectrometry analyses.

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