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Constitutive Uptake and Degradation of Fatty Acids by *Yersinia pestis*

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Yersinia pestis was found to utilize palmitic acid as a primary carbon and energy source. No inhibition of growth by palmitic acid was observed. Comparison of palmitic acid uptake by cells pregrown either with or without palmitic acid demonstrated that fatty acid uptake was constitutive. High basal levels of two enzymes of β-oxidation, β-hydroxyacyl-coenzyme A dehydrogenase and thiolase, and the two enzymes of the glyoxylate shunt, isocitrate lyase and malate synthase, were found in cells grown in defined medium with glucose. Elevated levels of all four enzymes were found when cells were grown with acetate as a primary carbon and energy source, and even higher levels were observed when palmitic acid was provided as a primary carbon and energy source. High-pressure liquid chromatography was used to demonstrate that, in the presence of glucose, uniformly labeled [^{14}C]palmitic acid was converted to intermediates of the tricarboxylic acid cycle and glyoxylate shunt. Pregrowth with palmitic acid was not required for this conversion. Strains lacking the 6- or the 47-megadalton plasmid did not take up [^{3}H]palmitic acid but did possess levels of enzyme activity comparable to those observed in the wild-type strain.

Fatty acids are found in high concentrations in human serum (8) and leukocytes (7). Some pathogenic microorganisms, such as *Mycoplasma* (18, 21) and *Mycobacterium* (19), are known to require fatty acids for growth. In contrast, fatty acids have been reported to be toxic to a number of bacteria. Accordingly, the killing of bacteria by fatty acids has been proposed as a host defense mechanism (7, 16). Fatty acids were identified as an anti-*Yersinia pestis* factor found in mice and guinea pigs, with C-16 fatty acids having the greatest antibacterial effects (5). However, it is difficult to reconcile the reported fatty acid sensitivity of *Y. pestis* with the known ability of this bacterium to survive and multiply in the fatty acid-rich environment provided by the mammalian host.

Despite the reported fatty acid sensitivities, some bacteria, such as *Escherichia coli*, are capable of utilizing exogenous fatty acids as a carbon and energy source or for synthesis of membrane components or both (17). The rapid synthesis of membrane components is believed to enhance survival within phagocytes (15). The oxidation of fatty acids results in the formation of acetyl-coenzyme A (CoA), which may enter into the tricarboxylic acid (TCA) cycle or glyoxylate shunt. *E. coli*, for instance, possesses a specific uptake system for fatty acids which is coordinately controlled with the enzymes of β-oxidation (17, 20, 23) and coregulated with the enzymes of the glyoxylate bypass (13). One class of *E. coli* mutants constitutive for fatty acid uptake and degradation (*fadR*) also possess high levels of the two enzymes of the glyoxylate shunt (13). *Y. pestis* is unusual among the *Enterobacteriaceae* in that it normally maintains high levels of the glyoxylate shunt enzymes (11).

The similarity of the glyoxylate shunt enzyme activities in *Y. pestis* and *E. coli fadR* mutants suggests that *Y. pestis* might be capable of utilizing fatty acids. In view of the potential advantages of fatty acid utilization during infection, the reported toxicity of palmitic acid was reexamined, and the possibility that palmitic acid might be utilized by *Y. pestis* was tested. Our results demonstrate that palmitic acid is not toxic to *Y. pestis* and that this organism is constitutive for fatty acid uptake, possesses high basal levels of representative enzymes involved in fatty acid degradation, and can use palmitic acid as a primary carbon and energy source. Mutants lacking the 47-megadalton (Mdal) or 6-Mdal plasmid were unable to take up palmitic acid.

MATERIALS AND METHODS

Bacteria and growth conditions. *Y. pestis* EV76 (Pgm$^-$ Vwa$^+$ Fra$^+$ Pst$^+$ Cal$^+$) and a derivative of EV76 which lacks the 6-Mdal plasmid, EV76X (Pgm$^-$

Vwa$^+$ Fra$^+$ Pst$^-$ Cal$^+$), were obtained from R. R. Brubaker, Michigan State University, East Lansing. Strain EV76C1 (Pgm$^-$ Vwa$^-$ Fra$^+$ Pst$^+$ Cal$^-$), which lacks the 47-Mdal plasmid, was derived from strain EV76 in this laboratory. The *E. coli* K-12 derivative LS7070 (*fadR*) was obtained from W. D. Nunn, University of California, Irvine. Cells were grown at 26°C on a gyrating shaker bath at 200 rpm in 250-ml Erlenmeyer flasks containing 25 ml of medium and growth monitored by optical density at 620 nm (OD_{620}). A casein hydrolysate medium was prepared with N-Z amine Type A (Sheffield Products, Memphis, Tenn.) as described by Darveau et al. (4). A modification of the defined medium of Higuchi et al. was used as previously described (2, 9), with glucose as a primary carbon and energy source. This medium is referred to as defined medium with glucose. A minimal medium with the same components but with reduced levels of amino acids (10) was used when acetic or palmitic acid was used as a primary carbon and energy source. Carbon sources were sterilized separately and added to a final concentration of 1% glucose or acetic acid or 0.1 or 0.01% palmitic acid. All media incorporated 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.).

Enzyme assays. Cells grown to an OD_{620} of 1.0 to 1.4 (3.6×10^8 to 5.1×10^8 colony-forming units per ml) were collected by centrifugation, and lysates were prepared with a French pressure cell as previously described (10). Protein concentrations were determined by the binding of Coomassie brilliant blue as described by Bradford (1). Isocitrate lyase (EC 4.1.3.1) activity was determined by the method of McFadden and Howes (14). Malate synthase (EC 4.1.3.2) activity was determined by a modification of the citrate-condensing enzyme assay of Srere et al. (22). Loss of acetyl-CoA was measured after incubation for 10 min at 30°C by the addition of 5,5′-dithiobis-2-nitrobenzoate and determination of change in absorbancy at 412 nm ($E_{412} = 14.13 \times 10^3$, according to Collier [3]). β-Hydroxyacyl-CoA dehydrogenase (L-3-hydroxyacyl-CoA:NAD oxidoreductase [EC 1.1.1.35]) and thiolase (acetyl-CoA:acetyl-CoA *C*-acetyltransferase [EC 2.3.1.9]) activities were determined as described by Weeks et al. (23), except lysates were prepared in 0.1 M morpholine propane sulfonic acid buffer (pH 7.7) containing 5 mM $MgCl_2$ and 1 mM EDTA.

Palmitic acid uptake. Cells were grown in casein hydrolysate medium either with or without palmitic acid (100 μg/ml) to an OD_{620} of 0.6 to 0.8, collected by centrifugation for 5 min at $3,000 \times g$ at 26°C, washed twice in fresh medium without palmitic acid, suspended to an OD_{620} of 6.0, and then equilibrated at 26°C for 10 min. Five μCi of [9,10-^{3}H]palmitic acid (16.8 Ci/mmol in absolute ethanol-benzene [1:1]; New England Nuclear Corp., Boston, Mass.) was dried on the bottom of a 250-ml Erlenmeyer flask under dry nitrogen gas, and then 22.5 ml of filtered (0.22-μm membrane filter; Millipore Corp., Bedford, Mass.) casein hydrolysate medium was added to the flask. After equilibration at 26°C with shaking for at least 20 min, 2.5 ml of the cell suspension was added to the labeling medium, and 50-μl samples were taken immediately and at 10-min intervals. Samples were added to 2.5 ml of medium containing 100 μg of palmitic acid per ml at 4°C, and the cells were collected immediately by filtration onto 0.45-μm membrane filters (Millipore), washed twice with cold medium plus palmitic acid, placed in scintillation vials, and dried under reduced pressure for 15 min at 60°C. Samples were counted in 3a70b scintillation fluid (Research Products International, Mount Prospect, Ill.) in a refrigerated Packard Tricarb liquid scintillation spectrometer. As controls for nonspecific binding of fatty acid to membrane filters, 2.5 ml of medium without cells was added to the labeling medium, and samples were taken as described above. No significant differences were observed between these controls and the zero time samples. Uptake, expressed as nanomoles per milligram of cells (dry weight), was calculated after subtracting the nonspecific binding of [^{3}H]palmitic acid from the total activity in the samples.

Conversion of palmitic acid to TCA cycle intermediates and glyoxylate. Cells grown to an OD_{620} of 0.8 in the modified medium of Higuchi et al. containing glucose as a primary carbon and energy source were collected and washed as described above. Washed cells were suspended to a concentration of 3 mg/ml (dry weight) in the same medium, except acetic and citric acids were omitted. After equilibration for 10 min at 26°C, 1 ml of the cell suspension was added to a 15-ml Corex centrifuge tube containing 5.0 μCi of [*U*-^{14}C]palmitic acid (specific activity, 900 mCi/mmol; New England Nuclear Corp.). After 30 min of incubation at 26°C with agitation, the cells were collected by rapid centrifugation and resuspended in fresh medium, and organic acids were immediately extracted in three volumes of boiling absolute ethanol (80°C) for 3 min, followed by an additional 15 min at 50°C. Precipitates were removed by centrifugation at $10,000 \times g$ for 15 min at an ambient temperature, and the supernatant was dried at 60°C under vacuum. Extraneous lipids were removed by extraction with 50% chloroform, and phospholipids were removed by precipitation and centrifugation in 75% acetone. The supernatants were dried under vacuum at 60°C, and the residues were resuspended in glass distilled water and filtered (0.45-μm membrane filters; Millipore Corp.). The filtrates were subjected to high-pressure liquid chromatography with an Aminex HPX-87 column (Bio-Rad Laboratories, Richmond, Calif.) connected to a chromatography pump (Waters Associates, Inc., Milford, Mass.) at an average pressure of 800 lb/in^2. An 0.5 mM H_2SO_4 eluant was used at a flow rate of 0.6 ml/min. The absorbancy at 210 nm of each fraction was determined with a UV detector (Beckman Instruments, Inc., Fullerton, Calif.) coupled to an Autolab Mini-Grator (Spectra-Physics Inc., Santa Clara, Calif.). Organic acids were quantified by comparison with standards.

RESULTS

In contrast to earlier reports (5), we did not observe any adverse effects of palmitic acid on the growth of *Y. pestis*. A generation time of 1.5 h was observed for all *Y. pestis* strains during growth in casein hydrolysate medium plus 0.1% Triton X-100 or casein hydrolysate medium plus Triton X-100 and either 0.1 or 0.01% palmitic acid. Similarly, no significant difference in growth rate was observed when cells were grown in defined medium with glucose with or

without palmitic acid. *Y. pestis* EV76 grew in defined medium when palmitic acid was provided as a primary carbon and energy source.

Uptake of palmitic acid. When cells were grown for three to four generations in casein hydrolysate medium containing palmitic acid before uptake studies, [^{3}H]palmitic acid was taken up rapidly. Similar results were obtained with cells pregrown in the absence of palmitic acid. Sodium azide inhibited [^{3}H]palmitic acid uptake by greater than 95% (Fig. 1). These data suggest that *Y. pestis* has an energy-requiring constitutive uptake system for fatty acids. The apparent uptake of small quantities of palmitic acid by sodium azide-treated cultures probably represented the partition of fatty acids into cell membrane components. It is also possible that sodium azide did not completely inhibit the uptake process. Strains of *Y. pestis* lacking either the 6- or the 47-Mdal plasmid did not take up [^{3}H]palmitic acid (Fig. 1).

Activities of representative enzymes of β-oxidation and of the glyoxylate shunt. In *E. coli*, the fatty acid uptake system and the enzymes of β-oxidation and the glyoxylate shunt are apparently coregulated (12, 13). Accordingly, activities of representative enzymes of β-oxidation, β-hydroxyacyl-CoA dehydrogenase and thiolase, and the two enzymes of the glyoxylate shunt, isocitrate lyase and malate synthase, were determined for an *E. coli fadR* mutant (a repressor-minus mutant known to be constitutive for fatty acid uptake and degradation) and *Y. pestis* grown in selected media (Table 1). Cells grown in defined medium with glucose possessed measurable activities of all enzymes tested. With the exception of thiolase activity, the enzyme activities of cells grown on glucose were substantially higher in *Y. pestis* than in the *E. coli fadR* mutant. When wild-type *E. coli* K-12 was grown in defined medium with glucose, the levels of thiolase and β-hydroxyacyl-CoA dehydrogenase activity were less than 5% of those observed in the *E. coli fadR* mutant. When either *Y. pestis* or the *E. coli fadR* mutant was grown on acetic or palmitic acid as a primary carbon and energy source, the activities of all enzymes tested were observed to increase in concert. Enzyme activities of *Y. pestis* grown in defined medium with glucose were twofold lower than those observed in cells grown in minimal acetic acid medium, and the differences in the enzyme levels of cells grown in these media were more dramatic in the *E. coli fadR* mutant. The highest enzyme activities were observed with both organisms when cells were grown on palmitic acid. These data suggested that isocitrate lyase, malate synthase, β-hydroxyacyl-CoA dehydrogenase, and possibly thiolase may be coordinately regulated in *Y. pestis*. Coordinate regulation of these enzymes has been reported for *E. coli* (13). However, the dramatic increase in thiolase activity observed when *Y. pestis* was grown on palmitic acid suggests some additional control of this enzyme. Loss of either the 6- or the 47-Mdal plasmid did not significantly alter the levels of enzyme activity (Table 1).

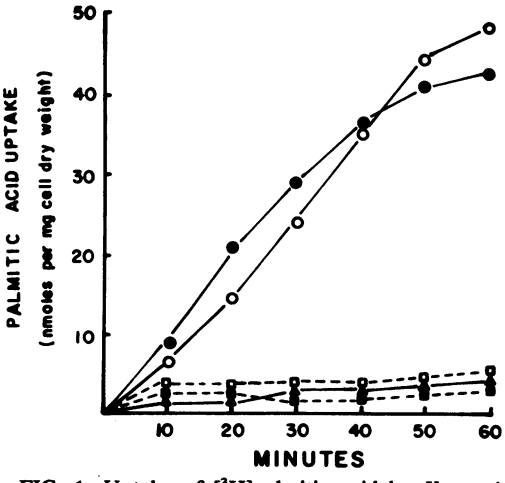

FIG. 1. Uptake of [^{3}H]palmitic acid by *Y. pestis* cells grown in casein hydrolysate medium. Cells were grown and assayed as described in the text. ●, Strain EV76 pregrown for four generations in medium containing 100 μg of palmitic acid per ml; ○, strain EV76 pregrown in the absence of palmitic acid; ▲, strain EV76 treated with 0.01% sodium azide; ■, strain EV76X; □, strain EV76C1. EV76X and EV76C1 were pregrown for four generations in medium containing 100 μg of palmitic acid per ml.

Conversion of palmitic acid to TCA cycle intermediates and glyoxylate. Strain EV76 was grown in defined medium containing glucose, concentrated, and labeled with [*U*-^{14}C]palmitic acid. After 30 min of incubation, 19% of the label was recovered in washed cells, consistent with constitutive uptake in this medium. When TCA cycle intermediates were extracted from these cells and separated by high-pressure liquid chromatography, a significant fraction of the label (5%) was found in the TCA cycle intermediates and glyoxylate (Table 2). These data are interpreted as demonstrating that, even in cells not preconditioned by growth on palmitic acid, palmitic acid is degraded to acetyl-CoA which enters the TCA cycle and glyoxylate shunt. These data also suggest that those enzymes of β-oxidation which were not assayed are present in levels sufficient to allow β-oxidation to occur.

DISCUSSION

Contrary to reports in the literature, we did not observe any toxic effects of palmitic acid when *Y. pestis* EV76 was grown in either casein hydrolysate medium or defined medium. The

TABLE 1. Specific activities of glyoxylate shunt enzymes and selected enzymes of β-oxidation

Organism	Primary carbon source[a]	Sp act (nmol/min per mg of protein) of:			Sp act (pmol/min per mg of protein) of thiolase
		Isocitrate lyase	Malate synthase	β-Hydroxyacyl-CoA dehydrogenase	
E. coli LS7070 (*fadR*)	Glucose	8	6.3	3	17
	Acetic acid	371	111.0	169	116
	Palmitic acid	471	111.0	233	259
Y. pestis EV76	Glucose	88	33.5	169	2
	Acetic acid	254	69.6	315	4
	Palmitic acid	335	122.0	1,210	8,590
	Xylose	74	25.6	161	4
Y. pestis EV76C1	Xylose	72	18.3	100	2
Y. pestis EV76X	Xylose	81	22.9	145	3

[a] Cells were cultured as described in the text or in casein hydrolysate medium containing 1% xylose.

discrepancy between our results and those of Eisler and von Metz (5) probably reflects differences in the strains or differences in the two systems used. Whereas those workers used the detergents Span 30 and Tween 80, we used Triton X-100 as a solubilizing agent in this study. In some experiments, the detergent Brij 58 was used, and results similar to those reported here were obtained. Eisler and von Metz (5) examined viability after 24 and 48 h in statically grown cultures, whereas we examined aerobically grown cells in the exponential and early stationary phases of growth (less than 24 h). It is possible that the toxic effects reported earlier were the result of a combined effect of their detergent fatty acid system or that the toxic effects of fatty acids become apparent only in older cultures or when cultures are generated under microaerophilic conditions. These possibilities were not examined.

Palmitic acid is utilized in the synthesis of cellular components (B. J. Moncla and W. T. Charnetzky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, K173, p. 166; manuscript in preparation). We have demonstrated here that *Y. pestis* is capable of using palmitic acid as a primary carbon and energy source. These observations suggest that the organism possesses an uptake system for fatty acids. Evidence for such an uptake system is shown in Fig. 1. The fatty acid uptake system found appeared to be constitutive in that pregrowth on palmitic acid was not a requirement for uptake.

TABLE 2. Incorporation of [^{14}C]palmitic acid into TCA cycle intermediates by *Y. pestis*[a]

Intermediate	Sp act (cpm/ng)
Oxaloacetic acid, ketoglutaric acid	560
Citric acid	39
Glyoxylic acid	9
Succinic acid	47
Fumaric acid	800

[a] Cells were pregrown and labeled as described in the text.

We determined the activities of enzymes which are known to be coregulated with fatty acid uptake in *E. coli*. The specific activities found for an *E. coli fadR* mutant in these experiments differ slightly from those reported previously (20), but the trends observed were essentially the same, even though media and culture conditions were varied to accommodate the growth requirements of *Y. pestis*. In defined medium with glucose, *Y. pestis* EV76 possessed significantly higher specific activities of β-hydroxyacyl-CoA dehydrogenase, isocitrate lyase, and malate synthase but lower levels of thiolase activity than the *E. coli fadR* mutant.

As expected, the levels of glyoxylate shunt enzymes, isocitrate lyase and malate synthase, were higher in both organisms when cells were grown on acetic acid than when cells were grown in defined medium with glucose. In addition, with both organisms, levels of thiolase and β-hydroxyacyl-CoA dehydrogenase activity found in cells grown on acetic acid were higher than those found in cells grown in defined medium with glucose. Since the enzymes of β-oxidation are not required during growth on acetic acid, these results are consistent with the reported coregulation of these enzymes with the glyoxylate shunt enzymes in *E. coli* (13) and with similar coregulation in *Y. pestis*.

Enzyme activities for the *E. coli fadR* mutant and *Y. pestis* grown on palmitic acid were significantly higher than those observed when cells were grown on acetic acid. However, the thiolase activity was disproportionately higher in *Y.*

pestis grown on palmitic acid. This was not observed in the *E. coli fadR* mutant. Lysates from *Y. pestis* grown on glucose or acetic acid were mixed with lysates from cells grown on palmitic acid. No evidence of activation or inhibition of thiolase activity by other components of the cell extracts was observed (data not shown). We can only speculate at this point that there may be additional control of thiolase synthesis in addition to, or in place of, those controls exerted on the synthesis of the other enzymes examined.

To confirm our observations of fatty acid uptake and degradation by this organism, we demonstrated the conversion of [*U*-^{14}C]palmitic acid to TCA cycle intermediates and glyoxylate by high-pressure liquid chromatography. These results provide clear evidence that a significant portion of the palmitic acid taken up is degraded to acetyl-CoA and metabolized through the TCA cycle and glyoxylate shunt. Implicit in these observations is a functionally complete system of β-oxidation which does not require induction by palmitic acid.

The data presented here demonstrate that *Y. pestis* is constitutive for fatty acid uptake and maintains high basal levels of the enzymes of β-oxidation. Furthermore, our data indicate that these systems are coregulated with the glyoxylate shunt enzymes. It has been shown that all strains of *Y. pestis* examined maintain high levels of the glyoxylate shunt enzyme isocitrate lyase under conditions which do not induce synthesis of this enzyme in other bacteria (11). The selective advantage of maintaining these systems at high levels is not known, but since the mammalian host provides a fatty acid-rich environment, the possibility that this characteristic enhances the virulence of this organism must be considered. Consistent with this possibility, strains lacking either the 6-Mdal plasmid, which has been associated with virulence and with the production of coagulase, fibrinolysin, and the bacteriocin pesticin (6), or the 47-Mdal plasmid, which is associated with virulence, calcium dependence, and V and W antigen production, are unable to take up fatty acids.

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