Medium- and long-chain fatty acid uptake and utilization by *Streptomyces coelicolor* A3(2): first characterization of a Gram-positive bacterial system

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The first characterization of fatty acid uptake in a Gram-positive bacterium is reported. *Streptomyces coelicolor* A3(2) utilizes fatty acids of different chain length (C_4 – C_{18}) as sole carbon and energy sources. *In vivo* β -oxidation studies and the assay of two enzymes of the β -oxidation cycle proved that fatty acid degradation is constitutive in this micro-organism. Uptake of the medium-chain fatty acid octanoate showed the characteristics of simple diffusion, whereas the uptake of palmitate, a long-chain fatty acid, occurred by both simple diffusion and active transport. After correcting for non-mediated transport, palmitate uptake measured over a wide range of concentrations followed Michaelis–Menten kinetics. The apparent K_m for palmitate was $97.8 \mu M$ and the V_{max} was $19.3 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$. Competition experiments showed specificity of the mediated transport component for long-chain fatty acids ($> C_{10}$). Metabolic inhibitors such as oligomycin, NaF and vanadate, and the ionophores gramicidin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) inhibited palmitate uptake to different degrees, consistent with the existence of an active transport mechanism. Uptake rates measured at different pH values indicated that both the ionized and the unionized forms of octanoate crossed the cytoplasmic membrane by simple diffusion. Palmitate in its ionized form appears to be transported by an active mechanism, whereas the unionized molecule diffuses through the membrane. When present in the medium, glucose stimulated the degradation of long-chain fatty acids by increasing the rate of uptake and the level of acyl-CoA synthetase.

Keywords: *Streptomyces*, fatty acids, transport, glucose stimulation, β -oxidation

INTRODUCTION

The genus *Streptomyces* consists of Gram-positive, obligately aerobic bacteria abundant in most soils. These micro-organisms exhibit a mycelial growth habit and rely for their nutrition on a variety of extracellular hydrolytic enzymes such as cellulases, hemicellulases, proteases and lipases (Korn-Wendisch & Kutzner, 1991). These proteins allow *Streptomyces* spp. to use a wide range of complex insoluble substrates such as cellulose, hemicellulose, agar, and oils as carbon

sources. While considerable data exist on these hydrolytic exoenzymes (Peczynska-Czoch & Mordarski, 1988), very little is known about the way their breakdown products enter the cell. Streptomycetes are also well able to utilize more readily available materials, such as simple sugars (Hodgson, 1982). Glucose is often preferred to other carbon sources, and as was shown in *S. coelicolor* A3(2), this preference is exerted either through repression of genes involved in the uptake of alternative carbon sources, including arabinose (Hodgson, 1982), glycerol (Smith & Chater, 1988) and galactose (Fornwald *et al.*, 1987), or by repressing the production of extracellular polysaccharide-degrading enzymes such as agarase (Hodgson, 1982; Servín-Gonzalez & Bibb, 1994), α -amylase (Virolle & Bibb,

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PMS, phenazine methosulphate.

1988) and chitinase (Miyashita *et al.*, 1991; Robbins *et al.*, 1992; Virolle & Bibb, 1988; Delic *et al.*, 1992).

Streptomyces used for industrial purposes are commonly grown on different oils and fats as carbon sources. Nevertheless no reports exist, to our knowledge, on how free fatty acids released from these substrates by the lipases (Pérez *et al.*, 1993) are imported and degraded by these micro-organisms. To study the ability of streptomyces to utilize different chain-length fatty acids as sole carbon and energy sources, and to characterize their uptake system biochemically, we have chosen *Streptomyces coelicolor* A3(2), genetically the best characterized species (Hopwood *et al.*, 1994).

METHODS

Bacterial strains, media and growth conditions. *S. coelicolor* A3(2) strains were M145 (SCP1⁺ SCP2⁻), J1501 (*bisA1 uraA1 strA1 pgl* SCP1⁺ SCP2⁻; Hopwood *et al.*, 1985) and M600 (SCP1⁺ SCP2⁻; M. Bibb, personal communication). *S. lividans* TK24 (Hopwood *et al.*, 1983), *S. glaucescens* ETH (wild-type) and *S. avermitilis* ATCC (wild-type) were kindly provided by Professor D. Hopwood (John Innes Centre, Norwich, UK). Spores were prepared on soya flour medium and stored as spore suspensions in 20% (v/v) glycerol at -20 °C. Bacteria were grown in liquid minimal medium (SMM, Takano *et al.*, 1992) or solid minimal medium MM (Hopwood *et al.*, 1985). Carbon sources were sterilized separately and added to the culture medium prior to inoculation. Fatty acids were neutralized with KOH and added at a final concentration of 0.1% in the presence of 1% (v/v) Brij 58. Glucose and other sugars were used at a final concentration of 1% (w/v).

Assay of fatty acid transport. Bacteria were grown overnight in SMM-Brij containing 0.1% oleate (C_{18:1}) and 0.5% Casamino acids at 30 °C and 250 r.p.m. Cells were harvested by centrifugation at 5000 g for 10 min at 4 °C, washed twice with SMM-Brij without Casamino acids, resuspended in the same medium, and briefly left on ice. Prior to assay, cells were preincubated for 10 min at 18 °C in a water bath, and depending on the study, 1-¹⁴C-labelled fatty acids were added to the cell suspensions.

For the uptake studies, after the addition of the 1-¹⁴C-labelled fatty acid, 0.5 ml samples were removed at different time points and rapidly filtered at room temperature through Amicon 0.45 µm pore size membrane filters. Filtered cells were washed with 5 ml SMM-Brij and put into scintillation vials containing 1 ml liquid scintillation fluid. Radioactivity in the vials was measured with a liquid scintillation counter. The binding of radiolabelled fatty acids to the filters was only just above background detectable levels.

For kinetic studies, different concentrations (from 10 to 800 µM, final concentration) of 1-¹⁴C-labelled fatty acids were added to the cell suspensions and, after 30 s, 0.5 ml samples were removed, filtered and treated as described above.

For the competition assay, cells were treated as in the uptake studies. Before the addition (to 200 µM final concentration) of labelled fatty acid, unlabelled fatty acids of different chain length were added at the same concentration and 0.5 ml samples removed after 30 s. One sample without the addition of unlabelled fatty acid was taken as a control.

In studies involving inhibitors or glucose, cells were preincubated under the conditions of transport followed by 5 min incubation in the presence of these effectors.

For studies involving pH, cells were resuspended in SMM-Brij with different buffers to obtain various pH values (4–10), followed by 10 min preincubation in the same buffer under the conditions of transport.

The results presented are the mean of at least three independent experiments.

Assay of β -oxidation. *In vivo* fatty acid degradation was monitored by determining the amount of ¹⁴CO₂ formed from 1-¹⁴C-labelled fatty acids. Cells were grown and washed as described for fatty acid transport, and then resuspended and preincubated for 10 min at 30 °C in SMM-Brij without Casamino acids and glucose. Rubber-stoppered 25 ml Erlenmeyer flasks containing centre wells with 0.2 ml 2 M NaOH to trap evolved CO₂ were used for the assay. 1-¹⁴C-labelled fatty acids were added to cell suspensions, and at different times 0.5 ml 2.5 M H₂SO₄ (for assay with long-chain fatty acid) or phosphate/citrate buffer pH 4.2 (for medium-chain fatty acid) was added to the cell suspensions to stop the reaction. Flasks were incubated for a further 60 min to ensure complete evolution and trapping of CO₂. The centre wells were removed and placed directly in scintillation vials containing 1 ml liquid scintillation fluid, and radioactivity was measured in a liquid scintillation counter. Values from control flasks lacking cells were subtracted as background for each condition assayed.

Enzyme assay. Acyl-CoA synthetase (EC 6.2.1.3) was assayed by a modification of the procedure of Tanaka *et al.* (1979). Cells were grown in SMM-oleate and SMM-glucose, and crude extracts were prepared by disruption in a sonicator. The reaction mixture contained 0.1 M Tris/HCl (pH 7.2), 1.6 mM Triton X-100, 0.15 M KCl, 10 mM MgCl₂, 10 mM ATP, 1 mM CoASH, crude extract (200 µg protein), and 0.2 µmol 1-¹⁴C-labelled fatty acid in 2 µl propylene glycol. The reaction mixtures were preincubated for 5 min at 35 °C and the reaction was initiated by the addition of crude extract. At different time points the reaction was stopped by adding 2.5 ml 2-propanol:heptane H₂SO₄ 1 M (40:20:1) and vortexing. After addition of 1 ml H₂O and 1 ml heptane, the organic phase was separated by centrifugation and removed. After three extractions the radioactivity present in the aqueous phase was measured in a liquid scintillation counter. Values from control experiments lacking crude extracts were subtracted as background for each condition assayed.

Acyl-CoA dehydrogenase (EC 1.3.99.3) activity was measured by a change in the A₅₃₀ of formazan dye. The reaction mixture contained 40 mM Tris/HCl buffer (pH 8.0), 0.05% (w/v) phenazine methosulphate (PMS), 0.2% (w/v) Triton X-100, 1 mM palmitoyl-CoA (Sigma), 0.4 mM nitroretetrazolium blue and cell extract, in a total volume of 0.1 ml. The reaction was started by addition of PMS solution and carried out at 37 °C. At different times the reaction was terminated by addition of 0.1 M HCl and 10% (v/v) Triton X-100, and the A₅₃₀ measured. One unit of acyl-CoA dehydrogenase activity was defined as the amount of enzyme required to oxidize 1 mmol palmitoyl-CoA min⁻¹.

RESULTS

Growth of *S. coelicolor* A3(2) on various fatty acids as sole carbon source

The ability of *S. coelicolor* A3(2) to grow on fatty acids of various chain lengths as sole carbon and energy source was studied in liquid minimal medium supplemented with different concentrations of various fatty

Table 1. Rate of β -oxidation of 300 μ M of labelled fatty acids by *S. coelicolor* M145 grown in SMM-oleate or SMM-glucose

The results are means of three independent determinations.

Carbon source	Rate of β -oxidation [nmol min ⁻¹ ml ⁻¹ (mg protein) ⁻¹]	
	[¹⁴ C]Palmitate	[¹⁴ C]Octanoate
Oleate	2.825	2.050
Glucose	4.500	1.950

acids with an even number of carbon atoms. *S. coelicolor* M145 grew on all of the fatty acids tested, from C₄ to C₁₈, with the best growth yields for most of these carbon sources at concentrations of 0.1% (w/v). Decanoate and dodecanoate, however, were toxic at these concentrations and for these fatty acids the best growth yields were obtained at 0.02% (w/v) (1.2 mM) and 0.05% (w/v) (2.5 mM) respectively. Similar observations were made in *Escherichia coli* but hexanoate and octanoate were toxic at concentrations higher than 5 mM (Nunn *et al.*, 1979). We were unable to determine the growth rates of the cultures for each of the fatty acids used, since the mycelia clumped markedly in the presence of these carbon sources, giving highly non-homogeneous cultures and preventing the accurate determination of growth parameters.

In vivo β -oxidation studies

To determine whether the β -oxidation enzyme system was constitutive or induced by fatty acids in *S. coelicolor*, we measured the rate of ¹⁴CO₂ released from labelled fatty acids incubated with M145 cultures pre-grown in SMM-oleate or in SMM-glucose (Table 1). Both fatty acids used in the assay, [¹⁴C]palmitate (C_{16:0}) and [¹⁴C]octanoate (C_{8:0}), were effectively oxidized by cells grown either in the presence or in the absence of the fatty acid. ¹⁴CO₂ was readily detected after 1 min incubation and the reaction was linear for at least 15 min under the conditions of the assay. Since ¹⁴CO₂ was detected as early as 1 min after the addition of fatty acid to glucose-grown cultures an induction of the β -

oxidation enzyme system by the fatty acid added seems unlikely. Surprisingly, the rate of palmitate β -oxidation by cultures grown on glucose was almost twofold higher than that of cultures grown on oleate, as deduced from the rates of ¹⁴CO₂ released from these cultures (Table 1). β -Oxidation of octanoate was also detected in cultures growing on either SMM-oleate or SMM-glucose, but the apparent stimulation of palmitate β -oxidation by glucose-grown cultures was not observed for octanoate (Table 1).

Activities of acyl-CoA synthetase and acyl-CoA dehydrogenase

The uptake and degradation of fatty acids to single acetyl-CoA units through the fatty acid β -oxidation cycle involves five enzymes: acyl-CoA synthetase, acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase (Clark & Cronan, 1996). To determine whether the enzymes of the β -oxidation cycle were expressed constitutively in *S. coelicolor* or induced in the presence of fatty acids, acyl-CoA synthetase and acyl-CoA dehydrogenase were assayed in crude extracts prepared from oleate- or glucose-grown cultures. The results in Table 2 clearly indicate that both proteins were present constitutively in *S. coelicolor*. The specific activity of the acyl-CoA dehydrogenase was very similar in extracts prepared from glucose- or from oleate-grown cultures; however, the levels of acyl-CoA synthetase, the enzyme which activates exogenous fatty acids into metabolically active CoA thioesters, were sixfold higher in cultures grown on glucose. The increase of palmitate β -oxidation in glucose-grown cultures (Table 1) could hence be partially explained by the increase in the level of acyl-CoA synthetase activity. However, as we have not measured all of the enzymes of the cycle, we do not know whether this represents the only enzyme of the cycle that increases in the presence of glucose.

Oleate utilization is stimulated specifically by glucose in solid MM

The higher activity of acyl-CoA synthetase and the higher rates of ¹⁴CO₂ released from cultures grown in glucose led us to analyse the effect of different carbon sources on the utilization of oleate in solid MM. Fig. 1 shows that glucose, but not galactose, stimulated oleate

Table 2. Acyl-CoA synthetase and acyl-CoA dehydrogenase activity in crude protein extracts prepared from cells of *S. coelicolor* M145 grown in SMM-glucose or SMM-oleate

The results are means of three determinations $\pm$ SE.

Carbon source	Acyl-CoA synthetase (pmol min ⁻¹ ml ⁻¹)	Acyl-CoA dehydrogenase [U (mg protein) ⁻¹]
Oleate	8.3 $\pm$ 0.5	35.0 $\pm$ 0.5
Glucose	95.0 $\pm$ 1.0	40.0 $\pm$ 0.5

Fig. 1. Breakdown of oleate (clear halo) by *S. coelicolor* M145 grown in solid MM with (a) oleate, (b) oleate/glucose or (c) oleate/galactose as sole carbon source.

consumption. Several other carbon sources, including xylose, fructose, arabinose, mannitol, acetate and glycerol, were also tested, but none of them stimulated the utilization of oleate. The same results were obtained with different strains of *S. coelicolor*, e.g. 1190, M600 and J1501, indicating that it was not a strain-related phenomenon. Other *Streptomyces* species, such as *S. lividans*, *S. glaucescens* and *S. avermitilis*, could also utilize oleic acid as a sole carbon source; however, only *S. lividans*, the most closely related species to *S. coelicolor* A3(2) (Stackebrandt *et al.*, 1992) showed a stimulation of oleate consumption by glucose.

Kinetics of fatty acid uptake

The rate of uptake of [14 C]palmitate and [14 C]octanoate, as examples of long- and medium-chain fatty acids, were studied in cultures of M145 grown on SMM-oleate or SMM-glucose. The rates of uptake of both fatty acids were linear for the first 60 s at 18 °C, and during this time a negligible loss of $^{14}\text{CO}_2$ due to β -oxidation was observed. After this period of time, evolution of $^{14}\text{CO}_2$ became significant, causing a decrease in the rate of cellular accumulation of labelled fatty acids.

Once the conditions for measuring initial velocities (v_i) had been established, the kinetics of transport of long- and medium-chain fatty acids were studied by measuring the rate of uptake of palmitate or octanoate at different concentrations (10–400 μM). The rate of palmitate uptake increased with increasing substrate concentration, and no saturation was observed. Between 10 and 100 μM palmitate, its rate of uptake was directly proportional to the external fatty acid level. These linear kinetics were confirmed by a linear regression analysis. Above 100 μM the rate of uptake increased abruptly (Fig. 2a), indicating the existence of a more complex kinetics with at least two components involved in the uptake system for palmitate. Addition of 250 μM oligomycin did not affect the uptake rate at low concentrations of the fatty acid, but it severely reduced the rate of uptake at concentrations above 100 μM . Linear kinetics were observed under inhibitory conditions, exhibiting the same velocity as the first part of the profile. The fact that linear uptake was observed in the presence of oligomycin provides strong evidence that the linear component of palmitate uptake was due to diffusion. When this linear component was subtracted from the uptake occurring in the absence of the inhibitor for each palmitate concentration, saturable uptake kinetics were observed (Fig. 2a), which conformed to Michaelis–Menten kinetics. Values for the Michaelis constant (K_m) and for the maximum velocity ($V_{\max}$), obtained by using a computer-assisted non-linear regression analysis, were $K_m = 97.8 \pm 23.3 \mu\text{M}$ and $V_{\max} = 19.3 \pm 1.7 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ (means $\pm$ SE). Attempts to fit the data to the Hill equation, or a double hyperbola, were both unsuccessful.

The rate of uptake of octanoate was directly proportional to the external concentration of the fatty acid, and no saturation was observed within the concen-

Fig. 2. Effect of substrate concentration on the uptake of palmitate by *S. coelicolor* in the absence or presence of 250 μM oligomycin. (a) Uptake occurring in the presence of oligomycin (○) was subtracted from uptake under non-inhibitory conditions (●) for each palmitate concentration, to give the mediated uptake (□). (b) Effect of substrate concentration on the uptake of octanoate by *S. coelicolor* in the absence (●) or presence (■) of 250 μM oligomycin.

Fig. 3. Relative uptake of labelled palmitate by *S. coelicolor* in the presence of various other fatty acids. The rate of uptake in the absence of competitors was taken as 100%. Data are means of two replicates.

Fig. 4. Effect of substrate concentration on the uptake of palmitate in the absence or presence of glucose. Uptake of palmitate from 50–500 μM solutions was assayed with (■) or without (●) glucose (1%, w/v) under standard conditions. Values obtained under inhibitory conditions (250 μM oligomycin) were subtracted for each of the concentrations assayed.

trations assayed (Fig. 2b). The K_d obtained from the slope of the curve was 0.12. A double reciprocal plot of these uptake data passed through the origin, suggesting that octanoate uptake corresponds to simple diffusion. In agreement with this, oligomycin failed to inhibit uptake of octanoate.

To determine the specificity of the long- and medium-chain fatty acid uptake systems, we compared the effect of unlabelled fatty acids on the uptake of 200 μM [^{14}C]palmitate or [^{14}C]octanoate. Fig. 3 shows that palmitate uptake was severely inhibited by other long-chain fatty acids, and to a small extent by a C_{10} fatty acid, whereas octanoate did not compete significantly with the uptake of palmitate. On the other hand, octanoate uptake was not inhibited by any of the fatty acids tested in this assay (data not shown). These results also support the idea that long-chain fatty acids have, besides a diffusion uptake mechanism, a mediated transport system, while medium-chain fatty acids simply diffuse into the cells.

Glucose stimulation of long-chain fatty acid uptake

Since utilization of long-chain fatty acids was clearly stimulated by glucose, we studied the effect of this monosaccharide on the uptake of palmitate and octanoate. For this purpose, we measured the rate of uptake of the two labelled fatty acids at different concentrations. Cultures were grown in SMM-oleate, and uptake was assayed with cells which were resuspended and pre-incubated for 5 min in the uptake buffer with or without glucose. The uptake of palmitate was clearly stimulated (nearly twofold) in the presence of glucose (Fig. 4); this stimulation became significant only above 100 μM fatty acid, coinciding with the activation of the mediated component of the transport mechanism (Fig. 2a). The

Table 3. Effect of metabolic inhibitors on fatty acid transport

Uptake of 400 μM labelled fatty acid was assayed in the presence of increasing concentrations of several inhibitors. Data are expressed as means $\pm$ SE of three replicates.

Inhibitor	Concn	Percentage inhibition of [^{14}C]palmitate uptake
CCCP	50 μM	24.6 $\pm$ 1.0
	100 μM	40.0 $\pm$ 1.0
Valinomycin	1 μM	37.7 $\pm$ 0.5
	5 μM	59.0 $\pm$ 1.0
Gramicidin	5 μM	45.0 $\pm$ 1.0
	50 μM	43.0 $\pm$ 1.0
Oligomycin	25 μM	18.0 $\pm$ 0.5
	250 μM	78.0 $\pm$ 0.5
Vanadate	50 μM	36.0 $\pm$ 1.0
	100 μM	41.0 $\pm$ 1.0
	500 μM	50.0 $\pm$ 1.0
NaF	1 mM	64.4 $\pm$ 1.0
	10 mM	78.0 $\pm$ 1.0

only kinetic parameter that changed in the presence of glucose was the V_{max} , which increased 1.9-fold, while the K_{m} remained unchanged. When galactose was utilized instead of glucose, no stimulation in the rate of palmitate uptake was detected (data not shown), consistent with the results obtained in solid MM (Fig. 1). The rate of uptake of octanoate was not altered by the presence of glucose (data not shown), agreeing with the data for β -oxidation of this fatty acid (Table 1). The stimulatory effect of glucose on the uptake and β -oxidation of fatty acids thus seems to affect exclusively the mediated component of the long-chain fatty acid transport system.

Effect of metabolic inhibitors on fatty acid transport

The energy requirements for fatty acid transport were studied by measuring the effect of several metabolic inhibitors on the uptake of octanoate and palmitate (Table 3). Gramicidin, an ionophore which forms channels in the membrane and induces changes in the two components of the protonmotive force, produced a maximum of 45% inhibition of long-chain fatty acid uptake when used at a concentration of 5 μM . The protonophore CCCP, which depletes the protonmotive force by increasing H^+ influx and thereby inducing acidification of the cytosol, also showed an inhibitory effect on the uptake of palmitate and the percentage inhibition was similar to that found for gramicidin. Vanadate, a specific inhibitor of the membrane H^+ -ATPases, inhibited palmitate uptake at all concentrations tested. Nonetheless, even at very high concentrations of vanadate (500 μM), only 50% inhibition was obtained. The strongest inhibitory effects were seen with substances that directly reduce the internal levels of ATP: the antibiotic oligomycin (an ATP synthase

Fig. 5. (a) Uptake rates of palmitate (●) and octanoate (■) were determined by using 200 μM of each of the fatty acids at various extracellular pH values. (b) Effect of substrate concentration (50–800 μM) on the uptake of palmitate at extracellular pH 4. (c) Effect of NaF on the uptake of palmitate at different concentrations and at various extracellular pH values. Uptake of palmitate at 50 (■, □) or 400 (●, ○) μM was assayed alone (■, ●) or in the presence of 10 mM NaF (□, ○).

inhibitor), and NaF (which inhibits glycolysis): both produced a maximum of 78% inhibition. These experiments clearly indicate that the uptake of palmitate is an

active process and that ATP hydrolysis is most probably the direct driving force. Octanoate uptake, however, was not inhibited by any of these ionophores or metabolic inhibitors, consistent with a simple diffusion mechanism for this medium-chain fatty acid.

Effect of pH on fatty acid uptake

The rate of long- and medium-chain fatty acid uptake was determined at different pH values to establish whether the ionized or the unionized form of the fatty acids was transported by the *Streptomyces* uptake system. The range of pH values used was outside those over which the organism grows, but nonetheless, as shown in Fig. 5(a), there were no significant variations in the rate of uptake of octanoate within the range of pH values assayed. However, for palmitate the highest rate of uptake was obtained at pH 4, decreasing significantly at higher pH values. The fact that both long- and medium-chain fatty acids have a pK_a of 4.8 (Weast, 1973) indicates that the transported species at pH 4 corresponds to the unionized form of the fatty acid, whereas at pH 7 most of the substrate is present in its ionized (anionic) form. The kinetic studies done with octanoate at pH 7 indicated that this fatty acid was most probably transported by a simple diffusion mechanism. Therefore the similar rates of uptake for the ionized and the unionized forms of this fatty acid suggest that both species are transported by the same mechanism. Interestingly, palmitate was transported more efficiently at pH 4 at the concentrations used in this assay (200 μ M), showing an important difference in the uptake of the ionized and the unionized forms of the fatty acid. This led us to check the kinetics of palmitic acid uptake at pH 4 (Fig. 5b). Initial uptake rates of the labelled fatty acid as a function of its concentration were linear and did not show saturation kinetics at least until 800 μ M. A double reciprocal plot of these data yielded a line that passed through the origin. These results suggest that the uptake of the undissociated form of this long-chain fatty acid occurs by simple diffusion, in strong contrast with the uptake mechanism of the ionized species, which seems to be transported by both simple diffusion and an active transport mechanism (Fig. 3a). To test this hypothesis further, we measured initial rates of palmitate uptake at two different concentrations (50 and 400 μ M), at different pH values and in the presence or absence of fluoride (Fig. 5c). These experiments showed that at 50 μ M there was no important difference in the rate of uptake at different pHs, and NaF did not inhibit incorporation of the labelled fatty acid. At 400 μ M, the rates of uptake at pH 4 and 10 were not modified by the presence of fluoride, while at pH 7 uptake was clearly inhibited.

DISCUSSION

The studies presented in this paper characterize the uptake and oxidation of long- and medium-chain fatty acids by *S. coelicolor* A3(2). It is worth mentioning that the only bacterium in which fatty acid transport and

degradation have been studied in detail so far is *E. coli* (for a review see Nunn, 1986; Black & DiRusso, 1994; Clark & Cronan, 1996). Interestingly, the fatty acid system characterized in this Gram-negative bacterium seem to differ in many respects from the one we describe here.

Our studies indicate that *S. coelicolor* strain M145 can effectively utilize exogenous fatty acids of different chain length, from C_4 to C_{18} , as sole carbon and energy sources. In contrast, *E. coli* can only grow on fatty acids of 12 or more carbons and then only after a distinct lag period, required for induction of the *fad* regulon (Overath *et al.*, 1967, 1969). Medium-chain fatty acids (6–11 carbon atoms), can be utilized by *E. coli* cells when previously induced with longer-chain fatty acids, or by strains in which expression of the *fad* regulon is constitutive due to mutations in the *fadR* gene (Klein *et al.*, 1971; Simons *et al.*, 1980). In contrast, other bacteria (e.g. pseudomonads) grow on fatty acids of all chain lengths, and in these cases, fatty acids of C_6 or longer are effective inducers of the β -oxidation pathway (Sato *et al.*, 1992). Our *in vivo* β -oxidation studies in cells grown in the presence or absence of fatty acids (Table 1), and the *in vitro* assays of two enzymes of the pathway, acyl-CoA synthetase and acyl-CoA dehydrogenase (Table 2), clearly indicate that *S. coelicolor* constitutively expresses the enzymes of the β -oxidation cycle, without the need for induction by a fatty acid of any chain length. Glucose repression in *S. coelicolor* A3(2) is known to affect expression of several genes involved in the uptake and/or utilization of many alternative carbon sources (Hodgson, 1982; Fornwald *et al.*, 1987). Interestingly, our data indicate that the β -oxidation pathway in this micro-organism, instead of being repressed by glucose was, at least for long-chain fatty acids, stimulated by this metabolite. This is also an important difference from *E. coli*, in which catabolite repression of the β -oxidation enzyme system has been described (Pauli *et al.*, 1974).

Kinetic studies on the uptake of octanoate showed that the concentration dependence of this medium-chain fatty acid displayed a linear component which corresponds to a diffusion-like mechanism (Fig. 2b). The transport of palmitate showed a somewhat more complex pattern. The biphasic kinetics obtained indicated the existence of two or more components in the transport system for this fatty acid (Fig. 2a). Interestingly, the active component of palmitate transport became apparent only above concentrations of 100 μ M, where the data conformed to simple Michaelis–Menten kinetics, whereas below this concentration the incorporation was directly proportional to the external concentration of palmitate. Uptake studies done in the presence of oligomycin showed concentration-dependent kinetics, probably corresponding to diffusion. The ability of oligomycin-treated cells to β -oxidize palmitate (data not shown) strengthens the idea of true diffusion of the fatty acid into the cell rather than simple non-specific binding. From these results we hypothesize that the mediated uptake of long-chain fatty acids occurs

through a carrier protein located in the cell membrane and that a critical concentration of the substrate (100 μ M) is required for the system to become active. The linearity of the palmitate uptake observed between 10 and 100 μ M could be explained as the first step in the uptake process in which the fatty acid intercalates between the phospholipids of the membrane from where, after reaching a certain concentration, it could be translocated into the cytoplasm or the inner layer of the cytoplasmic membrane by the carrier protein. In *E. coli* an outer-membrane protein, FadL, essential for long-chain fatty acid transport, has been described (Ginsburgh *et al.*, 1984; Black *et al.*, 1985; Black, 1988). However, the exact role of this protein in the uptake of long-chain fatty acids is still unknown (Clark & Cronan, 1996).

Competition experiments with different chain-length fatty acids demonstrated specificity of the mediated transport for long-chain fatty acids, but whether this specificity corresponds to the putative carrier protein or the acyl-CoA synthetase(s), which could be different for medium or long-chain fatty acids, remains to be determined. The inhibition of palmitate uptake by several metabolic inhibitors (Table 2), demonstration of a saturable component in the uptake of this fatty acid, and the competition assays, strongly support the idea of an active component in the uptake of long-chain fatty acids.

At present there is controversy as to the mechanism and rate of transbilayer movement of fatty acids in membranes. In principle, the physical transfer of fatty acids across the cytoplasmic membrane, even for long-chain fatty acids, does not require any energy or the involvement of any protein. Kamp & Hamilton (1992) have shown that unionized long-chain fatty acids can transit a phospholipid bilayer by a flip-flop mechanism with a half-life of < 2 s. Ionized fatty acids, in contrast, cross the bilayer with a half-time of several minutes. Thus conversion of fatty acids to the CoA thioester could explain the overall unidirectional uptake. To obtain more insight into this subject we measured the rates of palmitate and octanoate uptake at various pH values. For octanoate the rate of uptake was very similar at all pH values assayed (Fig. 5a). Oligomycin and NaF did not inhibit octanoate uptake either at pH 4 (data not shown) or at pH 7 (Fig. 5b). These results and the kinetics of octanoate uptake at pH 7 lead us to speculate that, independently of the ionized state of the molecule, octanoate is transported by simple diffusion. Palmitate, on the other hand, was imported at much faster rates in its unionized form (pH 4) (Fig. 5a, b). Kinetic studies at this pH, and the use of fluoride at two different concentrations of the fatty acid and at different pHs (Fig. 5b, c), strongly indicate that the unionized form of this long-chain fatty acid enters the cell by simple diffusion. The fact that at pH 7 approximately 20% of the palmitate enters the cell by simple diffusion, even when most of the fatty acid is in its ionized form, could be explained by a change in the apparent pK_a of a fatty acid when it is bound to a membrane; the pK_a of oleic

acid in egg phosphatidylcholine vesicles is 7.6 (Hamilton & Cistola, 1986). Therefore we concluded that palmitate (and probably other long-chain fatty acids), first bind to the cytoplasmic membrane, and the fraction that becomes protonated crosses it by simple diffusion, whereas the ionized fraction is translocated by a specific carrier protein through an active mechanism.

The stimulation of long-chain fatty acid utilization by glucose seems to occur at at least two levels: (a) by increasing the rate of uptake of the fatty acid (Fig. 4a), affecting the mediated component of the uptake system; and (b) increasing the levels of at least one of the enzymes of the β -oxidation cycle, acyl-CoA synthetase, at least sixfold. The fact that *in vivo* β -oxidation of palmitate increases only twofold in the presence of glucose suggests that the uptake of the fatty acid is the rate-limiting step. However, we cannot exclude the possibility that part of the acyl-CoA formed could enter into the synthesis of membrane phospholipids or triacylglycerols, which have been recently found in various *Streptomyces* species as storage molecules (Olukoshi & Packter, 1994). In such a case the limiting step would occur at the level of other enzymes involved in the catabolic pathway of fatty acids.

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