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Acyl-CoA sensing by FasR to adjust fatty acid synthesis in *Corynebacterium glutamicum*

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ABSTRACT

Corynebacterium glutamicum, like *Mycobacterium tuberculosis*, is a member of the *Corynebacteriales*, which have linear fatty acids and as branched fatty acids the mycolic acids. We identified *accD1* and *fasA* as key genes of fatty acid synthesis, encoding the β-subunit of the acetyl-CoA carboxylase and a type-I fatty acid synthase, respectively, and observed their repression during growth on minimal medium with acetate. We also identified the transcriptional regulator FasR and its binding sites in the 5′ upstream regions of *accD1* and *fasA*. In the present work we establish by co-isolation and gel-mobility shifts oleoyl-CoA and palmitoyl-CoA as effectors of FasR, and show by DNA microarray analysis that in presence of exogeneous fatty acids *accD1* and *fasA* are repressed. These results are evidence that acyl-CoA derivatives derived from extracellular fatty acids interact with FasR to repress the genes of fatty acid synthesis. This model also explains the observed repression of *accD1* and *fasA* during growth on acetate, where apparently the known high intracellular acetyl-CoA concentration during growth on this substrate requires reduced *accD1* and *fasA* expression for fine control of de novo fatty acid synthesis. Consequently, this mechanism ensures that membrane lipid homeostasis is maintained when specific nutrients are available resulting in increased acetyl-CoA concentration, as is the case with acetate, or when fatty acids are directly available from the extracellular environment. However, the genes specific to mycolic acid synthesis, which are in part shared with linear fatty acid synthesis, are not controlled by FasR, which is in agreement with the fact that they can not be supplied from the extracellular environment but that their synthesis fully depends on a constant supply of linear fatty acid chains.

1. Introduction

The rate of fatty acid (FA) synthesis is controlled within narrow limits to ensure that the supply of membrane phospholipids exactly matches the demand. In *Escherichia coli* transcriptional regulation for two genes of FA synthesis is known in detail which are *fabA* and *fabB*, encoding the key enzymes in unsaturated FA synthesis. These genes are repressed by FabR (Feng and Cronan, 2011), which possibly controls further genes of FA synthesis (Campbell and Cronan, 2001). Unsaturated acyl-CoA or acyl carrier protein (ACP) thioesters are considered as effectors for FabR. In addition to FabR, *fabA* and *fabB* are also controlled by FadR which acts as a transcriptional activator of the two genes. FadR has a dual function and also acts as a repressor of FA degradative genes (DiRusso et al., 1999). In presence of exogenous FAs FadR is released from the operator sequences of FA degradative genes by long-chain acyl coenzyme A (acyl-CoA) thioesters generated from the added FAs and serving as effectors (van Aalten et al., 2001). In *Bacillus subtilis* FA synthesis is controlled by the repressor FapR, which controls the *fab* regulon consisting of at least six biosynthesis genes (Martinez et al., 2010). FapR does not detect long-chain acyl-CoA thioesters as endproducts of the FA synthesis pathway, but instead detects malonyl-CoA, which fuels the elongation cycle during FA synthesis. In the presence of either malonyl-CoA or malonyl-ACP binding of FapR to its target sequences is prevented thereby providing a mechanism to balance the flux over the entire FA biosynthesis pathway. In *Streptomyces coelicolor* the transcriptional regulator FasR has been identified (Arabolaza et al., 2010). FasR controls the *fabDHPF* operon of FA synthesis and possibly acts as a transcriptional activator of the genes encoding proteins of the FA synthase. Thus, a wide variety of control mechanisms are realized in so far investigated microorganisms (Parsons and Rock, 2013).

In *Corynebacterium glutamicum* and related bacteria, lipid synthesis is significantly different from that of many other organisms.

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C. glutamicum belongs to the Actinobacteria and is grouped together with bacteria such as *Nocardia* and *Mycobacterium* species in the taxon *Corynebacteriales* (Dover et al., 2007). These bacteria have a FA synthase type I (FAS-I), which is one large polypeptide of approx. 260 kDa in size catalyzing the individual steps within the repetitive series of biosynthetic reactions, and which is typically present in mammalia. Its expression is modulated in *M. tuberculosis* by the transcriptional regulator FasR, controlling expression of the *fas-acpS* operon, with *fas* encoding FAS-I and *acpS* a phosphopantetheinyl transferase (Mondino et al., 2013). In addition to FAs, these bacteria contain mycolic acids originating from two FA molecules (Gande et al., 2004; Portevin et al., 2004). In *Mycobacterium* species, one of these FA chains can be further modified to a C_{56} fatty acid synthesized by FAS-II modules, which are additionally present (Bhatt et al., 2007). These are organized in the *kas* operon consisting of *fabD-acpM-kasA-kasB-accD6*. The transcriptional regulators FadR and MabR control expression of this operon (Biswas et al., 2013; Salzman et al., 2010).

We studied FA synthesis in *C. glutamicum*, which is used to produce L-glutamate on a scale of 1.5 million tonnes per annum (Eggeling et al., 2006). Production can be effected by uneven FA synthesis (Nampoothiri et al., 2002), with the actual efflux catalyzed by the mechanosensitive channel YggB (Nakamura et al., 2007). *C. glutamicum* has two FAS-I enzymes, with the essential FAS-IA being primarily responsible to stearate and oleate synthesis, and FAS-IB mostly to that of palmitate (Radmacher et al., 2005). The substrate of the FA synthases, malonyl CoA, is synthesized in *C. glutamicum* and *M. tuberculosis* by acetyl-CoA carboxylase composed of the three polypeptides AccD1, AccBC, and AccE (Gande et al., 2004, 2007). In addition, *C. glutamicum* and *M. tuberculosis* have further carboxylase subunits of the AccD-type, with both AccD2 and AccD3 forming together with AccBC and AccE another acyl-CoA carboxylase required for the condensation of two linear FAs to the branched mycolic acids (Portevin et al., 2004).

We initiated a study on *accD1* regulation, since a growth-dependent differential expression of this gene without added exogenous FAs on defined minimal media was observed (Hüser et al., 2003; Radmacher et al., 2005). This resulted in the identification of the transcriptional regulator FasR and its operator site *fasO* in front of FA synthesis genes (Nickel et al., 2010). However, we were not yet able to present a consistent model on control of the endogeneous pathway of FA synthesis in *C. glutamicum* since in vitro the regulator FasR interacted with *fasO* but apparently not requiring an effector. The present study was undertaken to elucidate this question.

2. Materials and methods

2.1. Access to FasR and LysG proteins

The FasR protein was expressed in *E. coli* by the use of pET16b–fasR and was isolated as described (Nickel et al., 2010). To enable LysG protein isolation, the primer pair lysG_NdeI (Fw) and lysG_EcoRI (Rev) was used to amplify *lysG* (NCgl1215) from the genomic DNA of *C. glutamicum* ATCC 13032. After digestion with NdeI/EcoRI, the fragment was cloned into pET28b(+), which had been treated in the same way. The final construct possesses an N-terminal hexa-histidine tag and a thrombin cleavage site with the sequence "MGSSHHHHHHSSGLVPRGSH". The construction was verified by sequencing. LysG protein was isolated from *E. coli* Bl21(DE3) pET28b-lysG after the culture had been pregrown for 3 h at 37 °C and was then further incubated for 16 h at 25 °C in the presence of 1 mM IPTG. The protein was isolated via Ni^{2+}-chelate affinity chromatography and elution with 50 mM Soerensen phosphate buffer pH8, 300 mM NaCl, 250 mM imidazole, 0.3 mM DTT. Fractions analyzed by SDS-PAGE were pooled and concentrated using Amicon Ultra-4 30 K centrifugal filters (Millipore Corporation, Billerica, MA01821).

2.2. GC–TOF-MS analysis

About 2.5 mg protein present in a volume of 3.5 ml was extracted with 7 ml chloroform in Pyrex tubes. After extraction and centrifugation the resulting chloroform phase was recovered and dried by evaporation. The sample was redissolved in pyridine for direct GC–MS analysis, or 50 μl MeOX (20 mg ml^{-1} *O*-methylhydroxylamine in pyridine) for derivatization prior to GC–MS analysis. In the latter case, the samples were incubated for 90 min at 30 °C followed by the addition of 80 μl of MSTFA (*N*-acetyl-*N*-(trimethylsilyl)-trifluoroacetamide) and a further incubation for 90 min at 40 °C. The analysis was done on an Agilent 6890N gas chromatograph using a 30 m Varian Factor-Four VF-5ms column + 10 m guard column coupled to a Waters Micromass GCT Premier high-resolution time-of-flight mass spectrometer. The ToF MS was operated in positive electron impact [EI]+ mode at an electron energy of 70 eV, with all further details as described (Paczia et al., 2012).

2.3. Electrophoretic mobility shift assays

In order to test the binding of FasR to *fasO* the *accD1* control region was amplified in a 2-step PCR. First *accD1* was amplified with the primer pairs FL-KI-accD1-for and FL-KI-accD1-rev from genomic DNA. The product was purified from an agarose gel and used in the second PCR as a template for amplification with the primer pairs Cy3-acn-Prom-5-for and FL-KI-*accD1*-rev. The resulting product was purified using the MinElute Kit (Qiagen) and diluted in binding buffer consisting of 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA and 5% (v/v) glycerol to give a concentration of 20 μg μl^{-1}. Reactants were mixed to give final concentrations as indicated in the figures, incubated at room temperature for 30 min and then loaded onto a 15% native polyacrylamide gel. After electrophoresis, the DNA was detected using a fluorescence scanner (Typhoon Trio, GE Healthcare) at an excitation of 532 nm and an emission of 580 nm.

2.4. DNA microarray analysis

For RNA preparation, cells were pre-cultivated overnight in CGXII-glucose ± 1 mM oleate, and then used to inoculate fresh CGXII-glucose ± 1 mM oleate. The minimal medium CGXII-glucose used is as described (Eggeling and Bott, 2005). After the cultures had reached an OD_{600} of 5–6, 20 ml of the cultures was poured into ice-containing tubes precooled to −20 °C and cells were harvested by centrifugation (3 min, 4200 × *g*, 4 °C). The cell pellet was directly used for RNA isolation as described before (Nickel et al., 2010). All DNA microarray analyses were performed with custom-made DNA microarrays based on 70-mer oligonucleotides obtained from Operon Biotechnologies. We defined the experimental data as significant if the mRNA level showed an at least threefold change in at least two of three replicates and the *P*-value was ≤0.05.

3. Results

3.1. Co-isolation of fatty acid derivatives with FasR

FasR of *C. glutamicum* is a TetR-type regulator, which shows strong structural similarity to EthR from *M. tuberculosis* (Remmert et al., 2012). EthR is a repressor of *ethA*, a gene encoding a monooxygenase required for the activation of the prodrug ethionamide.