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Control of Fatty Acid Synthesis in Bacteria

LEONARD MINDICH

Department of Microbiology, The Public Health Research Institute of the City of New York, Inc., New York, New York 10016

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When glycerol-requiring auxotrophs of *Bacillus subtilis* are deprived of glycerol, the synthesis of fatty acids continues at an apparent rate of 20 to 50% that of supplemented cultures. The newly synthesized fatty acids are not incorporated into phospholipid and accumulate as free fatty acids. These molecules undergo a much more rapid turnover than phospholipid fatty acids, and the rate of turnover is sufficient to indicate that the rate of fatty acid synthesis in glycerol-deprived cultures is similar to that in supplemented ones. The average chain length of the free fatty acids is greater than that of the phospholipid fatty acids. Cells deprived of required amino acids also show a diminution in the apparent rate of fatty acid synthesis; however, in this case, the fatty acids accumulate in phospholipid, and no increase of the free fatty acid fraction is observed. It is argued on the basis of these findings that the control of lipid synthesis does not operate at the level of transacylation but must act on one or more of the reactions of the fatty acid synthetase.

The synthesis of fatty acids has been studied in both eukaryotic and prokaryotic organisms. Although there has been excellent progress in the elucidation of the individual steps of fatty acid synthesis, there has been rather small advancement made in determining the manner in which the process is regulated (8) both in terms of the extent of synthesis and the final composition of fatty acids. Acetyl coenzyme A (CoA) carboxylase is the rate-limiting enzyme in fatty acid synthesis in vitro, and many of the schemes for regulating fatty acid synthesis involve the regulation of the activity of this enzyme. Regulation of the rate of fatty acid synthesis can involve variations in the amount of enzyme per cell, feedback inhibition by products of fatty acid synthesis such as fatty acids or their derivatives, or modulation of activity by the allosteric interaction of other molecules with the components of the fatty acid synthetase.

Evidence is presented in this paper that the rate of fatty acid synthesis in at least two species of bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, is not regulated by feedback inhibition mechanisms involving free fatty acids, nor is the control of phospholipid synthesis at the transacylation step. We have investigated the synthesis of fatty acids in bacterial mutants that are defective in glycerol biosynthesis (10, 11). These organisms cease net phospholipid synthesis when deprived of glycerol. However, they continue to incorporate radioactivity from glucose or acetate into lipid, albeit at a diminished rate (10, 11, 16). We have found that fatty acids are made at normal rates in the absence of complex lipid formation and that the apparent diminution of the synthetic rate is due to the concurrent degradation of these fatty acids.

MATERIALS AND METHODS

Bacterial strains and media. Two strains of *B. subtilis* were used: strain 168-2, obtained from David Dubnau; and strain B42, a mutant of strain SB1 that is auxotrophic for histidine (*his*), tryptophan (*try*), and glycerol (*glyc*) and lacks glycerol phosphate dehydrogenase (*glpD*) (10). *Escherichia coli* strain GR-1, auxotrophic for glycerol, was obtained from C. F. Fox (5). Cells were cultured in a defined medium consisting of salts, phosphate buffer, and amino acids as described previously (10). Media were changed by filtering and washing cells on membrane filters and resuspending the cells in prewarmed medium.

Isolation and analysis of lipids. Lipids were extracted by the method of Bligh and Dyer (1) and subjected to thin-layer chromatography (TLC) on Brinkman silica gel plates in petroleum ether-diethyl ether-acetic acid (70:30:2), followed by autoradiography and counting of material scraped off the plates in the areas corresponding to the radioactive spots (14). Standard mixtures containing mono-, di-, and triglycerides and palmitic acid (Applied Science

Laboratories, TLC-8) or β-hydroxy, β-keto- and α-β-unsaturated fatty acids (gift of M. Pullman) were run along with radioactive samples on silica gel plates. Samples were also chromatographed on Whatman SG-81 silica gel-loaded paper in solvent 1 of Wuthier (17). Radioactive spots were cut out after autoradiography. Radioactivity from TLC plates was determined in a Beckman LS2B scintillation counter in a mixture of dioxane, napthalene, and 2,5 diphenyloxasole; radioactivity from the paper was determined in a toluene-based mixture. Fatty acids were isolated from cells by saponification as described previously (10). Methyl esters of the phospholipid fatty acids were prepared by transmethylation in methanol-benzene-sulfuric acid (87.5:12.5:2) at 60 C for 3 hr or by reaction with boron trifluoride-methanol reagent [Applied Science Laboratories, Inc. (9)] after saponification. Methyl esters of free fatty acids were prepared with the boron trifluoride-methanol reagent. Fatty acids were decarboxylated by the procedure of Brady, Bradley, and Trams (2) and Goldfine and Bloch (4). Methyl esters of fatty acids were subjected to gas chromatography, using a column of ethylene glycol-succinate (15% Hi Eff 2BP by weight on Gas Chrom P; Applied Science Laboratories) with a 6-min period at 120 C and a programmed temperature rise of 7.5 degrees per min until 170 C was reached. Chain length was determined by comparison with standard mixtures and with the known composition of *B. subtilis* fatty acids (6). Samples of the methyl esters were collected for determination of radioactivity by placing pasteur pipettes lightly plugged with cotton on the outlet of the sample column before the detector (unpublished procedure of M. Pullman). Sample recoveries were 83%.

RESULTS

Accumulation of free fatty acids during glycerol deprivation. When a culture of *B. subtilis*, auxotrophic for glycerol, is deprived of glycerol, net phospholipid synthesis ceases (10). The incorporation of radioactivity from ^{14}C-glucose into lipid is immediately depressed to a rate that is approximately 20 to 50% of that in glycerol-supplemented cultures (10). To determine the nature of the lipid in which the radioactivity was accumulating, we chromatographed samples of lipid from cells grown with ^{14}C-acetate or ^{14}C-glucose in the presence and absence of glycerol. The distribution of radioactivity in various lipids is shown in Fig. 1. It is clear from Fig. 1B that phospholipid and neutral lipids are labeled in the presence of glycerol and that only neutral lipid is labeled in the absence of glycerol. The chromatogram shown in Fig. 1A, which was developed in a less polar solvent than that used in 1B, resolves the neutral lipids and clearly indicates that the neutral lipid that is labeled in the absence of glycerol has the same R_F as palmitic acid. The material in this spot was further identified as free fatty acid on the basis of the partitioning of the radioactivity between

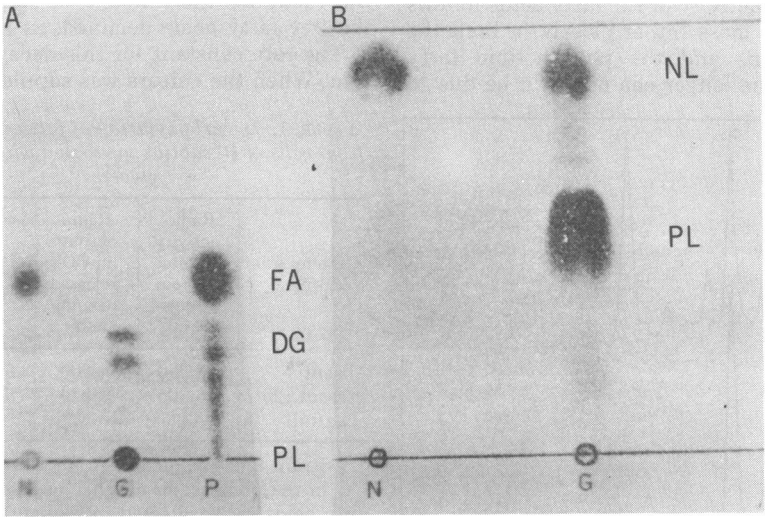

FIG. 1. *Autoradiographic patterns of B. subtilis lipids after chromatography. An exponentially growing culture of strain B42 was filtered, washed, and suspended in medium with 0.01 M sodium acetate-1-^{14}C (5 μCi/ml). One-half was incubated with glycerol for 45 min (G) and one-half without glycerol (N). The cells were then collected by centrifugation, and the lipids were extracted by the method of Bligh and Dyer (1) and chromatographed (A) on silica gel plates in petroleum ether-diethyl ether-acetic acid (70:30:2) along with ^{14}C-labeled palmitic acid (P); or (B) on Whatman SG-81 silica gel-loaded paper in the solvent of Wuthier (17). NL, neutral lipid; PL, phospholipid; FA, free fatty acid; DG, diglyceride.*

pentane and aqueous solvents at high and low *p*H and the behavior of methylated derivatives during gas-liquid chromatography. The radioactivity from deprived cells did not migrate on silica gel plates in a manner similar to unsaturated fatty acids, β-keto-fatty acids, β-hydroxy-fatty acids, monoglycerides, diglycerides, or triglycerides. The accumulation of radioactivity into various lipid classes in cells incubated in the presence or absence of glycerol was shown for *S. aureus* and *B. subtilis* in two papers (11, 16).

Lipids from the spot for the presumed free fatty acids were methylated with the boron trifluoride-methanol reagent, and the products were examined by gas-liquid chromatography. Figure 2 shows the distribution of methyl esters of the free fatty acids of a deprived culture and those from the phospholipids of a supplemented culture. The latter sample was methylated after saponification. Although the peaks in the sample from deprived cells are in the positions characteristic of methyl esters of fatty acids found in *B. subtilis*, the relative amounts are changed to a preponderance of longer-chain fatty acids. Whereas the normal lipids contain mostly C_{15} and C_{17} fatty acids, the free fatty acid fraction from the deprived cells is predominantly C_{17} and C_{19}, with a significant amount of fatty acid that might be C_{21}. The radioactivity of the fatty acids follows the pattern of mass rather closely in both the free fatty acids and the phospholipid fatty acids. Since the longer chains could be due to a lengthening of preformed chains, we determined the ratio of radioactivity in the terminal carboxyl groups of the fatty acids relative to that of the total molecules. Table 1 shows that the fraction of radioactivity in the terminal carboxyl group was approximately the same for lipids from deprived as well as supplemented cells. In fact, the ratio of the terminal to total radioactivity is close to what would be predicted on the basis of the average chain length of the free fatty acids in the deprived cells (18 carbon atoms) and of the phospholipid-associated fatty acids of the supplemented cells (16 carbon atoms), if one assumes that the bulk of these molecules are synthesized from a five-carbon precursor that is lengthened by the addition of two-carbon units (7). If the fatty acids were labeled by the addition of radioactive acetate to preformed (phospholipid-derived) fatty acids, the ratio of total radioactivity to carboxyl radioactivity would have approached 1.

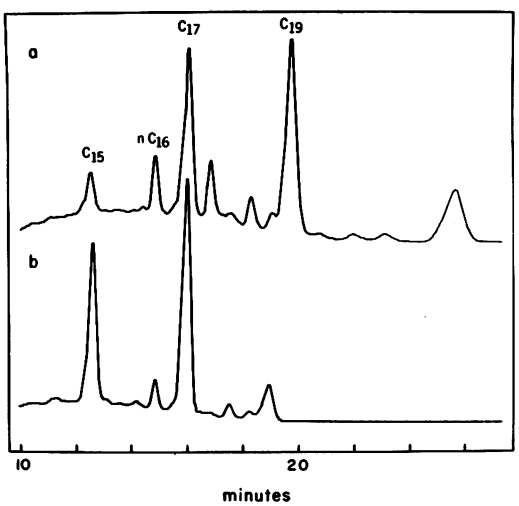

FIG. 2. *Gas-liquid chromatogram, on an ethylene glycol-succinate column, of the methyl esters of the fatty acids isolated from B. subtilis B42 phospholipids (b) and of the free fatty acids isolated from a similar culture deprived of glycerol for 45 min (a).*

Turnover of the free fatty acids. We found that the level of free fatty acid in the cell was the resultant of synthesis and breakdown of these molecules. A culture grown in the absence of glycerol for 45 min with ^{14}C-labeled acetate accumulated radioactivity in the free fatty acid fraction. If the culture was then filtered, and nonradioactive acetate was added to the medium, the amount of radioactivity in the free fatty acids declined, as shown in Fig. 3. The rate constant for this decay is 0.026 per min. When the culture was supplemented with

TABLE 1. *Decarboxylation of fatty acids isolated from cells of B. subtilis incubated with and without glycerol*[a]

Glycerol in culture	Radioactivity in total sample (counts/min)	Radioactivity in CO_2 released (counts/min)	Total/CO_2	
			Found	Expected[b]
Absent	8,270	1,200	6.89	6.5
Present (20 μg/ml)	16,365	3,029	5.40	5.5

[a] Strain B42 was incubated for 1 hr with acetate-*1*-^{14}C in the presence or absence of glycerol. The total fatty acids were collected by saponification and decarboxylated as described in the text. Total radioactivity was compared with that released as carbon dioxide.

[b] Expected on the basis of an average chain length of C_{18} for the deprived culture and C_{16} for the supplemented one. Since these fatty acids are synthesized from an initial five-carbon unit and elongated by two-carbon additions, we expect 5.5 two carbon units in the former case and 6.5 in the latter.

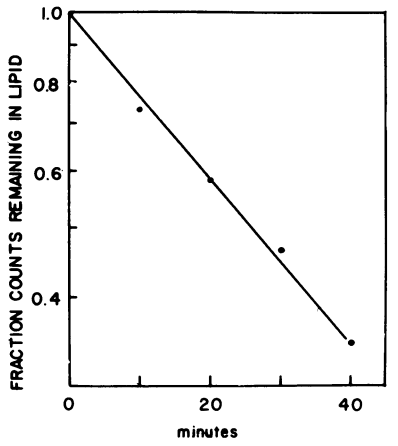

FIG. 3. *Turnover of fatty acids labeled in the absence of glycerol. A culture of B. subtilis B42 was incubated for 45 min with ^{14}C-acetate in the absence of glycerol to label free fatty acids. The cells were then washed and transferred to medium still lacking glycerol but containing 0.01 M acetate with no radioactivity. Samples were saponified, and radioactivity of the fatty acids was measured. Ordinate has a logarithmic scale.*

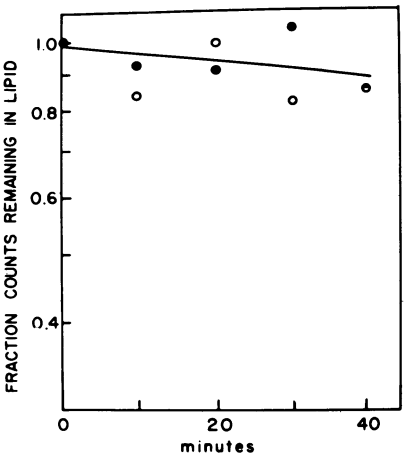

FIG. 4. *Turnover of fatty acids labeled in the presence of glycerol. A culture of B. subtilis B42 was incubated for 45 min with ^{14}C-acetate in the presence of glycerol. The cells were then washed and transferred to medium containing 0.01 M acetate with (○) or without (●) 20 μg of glycerol/ml. Samples were saponified, and radioactivity of the fatty acids was measured. Ordinate has a logarithmic scale.*

glycerol after filtration, the decline of radioactivity in the lipid fraction was less, and label was found in the phospholipid fraction of the cells. Radioactive acetate incorporated in the presence of glycerol has a very low rate of turnover during subsequent incubation in the presence or absence of glycerol (Fig. 4).

The rate of accumulation of radioactivity in the free fatty acid fraction of the deprived cells was 32 counts per min per min per ml. If the rate constant for turnover in these cells is 0.026/min and the amount of radioactivity in free fatty acids is 1,420 counts per min per ml at the time of the turnover measurement, then the rate of fatty acid turnover is 37 counts per min per min per ml. The rate of synthesis is the sum of the rate of accumulation and the rate of turnover, i.e., 69 counts per min per min per ml. The rate of accumulation is therefore 46% of the rate of synthesis. This figure agrees very well with the observed rates of fatty acid synthesis in glycerol-deprived cells relative to supplemented ones. We usually observe a rate of accumulation that is between 20 and 50% the rate of normal fatty acid synthesis.

This finding implies that the glycerol deprivation is not affecting the rate of fatty acid synthesis. The deprived cells are making fatty acids at their normal rate; however, these fatty acids are subject to breakdown and therefore accumulate at the observed low rate. We have seen the same results in glycerol auxotrophs of *S. aureus* (11). In a glycerol auxotroph of *E. coli* (5), we have found a much lower level of incorporation into free fatty acids; however, we found no such incorporation into glycerol-supplemented cells. Figure 5B shows the incorporation of ^{14}C-acetate into the free fatty acid fraction of glycerol-deprived *E. coli*, and Fig. 5A shows the incorporation into total phospholipids.

Effect of amino acid deprivation on fatty acid synthesis. It has been shown that amino acid deprivation leads to a reduction in the apparent rate of fatty acid synthesis in *E. coli* (15). We find the same result with *B. subtilis*. The fatty acids that are synthesized in the absence of required amino acids are incorporated into phospholipid and do not remain as free fatty acids; there is no increased appearance of free fatty acids during amino acid deprival. The normal level was found to be 2% of the total fatty acids both in strain B42 and in strain 168-2, which is prototrophic for glycerol. Figure 6 shows the incorporation of ^{14}C-acetate into free fatty acids and phospholipids under conditions of amino acid deprivation, glycerol deprivation, and chloramphenicol addition (70 μg/ml). One can see that glycerol deprival leads to free fatty acid accumulation, but this does not occur when protein synthesis is inhibited either by amino acid deprival or chloramphenicol. When cells are deprived of amino acids and glycerol simultaneously (*unpub-*

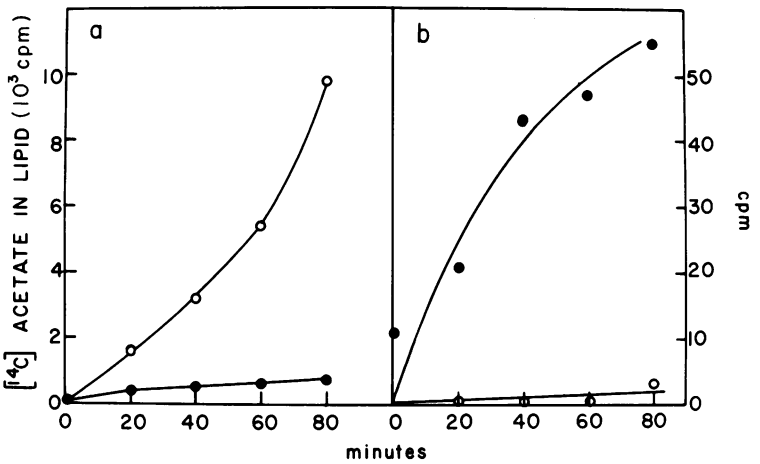

FIG. 5. *Incorporation of radioactive acetate into phospholipids and free fatty acids of E. coli strain GR-1 (5). An exponentially growing culture was filtered and resuspended in medium with 0.01 M sodium acetate-1-^{14}C (5 μCi/ml) with 20 μg of glycerol/ml (○) or without glycerol (●). Samples were taken at indicated times, and lipids were extracted and isolated by thin-layer chromatography. Accumulation of radioactivity into phospholipids is shown in (a) and into free fatty acids in (b).*

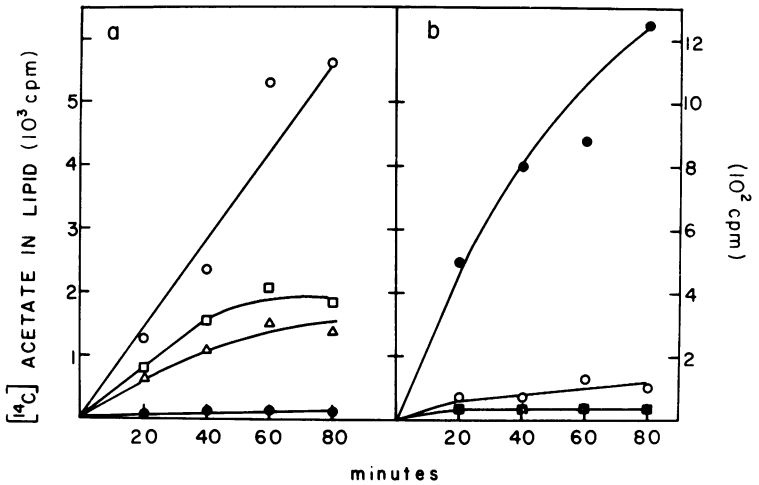

FIG. 6. *Incorporation of radioactive acetate into phospholipids and free fatty acids of B. subtilis B42 under conditions of inhibition of protein synthesis or glycerol deprivation. An exponentially growing culture was filtered and resuspended in medium with 0.01 M sodium acetate-1-^{14}C (5 μCi/ml) with amino acids and glycerol (20 μg/ml) (○), with amino acids but lacking glycerol (●), with glycerol but lacking tryptophan and histidine (□), or with glycerol, amino acids, and chloramphenicol (70 μg/ml) (△). Samples were taken at indicated times, and lipids were extracted and isolated by thin-layer chromatography. Accumulation of radioactivity into phospholipids is shown in (a) and into free fatty acids in (b).*

lished data), the rate of fatty acid accumulation does not decrease below that for glycerol deprival alone, and the fatty acids accumulate in their free form.

The inhibition of fatty acid synthesis observed after amino acid deprivation is quickly reversed by the readdition of amino acids. This is illustrated in Fig. 7, where it can be seen that, upon readdition of amino acids to a deprived culture, there is a rapid resumption of the normal rate of fatty acid synthesis.

DISCUSSION

The cell membrane is a complex and important structure. The synthesis of the membrane components must be under some sort of regu-

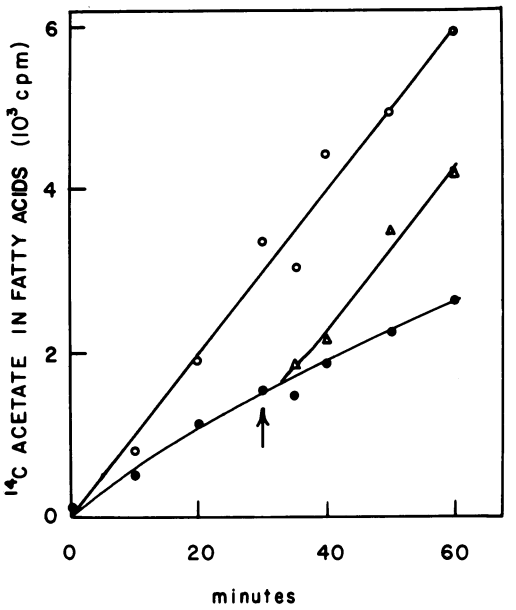

FIG. 7. *Incorporation of radioactive acetate into fatty acids of B. subtilis B42 under conditions of amino acid deprival. An exponentially growing culture was filtered and resuspended in medium with 0.01 M sodium acetate-1-^{14}C with an amino acid mixture containing tryptophan and histidine* (○) *or lacking these amino acids* (●). *After 30 min, a sample of the deprived culture was resupplemented with tryptophan and histidine* (△). *Samples were removed at the indicated times and saponified, and radioactivity of the fatty acids was measured.*

lation so as to keep the composition and quantity of the membrane within acceptable limits for the economy of the cell. We have shown previously (10, 11) that the synthesis of membrane proteins is not regulated by the progress of lipid synthesis or the lipid content of the membrane. In this paper we have demonstrated that fatty acid synthesis is not under the control of complex lipid synthesis. Glycerol auxotrophs that are deprived of glycerol and therefore are not synthesizing complex lipids accumulate free fatty acids at approximately 20 to 50% the rate of fatty acid synthesis in supplemented cultures. The actual rate of synthesis appears to be the same as that in the supplemented cultures. The fatty acids synthesized in the deprived cultures appear as free fatty acids. Ordinarily the cells have only a very small proportion of free fatty acids, about 2%; in the deprived cultures this proportion can reach as high as 20%.

Whereas the phospholipid-bound fatty acids are stable during glycerol deprivation, the pool of free fatty acids is turning over quite rapidly. The free fatty acids are also longer in chain length than the phospholipid-bound fatty acids. This supports the possibility that the determinant of chain length is the transacylase and that, in the absence of an acceptor, the chains are allowed to elongate further before being freed from the acyl carrier protein on which they are synthesized (8). It is possible that the transacylase is normally the means by which fatty acid is split from the acyl carrier protein but that the specificity is altered when water instead of glycerol phosphate is the acceptor. It is also possible that a different enzyme system is responsible for the cleavage reaction. It seems clear as a result of these findings that, at least for *B. subtilis* and *S. aureus*, neither glycerol phosphate nor a feedback mechanism (3) controls fatty acid synthesis.

The results with *E. coli* are of interest in that we find much less accumulation of free fatty acids during glycerol deprivation in this species than was found with the two gram-positive species. These results can be reconciled by several possibilities. The first would be that there is a more rapid turnover of the free fatty acids in *E. coli* than in *B. subtilis* and *S. aureus*. However, a measurement of the turnover rate in *E. coli* shows that it is not sufficient to explain the lack of accumulation. The accumulation of fatty acids would also be expected to be much greater in *E. coli* grown on glucose because of the catabolic repression of the fatty acid degradation system (13). Although more free fatty acids accumulate in *E. coli* grown with glucose as a carbon source as compared to cells grown on amino acids, the level of accumulation is still much lower than that found for the gram-positive cultures. The alternative possibilities would be that (i) the acyl carrier protein derivatives of the fatty acids are more stable in *E. coli* and the synthesis of new fatty acids is limited by the availability of acyl carrier protein; (ii) the fatty acids are synthesized by being incorporated into nonextractable compounds such as lipid A of the cell envelope (12); or (iii) a feedback control mechanism does operate on *E. coli*, and the low levels of free fatty acids observed are sufficient to prevent further synthesis.

Another difference between *B. subtilis* and *E. coli* in the control of fatty acid synthesis is the response to chloramphenicol. Sokawa et al. (15) demonstrated that stringent cultures of *E. coli* make fatty acids at a reduced rate when deprived of amino acids. They found that chloramphenicol partially reversed this effect in a manner somewhat similar to the control of

ribosomal ribonucleic acid (RNA) synthesis. We found that amino acid deprivation reduces the rate of fatty acid synthesis in *B. subtilis* B42, which is stringent for RNA synthesis, but that chloramphenicol does not reverse the effect of amino acid deprival on fatty acid synthesis although it does reverse the effects on RNA synthesis (*unpublished data*). Moreover, we found that chloramphenicol inhibits fatty acid synthesis even in the presence of amino acids. Rifamycin and actinomycin D, antibiotics which inhibit RNA synthesis and consequently protein synthesis, also inhibit fatty acid synthesis. Although it is tempting to implicate the same regulatory mechanism in both RNA and fatty acid synthesis, it appears, at least for *B. subtilis*, that although similarities exist there is not an identical control mechanism. It seems more likely that protein synthesis is necessary for normal rates of fatty acid synthesis. The observations (8) that acetyl CoA carboxylase and fatty acid synthetase show changes in level in starved animals is perhaps a similar phenomenon.

The low level of accumulation of labeled free fatty acids during amino acid deprivation of *B. subtilis* B42 is probably not the result of control at the level of the transacylase reaction, since it is clear that the free fatty acids do accumulate in this organism when glycerol is missing. The most likely means of control would therefore be a change in the levels of the fatty acid synthetase enzymes resulting from reduced rate of synthesis along with turnover or a modulation of the enzymatic activities by as yet unknown effector molecules.

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