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Transcriptional and translational leakiness in expression of a fungal cyclopropane fatty acid synthase in the *Escherichia coli* pET-vector expression system

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Abstract

We expressed the putative cyclopropane synthase Cfs1 from the mushroom *Coprinopsis cinerea* and the native bacterial Cfa enzyme in *Escherichia coli* as fusion proteins with an N-terminal 10xHis-tag and a proteolytic Factor Xa site, using the commercial pET-16b vector with the T7*lac* promoter for expression. Proteomics detected both proteins with high Mascot scores via trypsin-digested peptides in total cytoplasmic protein samples from transformed BL21(DE3) bacteria, induced by IPTG (isopropyl β-D-1-thiogalactopyranoside) for T7 RNA polymerase production from theT7 phage gene 1 present in the host genome under control by the *lacUV5* promoter. In lower amounts,Cfs1 and Cfa were also found in protein samples from non-induced cells based on the known transcriptional leakiness of the pET-system in expression of T7 RNA polymerase. As a novelty, the results on Cfs1 from proteomics suggest that the sequence at the pET-16b-*cfs1* fusion point CTCGA*GGAT CC*TATG (*BamHI* cloning site in italic, native *cfs1* **ATG** start codon in bold) serves in transcripts as a minor RBS (ribosome binding site) to inadvertently initiate protein synthesis on the native *cfs1* start codon. The first peptide MPAHHHPSSSAPCVSFPSSSK of the native *Cfs1* protein was found in *E. coli* which is only possible if the normal *cfs1* ATG was recognized as the start codon in protein biosynthesis. The GAGG-stretch in the above DNA sequence with a 7-bases-aligned spacing to the native *cfs1* start codon features a partial Shine-Dalgarno (SD) sequence.

Key words: pET-vector, heterologous protein expression, basal transcription, translational leakiness, Shine Dalgarno (SD) sequence.

Introduction

Coprinopsis cinerea is an edible mushroom that is regularly used as a model species in research on *Agaricomycetes* (1). Several mutants in fruiting body development were analyzed to identify gene functions required for the fruiting process. Among, gene *cfs1* for a potential cyclopropane fatty acid (CFA) synthase was found to be essential for fruiting. When defective, the fungal mycelium on the contrary grows still normal (2). In the grown mycelial cultures of the wildtype, microscopic primary hyphal knots with multiple short stunted side branches are formed in the dark by localized intense hyphal branching. When further cultured in the dark, primary hyphal knots can transform into multicellular sclerotia which are compact round resting structures with an outer brown rind (3,4). Alternatively when receiving a light signal, the highly branched primary hyphal knots in wildtype strains can enter the fruiting pathway and convert into compact secondary hyphal knots (5,6) in which mushroom

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cap and stipe tissues will differentiate (6,7). However, a *cfs1* mutant is unable to perform this light-induced step of secondary hyphal knot formation, unless the gene defect has been complemented with a wildtype gene copy via transformation (2). In accordance, expression of the *cfs1* gene is also light-induced, together with secondary hyphal knot formation and it continues in further development (2,5).

CFA synthases are known from many bacteria (8-10). Of these, the *Escherichia coli* enzyme *Cfa* is best characterized (11-16). CFA synthases, in full enzymatic name cyclopropane-fatty-acyl-phospholipid synthases (EC 2.1.1.19), catalyze the post-synthetic transfer of a methylene group from *S*-adenosyl methionine (SAM) across *cis*-double bonds in mono-unsaturated fatty acyl chains in phospholipids, thereby replacing the double bond with a methylene bridge to generate a CFA (8-16). More specifically, *E. coli Cfa* cyclopropanizes unsaturated double bounds in C16:1 (9) and C18:1(9) fatty acid chains (in palmitoleic and *cis*-vaccenic acid, respectively) in membrane-localized phopholipids at the Δ_9 positions. Thereby, 17:0*cyclo* and 19:0*cyclo* CFAs are yielded (8,9,11-15,17), while other enzymes may only form 17:0*cyclo* CFAs (9). Cyclo propanation of unsaturated fatty acids (UFAs) alters membrane fluidity, permeability and integrity stability. This provides resistances to cells to withstand multiple physical and chemical environmental stressors such as acids, high salt, low or high temperature, and others (8,15,18-24). Manifold interests in CFAs exist then in applications in biotechnology and medicine. CFAs might be exploited in stress responses to improve microbial growth in fermentations in presence of inhibitors and under acid stress (25-27), and for better survival of probiotics (28,29). CFAs can also be target for antibiotic drugs designed against pathogens (30,31). As ingredients, they may increase the value of edible oils and of health-promoting food (32,33). Further, they serve in production of feed stocks for biofuels and lubricants (34,35).

Certain protists (30,36,37), fungi (2,38) and plants (39-42) also have genes for Cfa-like enzymes and in some instances, developmental defects by mutations have been recorded (2,37,42). *Leishmania* and plant enzymes have been reported to undergo similar cyclopropanation reactions than *E. coli* Cfa, but in part on other fatty acids (36,37). Of fungi, no such enzymatic reaction has yet been shown by experiments. In this study, we expressed *C. cinerea* Cfs1 as a 10x His-tag-fusion protein under control of a T7*lac* promoter in *E. coli*, using the native *cfa* gene in parallel as a control gene and making use of the commercial *E. coli* pET-vector expression system (43). The Cfs1 protein was abundantly expressed in the *E. coli* B strain BL21(DE3), under isopropyl β-D-1-thiogalactopyranoside (IPTG) induction of the chromosomally inserted T7 RNA polymerase gene controlled by the efficient *lac UV5* promoter. However, we also observed protein expression under non-inducing conditions and, surprisingly, expression of unfused Cfs1 suggesting coincidental translation on the transcripts from the native ATG start codon cloned downstream to a cryptic ribosome binding site (RBS). Our work reveals therefore types of leakiness in the pET system at both levels, transcription and translation.

Materials and methods

Vector constructs: As formerly reported (44,45), full-length cDNA of the *C. cinerea* wildtype gene *cfs1* subcloned in vector pBluescript KS(-) in *E. coli* DH5α was PCR-amplified with primers PETcfs1 (5' TGATA*GGTACC*T**ATG**CCGGCC CACCAC 3') and M13 forward(-20) (5' GTAAAACG ACGGCCAG 3'), and the genomic sequence of the functional *E. coli cfa* gene (GenBank NC_000913) with primers Bam.cfaF (5' CAC CGACCAGTGA TGGAG AAACT*G GATCCG***ATG** AGTTCATCGTGTATAG 3´) and Bam.cfaR (5' CTTG GCGC ACGCGTAGGAT*GGATCC*AAC ATTGAAATCGATCCAC 3´). *Bam*HI restriction sites are in italic, native start codons in bold. Amplified *Bam*HI-digested fragments were subcloned into the *Bam*HI site of the expression

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vector pET-16b. Cloning in sense direction in frame with the sequence for the 10xHis-tag (Fig. 1) resulted in sense vectors pET-16b*cfs1* and pET-16b*cfa*. Cloning in antisense direction gave antisense vectors pET-16b*cfs1*-inv and pET-16b*cfa*-inv. All constructs were transformed into the Lon- and OmpT protease-deficient *E. coli* B strain BL21(DE3) with a genomic *lacUV5* promoter-controlled T7 RNA polymerase gene as functional strain for expression in the pET-system (43). All constructs were further transformed into the protease-proficient *E. coli* K12 wildtype strain ZK126 and its mutant YYC1272 (*cfa:kan* of ZK126) with an inactivated *cfa* gene (46).

Protein production: Selected BL21(DE3) clones were cultivated for 2.5 h in 3 ml LB/amp medium (100 mg ampicillin/l LB) at 37 °C and 200 rpm to an $OD_{600\ nm}$ of 0.2. Cells were transferred into 100 ml LB/amp in 500 ml Erlenmeyer flasks for 3 h growth at 37 °C and 200 rpm until an $OD_{600\ nm}$ of ≅0.5. Cultures were split into two, with 50 ml LB/amp each in 100 ml Erlenmeyer flasks. One culture was induced by 1 mM IPTG as described in the pET-vector manual (43) and the other remained non-induced during further growth of all cultures for 2 h at 37 °C and 200 rpm up to an $OD_{600\ nm}$ of ≅1.0. Flasks were then placed onto ice for 5 min. Each one ml of cells was harvested from well-mixed cultures and centrifuged. Supernatants were discharged and the pellets drained by tapping the inversed tubes onto a paper towel to remove excess medium. Pellets were completely resuspended by mixing in 100 µl of 1x phosphate-buffered saline (PBS). Then, for lysis of cells, 100 µl of 4x SDS (sodium dodecyl sulfate) sample buffer (250 mM Tris-HCl pH 6.8, 6% SDS, 300 mM DTT (dithiothreitol), 30% glycerol, and 0.02% Bromophenol blue; Cat. No. 70607-3; Novagen, Darmstadt, Germany) was added to a sample and sonicated with a microtip until the pellet was completely dissolved. Immediately after, samples were heated for 3 min at 85°C to denature the proteins and then stored at –20°C until further use (47).

For total cytoplasmic protein isolation from acid-stressed ZK126 and YYC1272 clones, strains were cultured and incubated at pH 3.0 and pH 7.0 in acid tests as described before (46). Single colonies were picked from freshly streaked LB/amp agar plates and inoculated into 25 ml LB/amp in 100 ml Erlenmeyer flasks to grow overnight at 37 °C and 200 rpm on a shaker to reach an OD_{600nm} of 2.0. Four ml of the precultures were then transferred into 25 ml LB/amp in 250 ml Erlenmeyer flasks (4 ml preculture corresponded to an OD_{600nm} of 0.3 to 0.4 in the main culture) and incubated at 37 °C for 3 h at 200 rpm on a shaker to reach an OD_{600nm} of about 1.2 for the actual acid test of the main culture. One ml aliquots per test sample were centrifuged for 10 s at full speed and supernatants were discarded. Pellets were washed one time with LB medium (pH 7.0). One ml LB/amp at appropriate pH (control cells at pH 7.0; acid shock cells at pH 3.0 adjusted by 6N HCl) was added per sample for 1 h incubation of resuspended cells on a shaker at 200 rpm and 37 °C. Then, proteins were isolated from the samples as described above.

***Protein gelectrophoresis*:** SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed in a Multigel-Long chamber (Biometra GmbH, Göttingen, Germany) using 4 % stacking and 12 % resolving gels as described (47). Per well, 25 or 30 µl protein samples were loaded into the gels. Protein size marker #SM0431 from Fermentas (Vilnius, Lithuania) was used. Electrophoresis was first conducted at a constant current of 15 mA until the samples reached the resolving gel and then at 25 mA for migration of proteins into the resolving gels. Afterwards, gels were fixed in 12 % trichloroacetic acid (TCA) for at least 1 h, stained overnight in colloidal Coomassie solution (10 % phosphoric acid (v/v), 10 % ammonium sulfate (w/v), 0.12 % Coomassie Brilliant Blue G250 (w/v; Serva, Heidelberg, Germany) in a 4:1 water/methanol solution). Gels were washed with water until an optimal contrast for bands was achieved.

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Protein digests, mass spectrometry and Mascot protein identification. Bands of interest were cut out of gels with a razor blade and proteins within gel pieces were trypsin-digested as described before (48). Tryptic peptides were dissolved in 2 % formic acid and separated on a 12 cm capillary column (ID 180 µm) packed with 3 µm particles of Reprosil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany). Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI–MS/MS) spectra were acquired using an LC 1100 series high performance liquid chromatograph (Agilent, Waldborn, Germany) coupled to an Esquire 3000+ ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Resulting raw data were analyzed with Data-Analysis v. 3.0 software (Bruker Daltonik GmbH). Proteins were identified by searching against a database of the annotated genome of *C. cinerea* Okayama 7 #130 combined with the SwissProt database (http://us.expasy.org/sprot/) and against NCBIProt, using Mascot software (www.matrixscience.com) and as settings C-carbamidomethylation as fixed modification, M-oxidation as variable modification, peptide mass tolerance 1.2 Da, peptide charge 1+, 2+, and 3+, MS/MS tolerance 0.6 Da, and 1 missed cleavage allowed (49). Molecular weights of pure amino acid chains were calculated with ExPaSy Compute pI/Mw (https://web.expasy.org/compute_pi/).

Results and discussion.

The structure of the fusion proteins. In-frame-cloning of the complete *cfa* coding sequence of *E. coli* and of the full length *cfs1* cDNA from *C. cinerea* into the *Bam*HI-site of pET-16b should result in fusion proteins which are 26 aa (amino acids) longer (Fig. 1) and by 3.21 kDa heavier compared to the native proteins Cfa (382 aa; theoretically calculated molecular mass of 43.94 kDa) and Cfs1 (488 aa; theoretically calculated molecular mass of 54.4 kDa). The extra N-terminally added aa present a 10xHis-tag for protein purification over Ni-NTA columns (43), a

Fig. 1

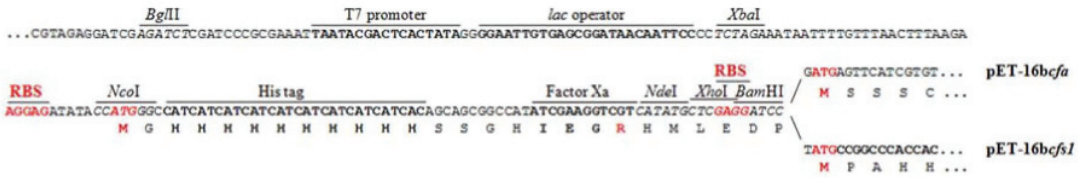

Fig. 1. Cloning region of *E. coli* gene *cfa* and *C. cinerea* gene *cfs1* in vector pET-16b. Part of the sequence of vector pET-16b (43) with marked restriction enzyme sites (sequences shown in italic), the T7 promoter for recognition by the T7 RNA polymerase followed by the *lac* operator (together known as T7*lac* promoter), the normal vector Shine-Dalgarno (SD) sequence (marked as RBS = ribosome binding site in red) followed by the vector ATG start codon (in red) for fusion protein production and the DNA sequences for the 10xHis-tag (marked in bold) and the Factor Xa recognition motif IEGR (marked in bold; residue R marked in red represents a potential enzymatic cleavage site in trypsin digests of fusion proteins) and a series of three cloning sites (for restriction enzymes *Nde*I, *Xho*I and *Bam*HI). Note the cryptic RBS overlapping the *Bam*HI cloning site (marked also in red) at an aligned seven bases distance to the native ATG start codons of the genes *cfa* and *cfs1* as cloned in pET-16b. The normal ATG start codons of genes *cfa* in pET-16b*cfa* and *cfs1* in pET-16b*cfs1* are marked in red together with the encoded first native methionine residues (M).

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Factor Xa site IEGR for proteolytic cleavage of the N-terminus after residue R (43) and the sequence HMLEDP resulting from translation of the vector sites in series for the restriction enzymes *Nde*I, *Xho*I and *Bam*HI, respectively (Fig. 1). The subsequent sequences in the fusion proteins are then those of the native enzymes, continuing protein translation from the normal ATG start codon of the genes (marked in Fig. 1 for both sense-constructs).

Protein identification after expression in* E. coli *B strain BL21(DE3). Vector-transformed *E. coli* BL21(DE3) clones were cultivated for protein isolation as described in the methods. Aliquots of all protein samples were separated by SDS-PAGE and stained by Coomassie (Fig. 2). Intensities of most protein bands in the gel were comparable between the different probes, including in the samples of clones that were transformed with the antisense constructs. In the samples with the sense-plasmids pET-16b*cfa* and pET-16b*cfs1* however, stronger blue bands were clearly visible at gel positions of around 45 kDa and 55 kDa, respectively. A slightly stronger band of corresponding size was also detectable by eye in the IPTG-non-induced sample of BL21(DE3) with the pET-16b*cfa* plasmid but not in the sample of BL21(DE3) with the pET-16b*cfs1* plasmid (Fig. 2). The theoretical masses of the native *E. coli* protein Cfa and the native *C. cinerea* protein Cfs1 corresponded roughly to the size of the two strong bands seen in Fig. 2 in the sense-vector samples induced by IPTG. The fusion proteins produced recombinantly from the pET-16b vector should, however, be slightly larger than the native proteins by the extra 3.21 kDa obtained through the N-terminal addition of the artificial sequence MGHHHHHHHHHHSSFHIEGRHMLEDP (see Fig. 1).

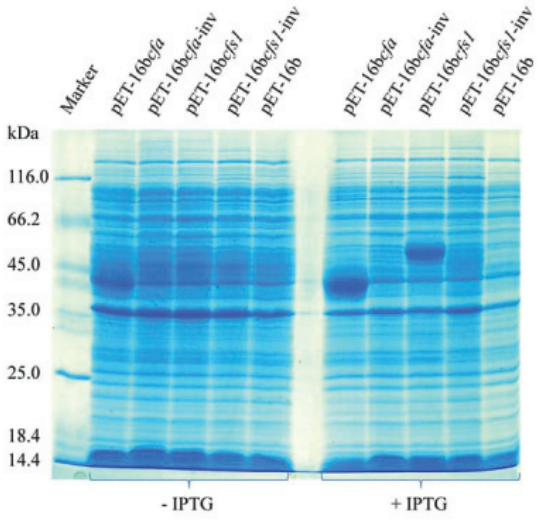

Fig. 2. 12 % SDS-PAGE gel of protein extracts from *E. coli* BL21(DE3) cells transformed with different pET-16b expression plasmids as indicated above the gel lanes. Note the strong 45 and 55 kDa bands in the IPTG-induced samples of BL21(DE3) carrying pET-16b*cfa* and pET-16b*cfs1*, respectively. Protein extracts (30 µl each) were from non-induced (-IPTG; left) and ITPG-induced cultures (+ IPTG, right).

The strong bands in the IPTG-induced samples and bands of corresponding regions from the non-induced samples were cut out of the gel and trypsin-digested for ESI-MS/MS analysis. Mascot-searches then identified proteins Cfs1 and Cfa with very high scores (768 and 296) and protein coverages (47 and 37 %) in the IPTG-induced samples but also, among other well-expressed *E. coli* proteins, with good scores (113 and 114) and protein coverages of 11 and 27 % in the non-induced samples (Table 1). The proteomics data thus clearly documented the successful expression of the heterologous *C. cinerea* protein Cfs1 from the fungal gene cloned in *E. coli*. Cfa outperformed other similar-sized proteins in abundant expression in the IPTG-induced pET-16b*cfa* sample harvested at a later logarithmic growth and was highly expressed in the non-induced sample as well (see Mascot scores and peptide numbers of best proteins in Table 1). It is of relevance that *E. coli* strain BL21(DE3) possesses a native *cfa* gene. Typically in *E. coli* cultivation, *cfa* is moderately expressed with short-lived protein products not before a strain enters

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stationary growth phases (50,51). Finding very high amounts of Cfa here in the BL21(DE3) samples suggests that the protein expression derived from the cloned *cfa* gene.

Detection of Cfs1 and Cfa in non-induced cells indicates leakiness in the pET-system in expression from the T7*lac* promoter. Similar observations of T7*lac* promoter leakiness had been reported before (43,52-57). According to the literature, leakiness in transcription depends on the *E. coli* strain, whether it has a T7 RNA polymerase gene and how well expression of the polymerase is suppressed (57). In the artificial pET-16b-system, induced expression of the T7 RNA polymerase gene is controlled by the *lacUV5* promoter via IPTG addition, but this promoter has also some basal activity in absence of IPTG (43).

Expression without a T7 RNA polymerase in* E. coli *K12 strains ZK126 and YYC1272: All sense and anti-sense pET-constructs were also transformed into wildtype *E. coli* ZK 126 and the *cfa*-mutant strain YYC1272, respectively. Cultures were grown at 37 °C as described in the method section and acid-challenged for 1 h.Then, intracellular proteins were isolated from harvested cells and separated on 12 % SDS-PAGE gels. Regardless of the *E. coli* strain, the transformed vector used, or the pH applied to the cultures, banding patterns in all samples were very similar (Fig. 3A and B). Bands of sizes of about 45 kDa and 55 kDa were excised from the gels, trypsin-digested and resulting peptides separated in mass-spec analysis for protein identification.

Mascot searches identified for the 45 kDa gel regions from the *E. coli cfa* mutant strain YYC1272 with good scores and at least two peptides per protein repeatedly: phosphopyruvate hydratase/enolase (mostly at pH 3.0), glutamate decarboxylase, elongation factor Tu (as in BL21(DE3); Table 1), isocitrate lyase (as in BL21(DE3); Table 1), NADP-dependent 6-phosphoglucanate dehydrogenase (mostly a pH 3.0), and phosphoglycerate kinase pgk (mostly at pH 7.0), and citrate synthase (mostly at pH 7.0; as in BL21 (DE3); Table 1). Proteins identified from the 55 kDa mass range included 60 kDa chaperone 1 GroEL, periplasmic oligopeptide-binding protein OppA (as in BL21 (DE3); Table 1), adhesin Ag43 (mostly at pH 3.0), PTS *N*-acetyl glucosamine transporter subunit IIABC (mostly at pH 3.0), nitrate reductase subunit beta (mostly at pH 3.0), trigger factor (mostly at pH 7.0),

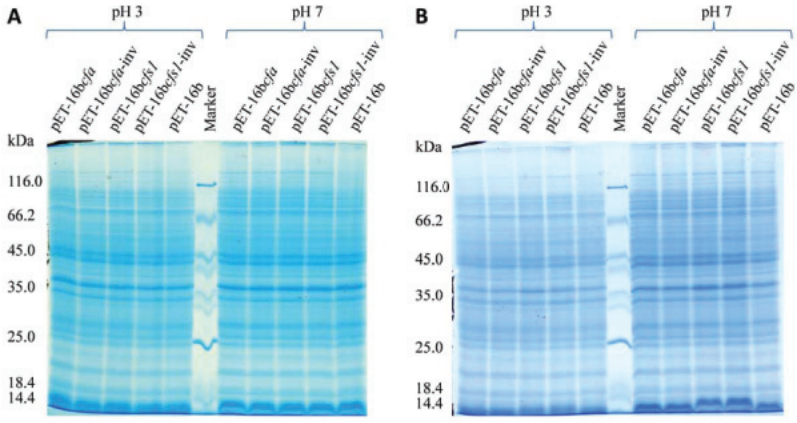

Fig. 3. 12 % SDS-PAGE gels of protein extracts from A. *E. coli* ZK126 and B. YYC1272 cells transformed with different pET-16b expression plasmids as indicated above the lanes.
Protein extracts (25 µl each) from cultures were incubated either at pH 3.0 (left) or at pH 7.0 (right).

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pyruvate kinase I (mostly at pH 3.0), trehalose-6-phosphate hydrolase TreC (mostly at pH 3.0), glyceraldehyde-3-phosphate dehydrogenase A, malate synthase A, and long-chain-fatty acid transporter FadL (mostly at pH 7.0). Several of these proteins in similar patterns of distribution were also detected in the corresponding samples taken from wildtype strain *E. coli* ZK126 cultures. However, neither *E. coli* Cfa nor *C. cinerea* Cfs1 peptides were detected in any of the bands excised from the two strains.

Since wildtype strain *E. coli* ZK126 contains a functional native *cfa* gene, it could actually have been expected to find the native acid-induced Cfa protein at least in the samples of the acid-stressed clones of this strain (46). While a range of highly expressed proteins was found in proteomics analysis of gel-excised bands, the sensitivity in detecting tryptic peptides from the bands might not have been sufficient enough to discover poorly expressed and very instable proteins. In the literature, it is reported that the *E. coli* Cfa protein is very short-lived and unstable in strain ZK126 (51). The same is probably true for Cfs1. Loos (44) formerly analyzed protein extracts from YYC1272 pET-16b*cfs1*-transformed cells in western blots with tetra-His-antibodies that recognize the 10x His-tag of pET-16b vector-encoded fusion proteins. She detected no band of the full-length Cfs1-fusion protein in SDS-PAGE separated protein extracts. Depending on IPTG, however, ladders of weak signals of smaller bands were found which indicated rapid breakdown of expressed 10xHis-tagged Cfs1 protein. In the experimental approach pursued here with a focus on bands of full-length protein sizes, any shorter degradation products of the 10xHis-tagged Cfs1 protein would not have been possible to discover.

Breakdown products of the Cfs1 fusion protein detected by western analysis in *E. coli* YYC1272 in the absence of a T7 RNA polymerase gene proofed leaky expression of Cfs1 in the strain (44). As an additional effect in the *E. coli* pET-16b vector expression system, the addition of IPTG removes the blockage of transcription by the LacI repressor bound to the *lac* operator directly downstream to the T7 promoter (Fig. 1), (43). The leaky expression of Cfs1 in *E. coli* YYC1272 then probably resulted from leaky transcription of the cloned *cfs1* gene from a pseudo-promoter for the normal *E. coli* RNA polymerase, mediated by some unidentified vector sequences upstream of the *lac* operator (44).

Translational leakiness bases on a cryptic SD sequence overlapping the* Bam*HI cloning site in the pET-16b vector: Fusion proteins produced from transcripts from the pET-vectors should all contain the N-terminally added artificial sequence MGHHHHHHHHHHSSFHIEGRHML EDP (Fig. 1). In protein digests with trypsin that cuts after R or K residues unless a proline follows (58), the 20 amino acid-long peptide MGHHHH HHHHHHSSFHIEGR might thus arise from the N-terminal fusion proteins. The short extra peptide sequence HMLEDP in contrast should remain N-terminally linked to M as the first native residue of the protein chosen for expression (Fig. 1). Accordingly, a peptide starting with the first native M should not be present in trypsin-digests of fused proteins encoded through cloning by pET-16b, unless the native start codon is somehow also recognized for translation by error. Indeed, we unexpectedly found the first peptide MPAHHHP SSSAPCVSFPSSSK of trypsin-digested Cfs1 in the gel-excised band from IPTG-stimulated B21(DE3) cells (Table 1, case '55 kDa band from IPTG-induced pET-16b*cfs1* transformed cells'), in a form as it can only come from an N-terminally non-fused Cfs1 protein.

In bacteria, ribosomes bind to specific recognition sequences, the Shine-Dalgarno (SD) motif (consensus *E. coli* AGGAGGU and motif GAGG in *E. coli* virus T4 early genes), on mRNAs and then start translation at an ATG of in average seven bases downstream to the RBS (59-61). This prompted us to search for a potential SD sequence in plasmid pET-16b*cfs1* in close vicinity upstream to the natural *cfs1* ATG start codon. In fact, the sequence at the pET-16b-*cfs1* fusion point CTCGA*GGATCC*T**ATG** (the *Bam*HI site used in

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Table 1 Mascot analysis of trypsin-digested protein bands cut from the gel shown in Fig. 2 with proteins isolated from recombinant *E. coli* BL21(DE3) cells

Hit	Protein	Mascot parameter	Sequence with peptide hits (marked in yellow)*
		45 kDa band from non-induced pET-16b*cfa* transformed cells	
1	Elongation factor Tu	Score 250 Mass 44860 Matches 20 (12) Sequences 12 (8) Coverage 39%	MLSPEGESTIVRNIAVSKEKFERTKPHVNVGTIGHVDHGK*TTLTAAITTVLA* *KTYGGAAR*AFDQIDNAPEEKARGITINTSHVEYDTPTRHYAHVDCPGHADYV KNMITGAAQMDGAILVVAATDGPMPQTREHILLGRQVGVPYIIVFLNKCDMV DDEELLELVEMEVRELLSQYDFPGDDTPIVRGSALKALEGDAEWEAKILELA GFLDSYIPEPERAIDKPFLLPIEDVFSISGRGTVVTGRVERGIIKVGEEVEI VGIKETQKSTCTGVEMFRKLLDEGRAGENVGVLLRGIKREEIERGQVLAKPG TIKPHTKFESEVYILSKDEGGRHTPFFKGYRPQFYFRTTDVTGTIELPEGVE MVMPGDNIKMVVTLIHPIAMDDGLRFAIREGGRTVGAGVVAKVLG
2	Cfa	Score 114 Mass 44337 Matches 25 (12) Sequences 9 (6) Coverage 27%	MSSSCIEEVSVPDDNWYRIANELLSRAGIAINGSAPADIRVKNPDFFKRVLQ EGSLGLGESYMDGWWECDRLDMFFSKVLRAGLENQLPHHFKDTLRIASARLF NLQSKKRAWIVGKEHYDLGNDLFSRMLDPFMQYSCAYWKDADNLESAQQAKL KMICEKLQLKPGMRVLDIGCGWGGLAHYMASNYDVSVVGVTISAEQQKMAQE RCEGLDVTILLQDYRDLNDQFDRIVSVGMFEHVGPKNYDTYFAVVDRNLKPE GIFLLHTIGSK*KTDLNVDPWINK*YIFPNGCLPSVRQIAQSSEPHFVMEDWHN FGADYDTTLMAWYERFLAAWPEIADNYSERFKRMFTYYLNACAGAFRARDIQ LWQVVFSRGVENGLRVAR
3	Citrate synthase	Score 68 Mass 48383 Matches 2 (2) Sequences 2 (2) Coverage 11%	MADTKAKLTLNGDTAVELDVLKGTLGQDVIDIRTLGSKGVFTFDPGFTSTAS CESKITFIDGDEGILLHRGFPIDQLATDSNYLEVCYILLNGEKPTQEQYDEF KTTVTRHTMIHEQITRLFHAFRRDSHPMAVMCGITGALAAFYHDSLDVNNPR HREIAAFRLLSKMPTMAAMCYKYSIGQPFVYPRNDLSYAGNFLNMMFSTPCE PYEVNPILERAMDRILILHADHEQNASTSTVRTAGSSGANPFACIAAGIASL WGPAHGGANEAALKMLEEISSVKHIPEFFRRAKDKNDSFRLMGFGHRVYKNY DPRATVMRETCHEVLKELGTKDDLLEVAMELENIALNDPYFIEKKLYPNVDF YSGIILKAMGIPSSMFTVIFAMARTVGWIAHWSEMHSDGMKIARPRQLYTGY EKRDFKSDIKR
4	Succinyl-CoA ligase	Score 49 Mass 41652 Matches 2 (1) Sequences 2 (1) Coverage 7.5 %	MNLHEYQAKQLFARYGLPAPVGYACTTPREAEEAASKIGAGPWVVKCQVHAG GRGKAGGVKVVNSKEDIRAFAENWLGKRLVTYQTDANGQPVNQILVEAATDI AKELYLGAVVDRSSRRVVFMASTEGGVEIEKVAEETPHLIHKVALDPLTGPM PYQGRELAFKLGLEGKLVQQFTKIFMGLATIFLERDLALIEINPLVITKQGD LICLDGKLGADGNALFRQPDLREMRDQSQEDPREAQAAQWELNYVALDGNIG CMVNGAGLAMGTMDIVKLHGGEPANFLDVGGGATKERVTEAFKIILSDDKVK AVLVNIFGGIVRCDLIADGIIGAVAEVGVNVPVVVRLEGNNAELGAKKLADS GLNIIAAKGLTDAAQQVVAAVEGK
		55 kDa band from non-induced pET-16b*cfs1* transformed cells	
1	Trypto-phanase	Score 121 Mass 53139 Matches 13 (8) Sequences 7 (6) Coverage 17 %	MENFKHLPEPFRIRVIEPVKRTTRAYREEAIIKSGMNPFLLDSEDVFIDLLT DSGTGAVTQSMQAAMMRGDEAYSGSRSYYALAESVKNIFGYQYTIPTHQGRG AEQIYIPVLIKKREQEKGLDRSKMVAFSNYFFDTTQGHSQINGCTVRNVYIK EAFDTGVRYDFKGNFDLEGLERGIEEVGPNNVPYIVATITSNSAGGQPVSLA NLKAMYSIAKKYDIPVVMDSAR*FAENAYFIK*QREAEYKDWTIEQITRETYKY ADMLAMSAKKDAMVPMGGLLCMKDDSFFDVYTECRTLCVVQEGFPTYGGLEG GAMERLAVGLYDGMNLDWLAYRIAQVQYLVDGLEEIGVVCQQAGGHAAFVDA GKLLPHIPADQFPAQALACELYKVAGIRAVEIGSFLLGRDPKTGKQLPCPAE LLRLTIPRATYTQTHMDFIIEAFKHVKENAANIKGLTFTYEPKVLRHFTAKL KEV
2	ATP synthase sununit beta	Score 113 Mass 50308 Matches 10 (5) Sequences 5 (3) Coverage 17 %	MATGKIVQVIGAVVDVEFPQDAVPRVYDALEVQNGNERLVLEVQQQLGGGIV RTIAMGSSDGLRRGLDVKDLEHPIEVPVGKATLGRIMNVLGEPVDMKGEIGE EERWAIHRAAPSYEELSNSQELLETGIKVIDLMCPFAKGGKVGLFGGAGVGK TVNMMELIRNIAIEHSGYSVFAGVGERTREGNDFYHEMTDSNVIDKVSLVYG QMNEPPGNRLRVALTGLTMAEKFRDEGRDVLLFVDNIYRYTLAGTEVSALLG RMPSAVGYQPTLAEEMGVLQERITSTKTGSITSVQAVYVPADDLTDPSPATT FAHLDATVVLSRQIASLGIYPAVDPLDSTSRQLDPLVVGQEHYDTARGVQSI LQRYQELKDIIAILGMDELSEEDKLVVARARKIQRFLSQPFFVAEVFTGSPG KYVSLKDTIRGFKGIMEGEYDHLPEQAFYMVGSIEEAVEKAKKL

* Consecutive peptides for clear distinction are alternately shown in normal and in italic letters, respectively. Overlapping peptides of different length are underlined.

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Hit	Protein	Mascot parameter	Sequence with peptide hits (marked in yellow)*
3	Cfs1	Score 113 Mass 54787 Matches 5 (5) Sequences 4 (4) Coverage 11 %	MPAHHHPSSSAPCVSFPSSSKALQSSSLLSALSPRSWTISFGKVNPTASYTD VALTYLPLARNSILAVLEDAITVGRLTISDSEGDHQYGERQPGCNDVRLRIV NDNFWMRILLSGDVGFSEAYMIGDCEVQTGLKGAMDLWLDNQSGMEMTLSST VARISSAMTALYNSFLGQTKSQARLNAIASYDQSNELFKAFLSKEMMYSCAL WGENEGGVRGDLELGPTPGDLEAAQLRKLHHVLRAARVKPGDRILEFGSGWG GLAIEAARTFGCEVDTLTLSIEQKTLAEERIAEAGLEGVIRVHLMDYREIPA EWEHAFDAFISIEMIEHVGPKYYNTYFKLVDFALKPQKAAAVITSSTFPESR YSSYQAEDFMRKYMWPNSSLPSATALITAAHTASQGRFTLQGVENHAAHYPR TLREWGRRLERNLTQELVARDYPSLKDNADYESFKRKWQYLFAYAGAGFSKG YITCHMLTFIRENDIPERCD
4	Glycerol kinase	Score 109 Mass 56480 Matches 15 (8) Sequences 8 (6) Coverage 18 %	MTEKKYIVALDQGTTSSRAVVMDHDANIISVSQREFEQIYPKPGWVEHDPME IWATQSSTLVEVLAKADISSDQIAAIGITNQRETTIVWEKETGKPIYNAIVW QCRRTAEICEHLKRDGLEDYIRSNTGLVIDPYFSGTKVKWILDHVEGSRERA HRGELLFGTVDTWLIWKMTQGRVHVTDYTNASRTMLFNIHTLDWDDKMLEVL DIPREMLPEVRRSSEVYGQTNIGGKGGTRIPISGIAGDQQAALFGQLCVKEG MAKNTYGTGCFMLMNTGEKAVKSENGLLTTIACGPTGEVNYALEGAVFMAGA SIQWLRDEMKLINDAYDSEYFATKVQNTNGVYVVPAFTGLGAPYWDPYARGA IFGLTRGVNANHIIRATLESIAYQTRDVLEAMQADSGIRLHALRVDGGAVAN NFLMQFQSDILGTRVERPEVREVTALGAAYLAGLAVGFWQNLDELQEKAVIE REFRPGIETTERNYRYAGWKKAVKRAMAWEEHDE
5	Outer membrane protein OppA	Score 94 Mass 53708 Matches 8 (5) Sequences 5 (4) Coverage 14 %	MKKLLPILIGLSLSGFSSLSQAENLMQVYQQARLSNPELRKSAADRDAAFEK INEARSPLLPQLGLGADYTYSNGYRDANGINSNATSASLQLTQSIFDMSKWR ALTLQEKAAGIQDVTYQTDQQTLILNTATAYFNVLNAIDVLSYTQAQKEAIY RQLDQTTQRFNVGLVAITDVQNARAQYDTVLANEVTARNNLDNAVEQLRQIT GNYYPELAALNVENFKTDKPQPVNALLKEAEKRNLSLLQARLSQDLAREQIR QAQDGHLPTLDLTASTGISDTSYSGSKTRGAAGTQYDDSNMGQNKVGLSFSL PIYQGGMVNSQVKQAQYNFVGASEQLESAHRSVVQTVRSSFNNINASISSIN AYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDATTTLYNAKQELANARYNYL INQLNIKSALGTLNEQDLLALNNALSKPVSTNPENVAPQTPEQNAIADGYAP DSRHQSFSKHPHALPPVTVITLSVTDDDDGASAPSERKAT
	45 kDa band from IPTG-induced pET-16b*cfa* transformed cells		
1	Cfa	Score 296 Mass 44337 Matches 33 (21) Sequences 14 (1) Coverage 37 %	MSSSCIEEVSVPDDNWYRIANELLSRAGIAINGSAPADIRVKNPDFFKRVLQ EGSLGLGESYMDGWWECDRLDMFFSKVLRAGLENQLPHHFKDTLRIASARLF NLQSKKRAWIVGKEHYDLGNDLFSRMLDPFMQYSCAYWKDADNLESAQQAKL KMICEKLQLKPGMRVLDIGCGWGGLAHYMASNYDVSVVGVTISAEQQKMAQE RCEGLDVTILLQDYRDLNDQFDRIVSVGMFEHVGPKNYDTYFAVVDRNLKPE GIFLLHTIGSKKTDLNVDPWINKYIFPNGCLPSVRQIAQSSEPHFVMEDWHN FGADYDTTLMAWYERFLAAWPEIADNYSERFKRMFTYYLNACAGAFRARDIQ LWQVVFSRGVENGLRVAR
2	Elongation factor Tu	Score 115 Mass 43427 Matches 12 (5) Sequences 6 (3) Coverage 17 %	MSKEKFERTKPHVNVGTIGHVDHGKTTLTAAITTVLAKTYGGAARAFDQIDN APEEKARGITINTSHVEYDTPTRHYAHVDCPGHADYVKNMITGAAQMDGAIL VVAATDGPMPQTREHILLGRQVGVPYIIVFLNKCDMVDDEELLELVEMEVRE LLSQYDFPGDDTPIVRGSALKALEGDAEWEAKILELAGFLDSYIPEPERAID KPFLLPIEDVFSISGRGTVVTGRVERGIIKVGEEVEIVGIKETQKSTCTGVE MFRKLLDEGRAGENVGVLLRGIKREEIERGQVLAKPGTIKPHTKFESEVYIL SKDEGGRHTPFFKGYRPQFYFRTTDVTGTIELPEGVEMVMPGDNIKMVVTLI HPIAMDDGLRFAIREGGRTVGAGVVAKVLG
	55 kDa band from IPTG-induced pET-16b*cfs1* transformed cells		
1	Cfs1	Score 768 Mass 54787 Matches 48 (35) Sequences19(15) Coverage 47 %	MPAHHHPSSSAPCVSFPSSSKALQSSSLLSALSPRSWTISFGKVNPTASYTD VALTYLPLARNSILAVLEDAITVGRLTISDSEGDHQYGERQPGCNDVRLRIV NDNFWMRILLSGDVGFSEAYMIGDCEVQTGLKGAMDLWLDNQSGMEMTLSST VARISSAMTALYNSFLGQTKSQARLNAIASYDQSNELFKAFLSKEMMYSCAL WGENEGGVRGDLELGPTPGDLEAAQLRKLHHVLRAARVKPGDRILEFGSGWG GLAIEAARTFGCEVDTLTLSIEQKTLAEERIAEAGLEGVIRVHLMDYREIPA EWEHAFDAFISIEMIEHVGPKYYNTYFKLVDFALKPQKAAAVITSSTFPESR YSSYQAEDFMRKYMWPNSSLPSATALITAAHTASQGRFTLQGVENHAAHYPR TLREWGRRLERNLTQELVARDYPSLKDNADYESFKRKWQYLFAYAGAGFSKG YITCHMLTFIRENDIPERCD

Transcriptional and translational leakiness in expression in the pET-vector system

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cloning is shown in italic; the native *cfs1* ATG start codon in bold; Fig. 1) contains a potential erroneous RBS. Within this sequence, there is a GAGG-stretch with a 7-bases-aligned spacing to the native *cfs1* start codon (as defined by alignment with the CCUCC sequence at the 3´end of the 16S rRNA (60)) which resembles a partial SD sequence. We conclude that it is this sequence that apparently can serve as a minor RBS in transcripts generated from pET-16b*cfs1*.

Translation from a downstream cryptic RBS in cloned sequences of a range of eukaryotic genes has several times been reported (62-68). As much as we deduce from searches in the literature, this work gives for the first time evidence for a cryptic RBS located upstream to a cloned eukaryotic gene in the pET-16b vector sequence.

Conclusions

In this study, the essential *C. cinerea* fruiting protein Cfs1 and the native *E. coli* Cfa protein have successfully been overexpressed in *E. coli* BL21(DE3) under IPTG induction from fusion genes cloned behind the T7*lac* promoter in the expression vector pET-16b. We further confirmed previously described transcriptional leakiness of the pET-16b expression system based on some basal transcription of the T7 RNA polymerase gene in absence of IPTG. This resulted in still good Cfs1 and Cfa expression from the fusion genes in pET-16b in absence of IPTG. Another type of transcriptional leakiness in *cfs1* expression occurred in *E. coli* YYC1272 in the presence of IPTG and the absence of T7 RNA polymerase by an unknown upstream pseudo-promoter in pET-16b for the normal *E. coli* RNA polymerase (44). To our best knowledge newly in this study, we found evidence for leakiness also in translation, mediated by a cryptic SD sequence within the polylinker of the pET-16b vector and directed towards production of the unfused Cfs1 protein from its native start codon.

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