A Pantothenate Kinase from *Staphylococcus aureus* Refractory to Feedback Regulation by Coenzyme A*

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The key regulatory step in CoA biosynthesis in bacteria and mammals is pantothenate kinase (CoaA), which governs the intracellular concentration of CoA through feedback regulation by CoA and its thioesters. CoaA from *Staphylococcus aureus* (SaCoaA) has a distinct primary sequence that is more similar to the mammalian pantothenate kinases than the prototypical bacterial CoaA of *Escherichia coli*. In contrast to all known pantothenate kinases, SaCoaA activity is not feedback-regulated by CoA or CoaA thioesters. Metabolic labeling of *S. aureus* confirms that CoA levels are not controlled by CoaA or at steps downstream from CoaA. The pantothenic acid antimetabolite N-heptylpantothenamide (N7-Pan) possesses potent antimicrobial activity against *S. aureus* and has multiple cellular targets. N7-Pan is a substrate for SaCoaA and is converted to the inactive butyldethia-CoA analog by the downstream pathway enzymes. The analog is also incorporated into acyl carrier protein and D-alanyl carrier protein, the prosthetic groups of which are derived from CoaA. The inactivation of acyl carrier protein and the cessation of fatty acid synthesis are the most critical causes of growth inhibition by N7-Pan because the toxicity of the drug is ameliorated by supplementing the growth medium with fatty acids. The absence of feedback regulation at the pantothenate kinase step allows the accumulation of high concentrations of intracellular CoA, consistent with the physiology of *S. aureus*, which lacks glutathione and relies on the CoA/CoA disulfide reductase redox system for protection from oxidative damage.

CoA is an essential cofactor that functions as the major acyl group carrier in intermediary metabolism and is synthesized in five steps from pantothenic acid (vitamin B₅) (1, 2). The first step in the pathway is the ATP-dependent phosphorylation of pantothenate by pantothenate kinase (3). All organisms characterized to date have a common mechanism to regulate the intracellular concentration of CoA through feedback inhibition of CoA or CoA thioesters. The primary sequences of the prototypical *Escherichia coli* pantothenate kinase (EcCoaA)† and the eukaryotic pantothenate kinases have little sequence similarity (Fig. 1). Nonetheless, the enzymes share the common property of being feedback-regulated by CoA and its thioesters, and this regulatory mechanism is primarily responsible for controlling the intracellular CoA concentration (4, 6, 9). The bacterial enzyme is more effectively regulated by CoA (6, 7), whereas the mammalian and *Aspergillus nidulans* pantothenate kinases are most potently inhibited by acetyl- and malonyl-CoA (7, 9–11). The mechanism of the feedback inhibition by CoA has been extensively investigated in *E. coli* and arises from competitive inhibition of ATP binding (5, 6). The details of this interaction are revealed by the high-resolution x-ray structures of the EcCoaA-ATP-ΔS (12), EcCoaA-CoA (12), and EcCoaA-ADP-pantothenate (13) complexes. Although the *E. coli* CoaA is considered the model bacterial pantothenate kinase, this isoform is not universally expressed in bacteria (14). *Pseudomonas aeruginosa* and *Helicobacter pylori* do not have recognizable pantothenate kinases in their genomes, although all other components of the biosynthetic pathway are present. *Staphylococcus aureus* and *Bacillus anthracis* possess an alternate isoform of pantothenate kinase that is more closely related to the mammalian enzymes than it is to EcCoaA (Fig. 1). SaCoaA was recently expressed and demonstrated to possess pantothenate kinase activity (15).

CoA is also the source of the 4'-phosphopantetheine prosthetic group present in a number of proteins that function as acyl/aminoacyl/peptidyl group carriers. Examples are the carriers proteins (ACP) of fatty acid synthases, polyketide synthases, and non-ribosomal peptide synthases (16). The transfer of the 4'-phosphopantetheine moiety of CoA to a conserved serine residue of these carrier proteins is catalyzed by dedicated phosphopantetheinyl transferases. Some of these transferases possess a restricted substrate specificity such as *E. coli* AcpS and EntD (17), whereas others are more promiscuous like *Bacillus subtilis* Sfp (17, 18). *E. coli* AcpS specifically modifies the ACP involved in fatty acid biosynthesis, although it also accepts substrates such as Dcp and some ACPs of type II polyketide synthases (17, 19, 20). Dcp is a small carrier protein required for the transfer of D-alanine to lipoteichoic acid in Gram-positive bacteria (21–23).

* The abbreviations used are: EcCoaA, *Escherichia coli* pantothenate kinase; N5-Pan, N-pentylpantothenamide; N7-Pan, N-heptylpantothenamide; ACP, acyl carrier protein; Dcp, n-alanyl carrier protein; MIC, minimal inhibitory concentration; ES-MS, electrospray mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; SaCoaA, *Staphylococcus aureus* pantothenate kinase; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; AcpS, acyl carrier protein synthase; TOF/TOF, tandem time-of-flight.

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The pantothenic acid analogs N5-Pan and N7-Pan inhibit E. coli growth (24–26). These pantothene antimetabolites are substrates and competitive CoaA inhibitors with respect to pantethione (13). N5-Pan is further metabolized through the CoA biosynthetic pathway to the cofactor analog ethylthiadiapho-CoA (25). More recently, the incorporation of these analogs into ACP was demonstrated, and the blockade of fatty acid synthesis by the accumulation of inactive ACP is the root cause for growth inhibition in the E. coli model system (26). N5-Pan and N7-Pan are also effective antimicrobial agents against S. aureus (15, 19); however, in this case Choudhry et al. (15) found that these compounds are inhibitors but not substrates for SaCoA. This suggests that the pantothene antimetabolites have a different mechanism of action in S. aureus than in E. coli. This study reports the unique regulatory properties of SaCoA and the mechanism of action of the pantothenamide antimetabolites in S. aureus.

**EXPERIMENTAL PROCEDURES**

**Materials**—Materials were purchased from the following suppliers: molecular biology reagents from Qiagen; restriction enzymes from New England Biolabs; DNA ligase from Promega; pCR2.1 vector from Invitrogen; pET-15b and pET-28a vectors and thrombin kit from Novagen; oligonucleotides for PCR from the Hartwell Center at the St. Jude Children’s Research Hospital; [1-14C]pantothenate (specific activity, 55 mCi/mmol), β-[3-3H]alanine (specific activity, 50 Ci/mmol), [1-14C]acetate (specific activity, 55 mCi/mmol), 1-β-(hexylamino) acid mixture (specific activity, 30–60 Ci/mmol) and [32P]ATP (specific activity, 6000 Ci/mmol) from American Radiolabeled Chemicals; Bradford dye-binding protein assay solution from Bio-Rad; lysostaphin from AMBI; 250-μm silicon gel H plates from Analtech, Inc.; ScintiSafe 30% and TqT DNA polymerase from Fisher; DE81 filters from Whatman; and HA filters from Millipore.

**CoaA and ACP Expression Vectors**—The coaA and acpP genes were amplified by PCR from S. aureus strain RN4220 genomic DNA, using TqT DNA polymerase and the following sets of forward and reverse primers, respectively: 5'-GCTAGCATGAAAATTTGGCATTGCAGCGTGGC and 5'-GGATCCCTATTTCCATTTGATTCGAC for coaA, 5'-CATATGGAAAAATTTGCCGCAATATTGAAAGGAATAC and 5'-GGATCCGTGCTGCAATGATACCGAGCTCCGAC for acpP. Amplified coaA was ligated into the pCR2.1 vector and subcloned into pET-BamHI restriction sites. The resultant plasmid pSCPc was transformed into E. coli strain BL21 Star(DE3) (Novagen). Similarly, acpP was first ligated into the pCR2.1 vector and then subcloned into pET-BamHI restriction sites. The resultant plasmid pSCPc was transformed into E. coli strain Rosetta(DE3) (Novagen). CoaA and ACP were expressed from pSCPc and pRL002, respectively, as His6-tagged fusion proteins.

**Effect of CoaA and Coa Thiosteres on E. coli and S. aureus CoaAs**—E. coli CoaA was expressed as a His6-tagged fusion protein from a pET-15b-derived vector and purified by immobilized metal ion affinity chromatography in a single step, as described under “Protein Purification and Preparation of ACP—E. coli AcpS was expressed from pET-15b-derived vector and purified by immobilized metal ion affinity chromatography in a single step, as described previously (5, 29).

**Analysis of Intracellular and Extracellular β-Alanine-derived Metabolites**—[3H]β-Alanine and [5-14C]pantothenate of increasing concentrations were prepared by diluting β-[3-3H]alanine and by radioactive β-alanine. This resulted in a 2-fold decrease of the isotope specific activity (from 50 to 0.78 Ci/mmol) for each successively higher concentration. S. aureus strain RN4220 was cultured in 1% treptone medium overnight at 37 °C and used as 1% inoculum into fresh medium (10 ml). 1-ml aliquots were transferred to 15-ml tubes containing increasing concentrations of [3H]alanine from 0.5 to 32 μM as indicated. After 4 h at 37 °C, cells were transferred to 1.5-ml tubes and harvested by centrifugation. The supernatant was removed and stored at −20 °C. The cell pellets were washed with phosphate-buffered saline and resuspended in 50 mM Tris-HCl, pH 7.5, 12.5 mM EDTA, 0.4 M NaCl, 5 mM β-mercaptoethanol, and ACP was eluted and stored at −20 °C.

**Measurement of the MIC**—The MICs of N5-Pan and N7-Pan against S. aureus strain RN4220 were determined by a broth microdilution method. Briefly, S. aureus was cultured at 37 °C in 1% treptone to mid-log phase, diluted 30,000 times, and used to inoculate (10 μl, 100 μl) in triplicate into 96-well microtiter plates. The plates were incubated at 37 °C for 18 h (bottom with low evaporation lid) containing fresh 1% treptone medium (100 μl) supplemented with the indicated concentrations of pantothenate analogs or Me2SO (negative control). The same experiments were repeated in the presence of 50 μM pantethione. After 20 h of incubation at 37 °C, the A595 of the cell suspensions in the wells was measured with a FusionTM universal microwave analyzer (Packard Instrument Co.). The optical density measured in negative controls was taken as 100% growth.
**Determination of IC₅₀ Values and Kinetic Parameters for the Pantotenate Analogues**—The inhibitory effect of N5-Pan and N7-Pan on the kinase activity of SaCoaA was estimated by measuring the amount of radioactive 4'-phosphopantetheine produced in standard reaction mixtures containing 45 μM tr-[14C]pantetheine (specific activity, 55 mCi/mmol), 100 μM ATP, 10 mM MgCl₂, 50 mM Tris-HCl, pH 7.5 (13), and increasing concentrations of each analogue as indicated. Reaction mixtures were incubated at 37 °C for 10 min and then stoped and analyzed as described previously (13). The kinetic constants were obtained from duplicate experiments by nonlinear regression analysis of initial velocities using Prism 4 (GraphPad Software). The constants for pantetheine and ATP were determined under standard conditions by fixing the ATP concentration at 250 μM and increasing the tr-[14C]pantetheine concentration from 5.6 to 90 μM or by fixing the tr-[14C]pantetheine concentration to 90 μM and increasing the ATP concentration from 15.6 to 250 μM. The possibility that Coa phosphorylated the substrate analogues N5-Pan and N7-Pan was investigated by omitting 32P-ATP and measuring the incorporation of 32P into the enzyme. Unlabeled ATP was substituted with [γ-32P]ATP (13). The kinetic parameters were determined in the presence of 250 μM [γ-32P]ATP (specific activity 1 Ci/mmol), 10 mM MgCl₂, 100 mM Tris-HCl, pH 7.5, and increasing concentrations of N5-Pan (0.63 to 20 μM) and N7-Pan (25 to 40 μM). Reaction mixtures were incubated for 30 min at 37 °C, and the products were recovered and determined as described previously (13).

**Growth Inhibition by N7-Pan and Cerulenin and Effect of Exogenous Fatty Acids—**Oleate and palmitate stock solutions (10 mg/ml) were typically used for protein measurements on this instrument. Deconvolution of the protein spectrum was accomplished using the mass entropy algorithm of the MassLynx software (Micromass Inc.) (33).

**RESULTS**

**Purification and Characterization of SaCoaA—**SaCoaA possesses an atypical primary sequence (10, 15, 34) that shares 18% identity with the murine CoaA isoform 1/β (MmPanK1β) and only 13% identity with EcCoaA, the prototype for bacterial pantothenate kinases (Fig. 1). Although SaCoaA is more related to the mammalian enzymes, it is not closely related to either pantothenate kinase isoform. SaCoaA was cloned, expressed as a C-terminal His₆-tagged fusion protein in E. coli, and purified to homogeneity by immobilized metal ion affinity and gel filtration chromatography (Fig. 2). SDS-PAGE analysis of fractions possessing pantothenate kinase activity revealed the presence of a 29-kDa protein, consistent with the subunit molecular size predicted from the amino acid sequence of SaCoaA (Fig. 2B). Characterization by gel filtration analysis also indicated a native molecular mass of 59 kDa for SaCoaA, consistent with the existence of a homodimer (Fig. 2A).

**Regulation of CoA Biosynthesis in S. aureus—**SaCoaA was assayed in vitro in the presence of different concentrations of CoA, acetyl-CoA, and malonyl-CoA and compared with the activity of EcCoaA (Fig. 3A). The ability of SaCoaA to phosphorylate pantothenate was completely unaffected by the presence of these pathway end products at concentrations that clearly inhibited EcCoaA (Fig. 3A). These data suggested that CoA biosynthesis in S. aureus was not controlled at the pantothenate kinase step. SaCoaA cells were labeled with radioactive β-alanine, a pantothenate precursor, and intracellular and extracellular radiolabeled metabolites were analyzed to determine whether CoA synthesis was regulated at a downstream step in the biosynthetic pathway (Fig. 3B). The intracellular CoA concentration increased proportionally to the extracellular β-alanine concentration. Neither radiolabeled pantothenate nor 4'-phosphopantetheine accumulated in the medium or inside the cells (Fig. 3, C and D). The absence of pathway intermediates accumulating in either intracellular or extracellular compartments indicated that CoaA biosynthesis in S. aureus was
not regulated by feedback inhibition either at SaCoaA or another downstream step.

Inhibition of SaCoaA and *S. aureus* Growth by Pantothenamide Antimetabolites—The pantothenate analogs N5- and N7-Pan inhibit both *S. aureus* and *E. coli* growth (13, 15, 25). The MICs of N5-Pan and N7-Pan against *S. aureus* strain RN4220 were determined by a broth microdilution method in the presence or absence of pantothenate (Fig. 4). The MIC values of N5-Pan and N7-Pan were estimated to be 0.16 and 25 μM, respectively. The addition of pantothenate at a concentration of 50 μM to the growth medium produced an 8- and 64-fold shift in the MICs of N5-Pan and N7-Pan, respectively. The reduced

| System  | MIC (μM)  |
|---------|-----------|
| SaCoaA  | 0.16      |
| MmPanK1β| 25        |
| EcCoaA  | 200       |

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**FIG. 1.** Sequence alignment of pantothenate kinases. Sequences were from *S. aureus* (SaCoaA, GenBank™ accession number NP_646871), *Mus musculus* (MmPanK isoform 81, GenBank™ accession number NP.076281), and *E. coli* (EcCoaA, GenBank™ accession number BAB38324). SaCoaA shares 13% identity with EcCoaA and 18% identity with MmPanK1β.

**FIG. 2.** Molecular properties of SaCoaA. A, elution profile of SaCoaA from the gel filtration column. Protein-containing fractions (—are assayed for pantothenate kinase activity (●) as described under “Experimental Procedures,” and the peak corresponding to SaCoaA was identified. The retention time was characteristic for a 59-kDa globular protein (inset). B, SDS-PAGE analysis of purified SaCoaA.

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The efficacy of the pantothenamides in the presence of pantothenate supported the idea that pantothenate metabolism is the primary target of these antimetabolites. N5-Pan and N7-Pan inhibited SaCoaA activity with IC50 values of 3.5 and 4.8 μM, respectively (Fig. 5A), and were found to be both inhibitors and substrates for EcCoaA (13, 25); however, Choudhry et al. (15) reported that the pantothenamides were not substrates for SaCoaA. We reinvestigated this issue.
of N5-Pan and N7-Pan to phosphorylated products by both
SaCoaA. The two pantothenamides were incubated in Tris-HCl (100 mM, pH 7.5), glycerol (5%), and increasing concentrations of a specific inhibitor, as indicated. The IC\textsubscript{50} values for N5-Pan and N7-Pan were 3.5 and 4.8 ng, respectively (Fig. 5B). Inhibition of SaCoaA by the pantothenamides was further corroborated by metabolic labeling experiments with \[^{14}C\]acetate or a mixture of \[^3H\]labeled amino acids. N7-Pan reduced by 85% the incorporation of the label into the lipid fraction and by 25% the incorporation of the label into the protein fraction, consistent with fatty acid synthesis being the primary target for N7-Pan.

**Isolation of N7-ACP and N7-Dcp**—We next investigated the possibility that the toxicity of N7-Pan was because of the formation of an inactive ACP by the transfer of the phosphopantothenamide from butyldethia-CoA to apoACP. The N7-ACP analog would be unable to form thioester bonds and therefore fail to function in fatty acid biosynthesis. The ACP fraction was partially purified from N7-Pan-treated cells and characterized. Different forms of ACP are resolved at alkaline pH on polyacrylamide gels containing low concentrations of urea (36, 37). ACPs carrying acyl chains migrate faster than ACP, and be-

**Effect of N7-Pan on Fatty Acid Biosynthesis**—N7-Pan proved to be the most potent inhibitor of \textit{S. aureus} growth and was used in all subsequent \textit{in vivo} studies. The addition of 1.6 \textmu m N7-Pan (10× MIC) to bacterial cultures at the early exponential phase (\(A_\text{600} = 0.1–0.2\)) did not result in an abrupt inhibition of cell growth in comparison to the negative control incubated with \textit{MeSO} (data not shown). Thus, the effects of the pantothenamides were not immediate, and the detection of a substantial difference in growth was observed when the N7-Pan-treated culture at the mid-log phase was diluted into medium containing the pantothenamide (Fig. 6B). Cerulenin inhibits \textit{S. aureus} growth by blocking fatty acid biosynthesis (35), and the MIC for this compound was 25 \textmu g/ml. Unlike N7-Pan, the addition of 100 \textmu g/ml (4× MIC) of cerulenin to a growing \textit{S. aureus} culture quickly reduced the rate of bacterial replication, which ceased after 1 h of incubation (Fig. 6A). We then tested the effect of adding 15 \textmu g/ml oleate and 40 \textmu g/ml palmitate to \textit{S. aureus} cells cultured in the presence of \textit{MeSO} or 1.6 \textmu g N7-Pan or 100 \textmu g/ml cerulenin. This combination of unsaturated and saturated fatty acids, known to restore the growth to cerulenin-treated \textit{S. aureus} (32), also allowed the partial recovery of cells incubated with N7-Pan (Fig. 6). These data clearly pointed to fatty acid synthesis as the primary target for N7-Pan. This further conclusion was corroborated by metabolic labeling experiments with \[^{14}C\]acetate or a mixture of \[^3H\]labeled amino acids. N7-Pan reduced by 85% the incorporation of the label into the lipid fraction and by 25% the incorporation of the label into the protein fraction, consistent with fatty acid synthesis being the primary target for N7-Pan.

**Table I** Kinetic parameters for pantothenate, ATP, and the pantothenate analogs N5- and N7-Pan

| Substrate | \(K_m\) | \(k_{\text{cat}}\) | \(k_{\text{cat}}/K_m\) |
|-----------|-------|-------------|-----------------|
| Pantothenate | 23 | 104 | 4.5 |
| ATP | 34 | 94 | 2.8 |
| N5-Pan | 3 | 9 | 3.0 |
| N7-Pan | 8 | 25.5 | 3.2 |

**Fig. 5. Inhibition of SaCoaA by the pantothenamides.** A, inhibition of SaCoaA activity by N5-Pan (●) and N7-Pan (○). Reaction mixtures (40 \textmu l) contained Tris-HCl (100 mM, pH 7.5), glycerol (5%), \[^{14}C\]pantothenate (45 \textmu M), ATP (100 \textmu M), MgCl\textsubscript{2} (10 mM), CoaA (20 ng), and increasing concentrations of a specific inhibitor, as indicated. The IC\textsubscript{50} values for N5-Pan and N7-Pan were 3.5 and 4.8 \textmu M, respectively. B, autoradiogram of a thin layer plate showing the conversion of N5-Pan and N7-Pan to phosphorylated products by both EcCoaA and SaCoaA. The two pantothenamides were incubated in Tris-HCl (100 mM, pH 7.5) containing glycerol (5%), \[^{32}P\]ATP (250 \textmu M, specific activity 1 Ci/mmol), MgCl\textsubscript{2} (10 mM), and either EcCoaA (100 ng) or SaCoaA (60 ng) as described under “Experimental Procedures.”

using a direct assay to monitor the transfer of \[^{32}P\]phosphate from \[^{32}P\]ATP to the two pantothenate analogs in the presence of the SaCoaA. Analysis of the reaction mixtures by thin layer chromatography and autoradiography revealed the formation of phosphorylated derivatives of N5-Pan and N7-Pan with \(R_f\) values of 0.45 and 0.47, respectively (Fig. 5B). The kinetic parameters for the natural substrates and the pantothenate analogs were determined and are listed in Table I. The pantothenamides had higher affinities for SaCoaA than pantothenate and lower \(k_{\text{cat}}/K_m\) values resulting in similar \(k_{\text{cat}}/K_m\) values for all the substrates. These data showed that the pantothenamides are processed as efficiently by SaCoaA as pantothenate.
Acid synthesis. Furthermore, the detection of N7-Dcp illustrated that these compounds also interfere with $\alpha$-alanine modification of the lipoteichoic acid component of the cell wall membrane complex in \textit{S. aureus}.

**DISCUSSION**

A key finding of this study is that SaCoaA differs from all described previously pantothenate kinases (6, 9–11) in that it is refractory to feedback inhibition by CoA and/or its thioesters (Fig. 3A). SaCoaA is more closely related to the mammalian pantothenate kinases than the EcCoaA (Fig. 1), and this atypical prokaryotic isoform is also found in other bacteria including \textit{B. anthracis}. Feedback regulation of EcCoaA \textit{in vivo} is illustrated in a metabolic labeling experiment using increasing amounts of labeled $\beta$-alanine in the medium (30). An upper limit to the intracellular CoA concentration is achieved at a $\beta$-alanine concentration of 4 $\mu$M, and pantothenate accumulates pointing to restriction of CoA formation at the pantothenate kinase step. Expression of a point mutation of EcCoaA that is refractory to CoA feedback inhibition results in uncontrolled pantothenate phosphorylation (4). A similar experiment with \textit{S. aureus} (Fig. 3B) shows that it produces CoA in proportion to the input of $\beta$-alanine with no evidence for regulation at SaCoaA or other downstream steps. The rationale for this distinct molecular property of SaCoaA is understood in the context of the physiology of the organism. Eukaryotes and most bacteria contain glutathione as the major low molecular weight thiol, which, together with the NADPH-dependent glutathione reductase, constitutes the primary thiol/disulfide redox system in nature (39, 40). This system is essential for maintaining the intracellular reducing environment and protects the organism from oxidative insults by functioning in the detoxification of peroxides, epoxides, and other products of reactive oxygen. \textit{S. aureus} lacks glutathione (41). Instead, CoA is the major intracellular thiol and together with a unique CoA disulfide reductase functions as the reducing system that performs the same role as the glutathione/glutathione reductase system (42–44). The CoA concentrations in \textit{S. aureus} reach millimolar levels (42), and the lack of CoA feedback regulation in SaCoaA allows CoA levels to rise to an upper limit set by the availability of pantothenate and cysteine. These observations lead to the conclusion that the CoA levels in \textit{S. aureus} are likely to be limited by the supply of pantothenate produced by the biosynthetic pathway encoded by the \textit{panB-E} genes (2). These key differences in the regulation of the CoA biosynthetic pathway account for the differential response of \textit{S. aureus} and \textit{E. coli} to the pantothenamides in the presence of pantotheate. The simultaneous addition of inhibitors plus 50 $\mu$M pantothenate to the growth medium resulted in an upward shift of the MICs (8-fold for N5-Pan and 64-fold for N7-Pan) against \textit{S. aureus}, consistent with pantothenate competitively inhibiting the phosphorylation of the pantothenamides. The same concentration of pantothenate only results in a 2-fold shift in the MIC of N5-Pan against \textit{E. coli} (26), suggesting that the lack of feedback regulation of SaCoaA allows synthesis and accumulation of larger amounts of CoA, thus more effectively overcoming the inhibitory effect of the pantothenamides in \textit{S. aureus}.

There are multiple targets for the pantothenamides in \textit{S. aureus} and several important differences between this Gram-positive pathogen compared with the \textit{E. coli} model system (26). N7-Pan (MIC = 0.16 $\mu$M) is considerably more potent than N5-Pan (MIC = 25 $\mu$M) in \textit{S. aureus}, but the efficacy of the two compounds is reversed in \textit{E. coli} because of the export of N7-Pan from the cell via a TolC-dependent pump (26). Thus, the differences in the cell wall and the outer membrane define the selectivity of pantothenamides in Gram-positive and Gram-negative bacteria. In both systems, the pantothenamides are phosphorylated by CoaA and incorporated into CoA and ACP analogs as outlined in the pathway shown in Fig. 7B. The accumulation of N7(N5)-ACP results in the inactivation of ACP and the inhibition of fatty acid synthesis. Fatty acid synthesis is the most critical pathway blocked in both cases as shown by the ability of exogenous fatty acids to ameliorate the toxic effects of the pantothenamides (Fig. 6) (26). However, exogenous fatty acids are more effective in reversing the growth inhibition in response to the highly selective fatty acid synthesis inhibitor cerulenin than the pantothenamides (Fig. 6A) suggesting the existence of other targets in \textit{S. aureus}. Also, it is
important to emphasize that the effect of the pantothenamides on cell growth is slower than with cerulenin because the pantotenamides must be incorporated into ACP and reduce the concentration of the carrier below what is required for fatty acid synthesis. In contrast, cerulenin is a fast acting covalent modifier of the condensing enzyme step (35) and blocks fatty acid synthesis immediately at the concentrations used in our studies.

A new finding in <i>S. aureus</i> is that N7-Pan is also incorporated into Dcp (Fig. 7). Dcp is required for the biosynthesis of D-alanyl-lipoteichoic acid, a macroamphiphile component of the Gram-positive cell wall-membrane complex (23). The hydrophilic backbone of teichoic acids is made up of polymers of glycerol or ribitol joined by phosphate groups, and the extent of esterification with D-alanine determines the net anionic charge and the properties of the cell wall (23). D-Alanine is bound to the phosphopantetheiny1 prosthetic group of Dcp as a thioester (21, 22, 38), the formation of which is catalyzed by D-alanine-Dcp ligase (38). Dcp inactivation would block D-alanine incorporation into the cell wall (22, 23, 38). This biochemical pathway is critical for the functions of the cell wall related to pathogenesis (23), but in the laboratory environment the lack of D-alanine modification is not lethal. The inhibition of D-alanine incorporation into teichoic acids by inactivation of the <i>dlt</i> operon in <i>S. aureus</i> increases not only the sensitivity of this pathogen to positively charged antimicrobial peptides such as defensins but also to vancomycin (45, 46). Similarly, inactivation of the gene encoding Dcp (<i>dltC</i>) in <i>Streptococcus mutans</i> abolishes D-alanine incorporation into teichoic acid resulting in

![Atypical CoaA from S. aureus](http://www.jbc.org/)

**Fig. 7.** Mass spectrophotometry and gel electrophoretic analysis of N7-ACP and N7-Dcp. A, ES-MS spectrum of purified ACP fraction isolated from N7-Pan-treated <i>S. aureus</i>. The expected mass for N7-ACP is consistent with the peak at 8,926.58 Da, whereas the expected mass for N7-Dcp is consistent with the peak at 9,439.67 Da. Peaks at the position of normal ACP and Dcp were not observed in the N7-Pan-treated extract. Gel electrophoresis (inset) of the protein fraction showed two major low molecular weight bands that were identified as indicated by MALDI-TOF/TOF sequencing as described under “Experimental Procedures.” B, metabolism of the pantotenamides to CoA analogs and prosthetic group transfer to the carrier proteins (CP) ACP or Dcp catalyzed by AcpS. The covalent modification of ACP and Dcp with ethylatedethia-CoA (N5-CoA) or butyldethia-CoA (N7-CoA) results in inactivation of these proteins.
increased acid sensitivity (47). Another important difference between S. aureus and E. coli is the role of CoA in cellular metabolism. Maintaining the reducing environment in the cell and detoxifying reactive oxygen species is not critical for survival in the laboratory, but they are important in the survival of the bacteria in the oxidative environment within animal hosts. This idea has been suggested previously (42–44), but the importance of this redox system in pathogenicity has not been directly tested.

In summary, the pantothenamide antimetabolites are substrates for SaCoA and are incorporated into downstream targets ACP and Dcp via their CoA analogs (Fig. 7B). The proximal cause for cell growth inhibition is the blockade of fatty acid synthesis because of the accumulation of inactive ACP, but the inhibition of Δ-alanine incorporation into the cell wall because of the inactivation of Dcp and the elimination of the CoA-dependent intracellular redox system are two additional significant cellular processes that are compromised by the pantothenamides. These findings are in sharp contrast to a previous report by Choudhry et al. (15), who conclude that N7-Pan and N5-Pan are inhibitors of SaCoA but are not substrates for this enzyme. It is likely that the indirect spectrophotometric assay employed by these investigators was not sensitive enough to detect N7-Pan phosphorylation and that they did not investigate its metabolism in intact cells.
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