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Incorporation of Specific Fatty Acid Precursors During Spore Germination and Outgrowth in *Bacillus thuringiensis*

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The selective incorporation of precursors specific for individual fatty acids in germinating and outgrowing spores of *Bacillus thuringiensis* is described. The specific precursors utilized were [^{14}C]butyrate, -isobutyrate, -valerate, and -isovalerate, which were incorporated into even-numbered normal-chain isomers, even-numbered iso-isomers, odd-numbered normal-chain acids, and odd-numbered isohomologs, respectively. This preferential incorporation by *B. thuringiensis* allows the terminal carbons of specific normal and branched-chain fatty acids, contained within the cytoplasmic membrane, to be labeled with ^{14}C and, potentially, ^{13}C.

Microbial systems have been very popular for the study of membrane structure and function (5, 6, 9). Interestingly, many bacteria have fatty acids characterized by the presence of a methyl branch at the ultimate or penultimate carbon. The gram-positive bacilli are especially prominent in this regard, and, commonly 85 to 90% of their overall fatty acids are branched (9, 10). The branched fatty acids are formed in these bacilli because branched primers are used to initiate fatty acid synthesis; the enzyme acyl coenzyme A (acyl-CoA)-acyl carrier protein transacylase has a strong preference for branched CoA esters of 4-6 carbon atoms instead of acetyl-CoA (3, 9). The methyl branches usually are derived from the three branched amino acids valine, leucine, and isoleucine via transamination to their α-keto acids and oxidative decarboxylation to the branched acyl-CoA primers (9). Consequently, there are three families of branched fatty acids, iso-even, iso-odd, and anteiso-odd, common to gram-positive bacilli, depending on the amino acid from which the branched acyl-CoA primer was derived.

A key question pertains to the physiological function of these branched fatty acids. They are essential for growth. Wegner and Foster (15) demonstrated that many rumen bacteria require isobutyric acid as a growth factor and fatty acid primer. Also, Willecke and Pardee (16) isolated a mutant of *Bacillus subtilis* with a defective α-keto acid dehydrogenase that required branched fatty acids or their precursors for growth. There was little specificity regarding which branched-chain fatty acid was required. Indeed, branched fatty acids from each of the three common classes were acceptable, as were numerous unnatural branched substitutes. Straight-chain fatty acids definitely were not suitable. Kaneda (8, 9) has concluded that the ratios among the three classes of branched fatty acids found in the cytoplasmic membrane of gram-positive bacilli are determined by precursor availability.

A plausible hypothesis to explain the required presence of branched fatty acids is that they serve the same function as unsaturated fatty acids in organisms which do not synthesize branched fatty acids (16), i.e., they determine membrane fluidity. However, there is still only meager information available on how the branched fatty acids influence membrane properties. One of the more promising methods of defining the lipid-lipid and lipid-protein interactions in membranes is ^{13}C nuclear magnetic resonance (1, 13). To accomplish such goals for the branched fatty acids requires a technique to incorporate ^{13}C-enriched precursors specifically into the branched portion of the membrane fatty acids. Accordingly, we have extended our previous studies on fatty acid and membrane synthesis during spore germination and outgrowth (10, 11) to include the incorporation of four- and five-carbon ^{14}C-precursors as primers for specific fatty acids. We used ^{14}C, rather than ^{13}C, in these studies because it is relatively inexpensive and readily available and because we wanted to determine whether germinating spores of *Bacillus thuringiensis* would selectively incorporate specific primers into individual fatty acids before we utilized the more expensive ^{13}C-precursors.

Spores of *B. thuringiensis* were germinated as previously described (10, 11). Four individual pulses of 30 min were employed for each of the

specific precursors. Because the radioactive precursors were added in low concentration without accompanying carrier, the overall fatty acid composition of the cells remained unchanged. Subsequently, the fatty acids were extracted and converted to their methyl esters, and the extent of precursor incorporation into specific fatty acids was determined on a gas chromatograph interfaced to a liquid scintillation counter (10, 14). Efficiency of recovery of radioactively labeled fatty acids was about 75% using this technique.

The results obtained, although previously observed in other species, had not been obtained under conditions of differentiation before. The data in Table 1 indicate that isobutyrate is incorporated exclusively into the iso-even fatty acids, isovalerate is incorporated into the iso-odds, butyrate is incorporated into the normal-evens, and valerate is incorporated into the atypical normal-odds. As indicated by the different pulse times, incorporation of these precursors differs during germination and during outgrowth. Of course, the normal-odd fatty acids are not synthesized unless precursors such as propionate or valerate are added exogenously (9, 10). The relative proportions within each family of fatty acids, e.g., i-C_{13}, i-C_{15}, i-C_{17}, were very similar to those we observed previously (10) with radioactive acetate. Also, we very recently observed efficient incorporation of ^{14}C-isoleucine into anteiso-isomers (data not shown).

Thus, we have demonstrated that during spore germination, the C_4 and C_5 precursors used in these experiments are incorporated into specific fatty acids, as expected, and therefore this procedure has potential for labeling the terminal carbons of the fatty acids present in membrane lipids. Recently, Ingram et al. (7) suggested the use of exogenous [^{13}C]propionate to label the terminal carbons of the fatty acids in *Escherichia coli*. Likewise, we have shown that a gram-positive bacillus such as *B. thuringiensis* can be similarly employed and we believe that the use of gram-positive bacilli should offer several significant advantages over gram-negative bacteria: (i) approximately 95% of the total fatty acids are localized in the cytoplasmic membrane of gram-positive bacilli (4); (ii) it is easier to form protoplasts; (iii) propionate added to *E. coli* competes with acetate to function as a primer (2, 7) and, consequently, is diluted in the process, whereas the addition of a ^{14}C-branched amino acid or [^{14}C]propionate to *B. thuringiensis* can result in the almost exclusive production of a single branched fatty acid (L. A. Bulla, Jr., unpublished data); (iv) also, because the gram-positive bacilli do not utilize acetyl-CoA as a primer for normal-fatty acids (9), the addition of exogenous butyrate or valerate leads to the undiluted formation of the normal fatty acids; (v) because the gram-positive bacilli are relatively unspecific in their branched fatty acid requirements (12, 16), a great many atypical branched primers also can be employed, e.g. 2-methyl valerate, 2-ethyl butyrate, or trimethyl acetate (8, 16); and (vi) the membrane interactions operative during vegetative growth of gram-posi-

TABLE 1. *Specific incorporation of radioactive precursors into individual fatty acids during germination and outgrowth of B. thuringiensis*[a]

Precursor	Pulse time (min)	^{14}C-precursor incorporation (cpm)[b]												
		n-C_{11}	i-C_{12}	n-C_{12}	i-C_{13}	n-C_{13}	i-C_{14}	n-C_{14}	i-C_{15}	n-C_{15}	i-C_{16}	n-C_{16}	i-C_{17}	n-C_{17}
Butyrate	0–30			1,800				27,000				55,000		
	30–60			2,000				67,000				137,000		
	60–90			12,000				125,000				179,000		
	90–120			25,000				221,000				365,000		
Isobutyrate	0–30		1,500				23,000				25,000			
	30–60		23,000				87,000				95,000			
	60–90		35,000				138,000				153,000			
	90–120		7,200				214,000				432,000			
Valerate	0–30					18,000				82,000				
	30–60	1,100				27,000				191,000				400
	60–90	3,100				45,000				317,000				900
	90–120	5,000				76,000				482,000				1,200
Isovalerate	0–30				35,000				30,000				23,000	
	30–60				65,000				127,000				23,000	
	60–90				76,000				176,000				37,000	
	90–120				131,000				316,000				75,000	

[a] Sixty milligrams of spores per 23 ml.

[b] Sodium [1-^{14}C]butyrate, sodium [1-^{14}C]isobutyrate, and sodium [1-^{14}C]valerate = 25 μCi/23 ml; sodium [1-^{14}C]isovalerate = 10 μCi/23 ml.

tive bacilli can be contrasted to those participating in the developmental phenomena of sporulation and spore germination.

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