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The Gene Cluster for Fluorometabolite Biosynthesis in *Streptomyces cattleya*: A Thioesterase Confers Resistance to Fluoroacetyl-Coenzyme A

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Summary

A genomic library of *Streptomyces cattleya* was screened to isolate a gene cluster encoding enzymes responsible for the production of fluorine-containing metabolites. In addition to the previously described fluorinase FlA which catalyzes the formation of 5′-fluoro-5′-deoxyadenosine from *S*-adenosylmethionine and fluoride, 11 other putative open reading frames have been identified. Three of the proteins encoded by these genes have been characterized. FlB was determined to be the second enzyme in the pathway, catalyzing the phosphorolytic cleavage of 5′-fluoro-5′-deoxyadenosine to produce 5-fluoro-5-deoxy-*D*-ribose-1-phosphate. The enzyme FlI was found to be an *S*-adenosylhomocysteine hydrolase, which may act to relieve *S*-adenosylhomocysteine inhibition of the fluorinase. Finally, *flK* encodes a thioesterase which catalyzes the selective breakdown of fluoroacetyl-CoA but not acetyl-CoA, suggesting that it provides the producing strain with a mechanism for resistance to fluoroacetate.

Introduction

Fluorine is the 13th commonest element in the earth's crust [1]. Despite the element's natural abundance, organically bound fluorine is relatively rare in nature. This contrasts markedly with the wide diversity of chlorine-containing natural products [2]. The apparent discrepancy between the natural abundance of fluorine and the rarity of naturally occurring organofluorine compounds can be explained by two principal considerations: first, the low solubility of mineral fluorine means that only a small fraction of the total mineral fluoride is available to organisms. Sea water, for example, contains only 1.3 ppm of fluoride in contrast to 19,000 ppm of chloride. Second, the high redox potential required for the oxidation of fluoride probably prevents it from being activated through a similar mechanism to that used by enzymes to effect chlorination. It is proposed that both haloperoxidases and halogenases generate HOCl as the active chlorinating species, thus explaining why these enzymes do not take fluoride [3, 4].

The most widespread known fluorinated natural product is fluoroacetate and it is found, often in high concentrations, in the leaves and seeds of a variety of tropical plants [5]. Despite considerable interest and effort, little progress has been made in understanding the process of fluorination in higher plants [6]. A suitable bacterial system offers a more accessible route of investigation. The production of fluorometabolites by bacterial species is particularly unusual despite the great diversity of bacterial natural products. Only two bacterial strains, both actinomycetes, have been identified with the ability to synthesize organofluorine compounds. Nucleocidin was originally isolated from *Streptomyces calvus* in 1956 [7]. More recent attempts to isolate the compound from this species have failed and hence prevented investigation of the biosynthetic route to this interesting natural product. The discovery in 1986 that *Streptomyces cattleya* was capable of producing both fluoroacetate (**5**) and 4-fluorothreonine (**6**) [8] provided an alternative system for the study of the mechanism of biological fluorination. The biosynthetic route to these fluorinated metabolites has been investigated by the feeding of ^{13}C- and ^{2}H-enriched precursors. Such studies have identified fluoroacetaldehyde (**4**) as a key common intermediate in the biosynthesis of both fluoroacetate (**5**) and 4-fluorothreonine (**6**) [8–12]. A PLP-dependent threonine transaldolase activity was subsequently purified from cell-free extracts of *S. cattleya* that was able to convert fluoroacetaldehyde (**4**) into 4-fluorothreonine (**6**) [13]. Similarly, an NAD^{+}-dependent fluoroacetaldehyde dehydrogenase capable of oxidizing fluoroacetaldehyde (**4**) to fluoroacetate (**5**) was purified from the same strain [14]. More recently, the key fluorination enzyme FlA was identified and shown to catalyze the initial fluorination of *S*-adenosylmethionine (SAM) (**1**) to give 5′-fluoro-5′-deoxyadenosine (5′-FDA) (**2**) [15]. The crystal structure of this enzyme has been determined [16]. 5′-FDA has thus been identified as the first fluorinated organic compound in the pathway to fluoroacetaldehyde (**4**). Very recently, 5-fluoro-5-deoxy-*D*-ribose-1-phosphate (5-FDRP) (**3**) has been proposed as the next intermediate of the pathway [17] (Figure 1). A nucleoside phosphorylase activity has been identified and partially purified from cell-free extracts of *S. cattleya* which is able to displace the nucleotide base from 5′-FDA (**2**) with phosphate to give 5-FDRP (**3**). Incubation of 5-FDRP (**3**) with a cell-free extract of *S. cattleya* resulted in the formation of fluoroacetate (**5**) [17]. However, the steps from 5-FDRP (**3**) to fluoroacetaldehyde (**4**) remain to be elucidated.

In this study, we report the cloning and sequence analysis of a locus containing genes clustered around the previously identified fluorinase gene *flA* from a genomic

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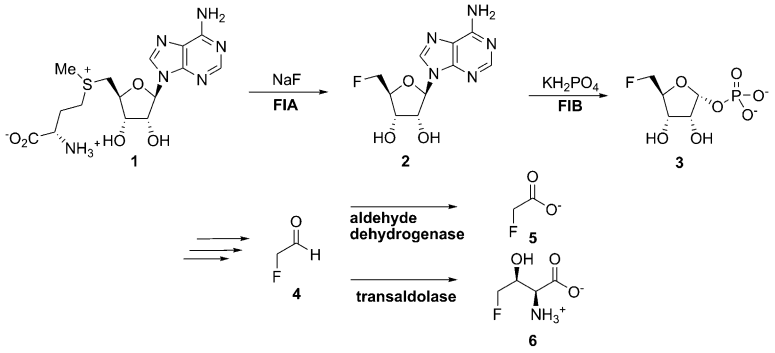

Figure 1. Proposed Biosynthesis of Fluoroacetate (**5**) and 4-Fluorothreonine (**6**)

DNA cosmid library isolated from *S. cattleya*. These genes are therefore likely to be involved in fluorometabolite biosynthesis in this organism. Of particular interest is the characterization of three genes: the *flB* gene encoding the 5′-FDA (**2**) phosphorylase responsible for conversion of 5′-FDA (**2**) into adenine and 5-FDRP (**3**), *flI* encoding an adenosylhomocysteine lyase proposed to relieve *S*-adenosylhomocysteine inhibition of the fluorinase, and a resistance gene, *flK*, for the specific degradation of fluoroacetyl-coenzyme A (fluoroacetyl-CoA) into fluoroacetate and coenzyme A (CoA).

Results and Discussion

Cloning of the *fl* Locus

A genomic library of *S. cattleya* was constructed in SuperCos 1 vector (Stratagene). This was screened by a PCR-based method with perfectly matched primers that hybridize to regions of the *flA* gene. One cosmid clone designated SC8E11 was isolated that gave PCR products of the expected size under conditions of high stringency for binding of the oligonucleotides to the cosmid template. A large-scale cosmid preparation was obtained. This was directly sequenced with the oligonucleotide primers used in the PCR screening reaction and the presence of the *flA* gene was confirmed by comparison of the sequence thus identified from the cosmid clone and published *flA* sequence [16]. The DNA sequence of SC8E11 (encoding a 34 kbp insert) was obtained in its entirety. Analysis of the DNA sequence of this clone showed it to encode several genes clustered around the fluorinase gene that are thought to be involved in fluorometabolite biosynthesis. These genes have been designated *fl* gene locus (Figure 2) and deposited in the EMBL nucleotide sequence database (accession number: AM055586).

Organization of the *fl* Locus

The genes of the *fl* locus are clustered in the middle of the insert of SC8E11 cosmid, and the *flA* gene located in the center of the locus encodes the previously characterized fluorinase FIA [18]. The limits of the *fl* locus remain to be determined by gene disruption experiments, so the contribution of the 12 identified open reading frames remains speculative. Clear housekeeping functions can be assigned to genes flanking this locus: a diaminopimelate decarboxylase (EC 4.1.1.20 lysine biosynthesis) and an arginyl-tRNA synthetase (EC 6.1.1.19 arginyl-tRNA biosynthesis) are found right upstream of the *fl* cluster. Genes identified downstream of the *fl* cluster are 1,4-α-glucan branching enzyme (EC 2.4.1.18 starch and sucrose metabolism), D-amino acid oxidase (EC 1.4.3.3 amino acid metabolism), and a cell cycle protein.

Biosynthesis of 5-Fluoro-5-Deoxy-*D*-Ribose-1-Phosphate

Immediately upstream of *flA* is a putative open reading frame (*flB*) that is divergently transcribed and predicted to encode a protein of 299 amino acids having a high degree of end-to-end similarity to authentic nucleoside phosphorylases. Our in vitro assays (see below) have demonstrated that the *flB* gene product catalyzed the phosphorolytic conversion of 5′-FDA (**2**) to adenine and 5-FDRP (**3**), which is in agreement with a recent study carried out by using a cell-free extract from *S. cattleya* [17]. FIB was overexpressed in *Escherichia coli* as a

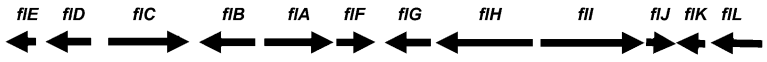

Figure 2. Map of the *fl* Gene Locus and Possible Functions for the Proteins Encoded by the Open Reading Frames

ORF	Start/Stop (bp)	Length (aa)	Function/Homology
E	127-795c	222	DNA binding regulatory protein
D	854-1504c	216	Dehalogenase/Phosphatase
C	1845-3038	397	MFS permease
B	3053-3952c	299	5′-FDA phosphorylase
A	4172-5071	299	5′-FDA synthase
F	5196-5753	185	DNA binding regulatory protein
G	5947-6651c	234	DNA binding regulatory protein
H	6648-8051c	467	Na^+/H^+ antiporter
I	8317-9786	489	Adenosylhomocysteine hydrolase
J	9802-10197	131	regulatory protein
K	10172-10591c	139	Fluoroacetyl-CoA thioesterase
L	10696-11373c	225	DNA binding regulatory protein

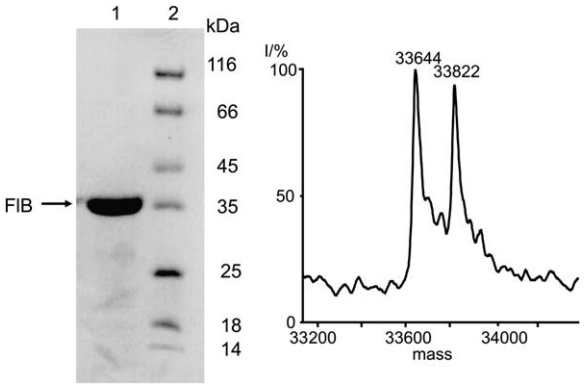

Figure 3. SDS-PAGE of FIB

Lane 1: purified recombinant FIB. Lane 2: protein molecular weight marker (MBI Fermentas); ESI-MS of FIB.

His-tagged protein and purified with His-Bind metal chelating resin (Novagen). From 1 liter of culture about 1 mg of purified FIB was obtained. The low yield is mainly due to the insolubility of the majority of the overexpressed protein. Purified FIB appeared on SDS-PAGE as an ~35 kDa protein (Figure 3).

The molecular weight of the protein determined by LC-ESI-MS is 33,644, which is consistent with the calculated value of 33,645. The peak at 33,822 represents the mass of the protein with an N-terminal gluconoylation (M+178) generated by *E. coli* [19]. Enzymatic assays have demonstrated that FIB is a nucleotide phosphorylase catalyzing the phosphate-dependent cleavage of 5′-FDA (**2**) to yield adenine and 5-FDRP (**3**). The generation of adenine was monitored by HPLC with UV and MS detection and comparison of the retention time of the generated adenine with an authentic reference (Figure 4A). The formation of the 5-FDRP (**3**) was confirmed with ^{19}F-NMR and ^{1}H-NMR (Figures 4B and 4C). In the ^{19}F-NMR, a new signal at −231.02 ppm is observed in the FIB enzyme assay just beside the signal for 5′-FDA (**2**) at −230.78 ppm, indicating the generation of a new fluororibose derivative. Furthermore, in the ^{1}H-NMR, a characteristic doublet of doublets coupling pattern for the anomeric proton of **3** at 5.69 ppm clearly indicates the attachment of the phosphate. This signal pattern matches that of the commercially available ribose-1-phosphate. The signals for H-C2, H-C3, and H-C4 of fluororibose-1-phosphate could also be observed clearly in the bioassay. However, both signals for H-C5 are hidden in the solvent signal.

Additionally, we performed an assay with FIB in the presence of adenosine. The ^{1}H-NMR of the ribose-1-phosphate generated closely matched that of the commercially available standard.

The formation of two new signals at 8.20 and 8.24 ppm in the aromatic region provides further evidence for the formation of adenine by using either 5′-FDA (**2**) or adenosine as a substrate for FIB. The combination of our MS and NMR data clearly indicates the production of 5-FDRP (**3**) even though the signals for H-C5 are obscured.

The purified recombinant FIB exhibited a K_m of 35 μM for 5′-FDA and a V_{max} of 15 μmol/min/mg protein (see Supplemental Data available with this article online). No 5′-FDA (**2**) cleavage was observed in the experiments with pyrophosphate instead of phosphate, confirming the requirement of phosphate ions for the enzyme activity. Adenosine is determined to be a poor substrate for FIB. At a substrate concentration of 60 μM in a 100 μl reaction, detection of adenine formation from adenosine in the presence of 100 ng of FIB required 30 min incubation at 30ºC (data not shown). Under the same assay conditions, however, 40 ng of FIB catalyzed the release of similar amounts of adenine from 5′-FDA within 1 min, demonstrating that the enzyme is selective for the fluorinated substrate.

Transmembrane Transport of Ions and Fluorometabolites

Two open reading frames within the *fl* locus are predicted to encode proteins that act as transmembrane

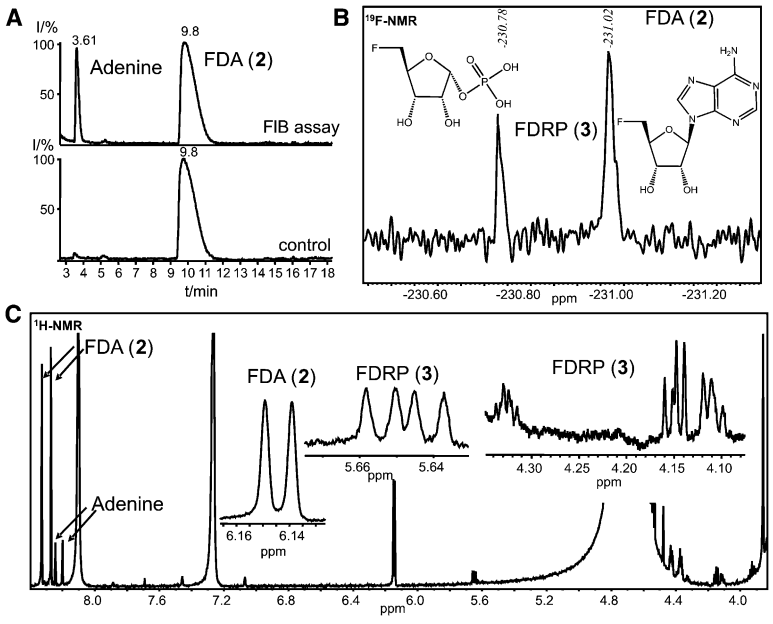

Figure 4. Characterization of FIB Activity by LC-ESI-MS, ^{19}F-NMR, and ^{1}H-NMR

(A) LC-MS trace of the formation of adenine by FIB.

(B and C) ^{19}F-NMR (B) and ^{1}H-NMR (C) after incubation of FIB with 5′-fluoro-5′-deoxyadenosine (5′-FDA, **2**), indicating the formation of FDRP (**3**).

transporters: *flH* encodes a protein of 467 amino acids with high similarity to transmembrane Na^+/H^+ antiporters. Na^+/H^+ exchange proteins eject protons from cells, effectively eliminating excess acid from actively metabolizing cells. Na^+/H^+ exchange activity is crucial for the regulation of cell volume and for the reabsorption of sodium chloride across renal, intestinal, and other epithelia [20]. The movement of fluoride ions across the cell membrane is thought to be as the un-ionized HF. Erythrocytes incubated in the presence of sodium fluoride accumulate fluoride anions in association with a decrease in intracellular pH [21]. It has been observed in a study on fluorometabolite production in resting cells of *S. cattleya* that the fluoride uptake was maximal at pH 6.0 and sharply declined at higher pH [10], indicating that a certain level of extracellular H^+ was required for transport of F^- into cells. *FlH* may therefore function as a Na^+/H^+ antiporter that buffers the accumulation of HF by exchange of an intracellular proton for an extracellular sodium ion and results in restoration of intracellular pH and net intracellular accumulation of NaF. The inferred translation product of *flC* is a 397 amino acid protein with significant sequence similarity to members of the MFS transporter family [22, 23]. These proteins are responsible for the transport of small metabolites across the cell membrane. The role of FlC remains to be established but it may be a candidate for the transmembrane transport of fluoroacetate (**5**) and/or 4-fluorothreonine (**6**).

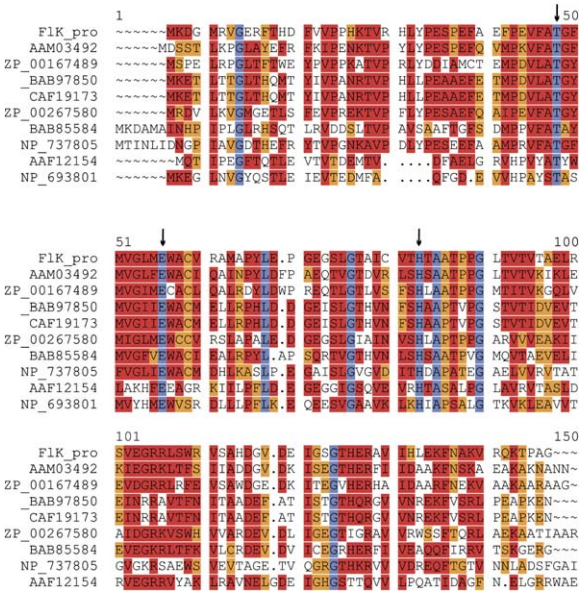

Figure 5. Multiple Protein Sequence Alignment of FlK with Related Proteins

The conserved Thr42, Glu50, and His76 are suggested to constitute a catalytic triad mechanism (highlighted by arrows).

Resistance

The deduced protein product of *flD* (216 amino acids) shows similarity to a number of haloacid-dehalogenase [24] (EC 3.8.1.2) and phosphatase/phosphomutase (EC 3.1.3.18) enzymes. *flD* could encode a resistance mechanism that is able to defluorinate fluoroacetate (**5**) and/or 4-fluorothreonine (**6**) acting as a resistance and regulatory gene product. It is also possible, however, that FlD may play a role in further metabolism of 5-FDRP (**3**) in the pathway leading to fluoroacetate (**5**).

The small protein (139 amino acids) encoded by the *flK* gene is a putative thioesterase as suggested by a BLAST search against the NCBI conserved domain database. FlK shows end-to-end homology (26%–54% of identity) to many predicted thioesterases or hypothetical proteins in bacteria, including an extremely halotolerant and alkaliphilic deep sea strain, *Oceanobacillus iheyensis* HTE831, and several thermophilic bacteria. FlK homologs with unknown functions are also found in a couple of thermo/acidophilic archaea as well as in eukaryotic organisms such as *Dictyostelium discoideum* and *Tetraodon nigroviridis*. The majority of FlK homologs have protein sizes similar to FlK, ranging between 127 and 180 amino acids. A protein sequence alignment of FlK with representative homologs is shown in Figure 5. Nine amino acid residues, Gly8, Thr42, Glu50, Leu61, Gly69, His76, Ala78, Gly83, and Gly116, in FlK appear to be well conserved in all these homologs. From this we speculate that FlK employs a variation of the classical catalytic triad-type mechanism [25] with Thr42 (instead of Ser), Glu50 (instead of Asp), and His76. Although proteases employing threonine instead of serine as part of the catalytic triad are known [26], there are no examples of thioesterases to our knowledge that utilize threonine in this role. It is tempting to speculate that having a secondary alcohol as the nucleophile might partly explain the exquisite selectivity shown by this enzyme.

To determine the function of FlK as a thioesterase, FlK protein was overexpressed in *E. coli* as a His-tagged protein and purified with His-Bind metal chelating resin (Novagen). The yield of the overexpressed recombinant FlK was about 10 mg per liter of culture. Purified FlK gave a band on SDS-PAGE with a molecular weight slightly less than 18 kDa (Figure 6A). The molecular weight of the FlK protein determined by LC-ESI-MS is 17,287 Da, in good agreement with the calculated molecular mass (17,288 Da) of the recombinant FlK without the first methionine residue that is often removed after expression of the protein by *E. coli*. Fluoroacetyl-CoA, the substrate used in the in vitro FlK enzymatic assays, was synthesized by activation of fluoroacetate sodium salt with oxalylchloride and DMF and subsequent reaction with coenzyme A. Fluoroacetyl-CoA slowly decomposes at 25ºC and pH 8.0 as previously reported [27]. Therefore, the reaction rates for FlK have been corrected for the nonenzymatic hydrolysis of the substrate.

The breakdown of fluoroacetyl-CoA to coenzyme A and fluoroacetate by FlK was followed by monitoring the UV signal at 412 nm of the generated coenzyme A after reaction with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) [28]. The formation of fluoroacetate by FlK was verified by direct observation of the ^{19}F-NMR fluoroacetate signal during the FlK assay (Figure 6C).

The purified recombinant FlK catalyzed the hydrolysis of fluoroacetyl-CoA with a K_m of 30 μM and a V_{max} of 15 μmol/min/mg protein (Figure 6B) but did not exhibit any activity toward acetyl-CoA at a concentration up to 1 mM. The observed high selectivity of the enzyme further supports its function as a resistance mechanism. The K_m and V_{max} values of FlK are similar to those of an acetyl-CoA hydrolase isolated from pig heart, which

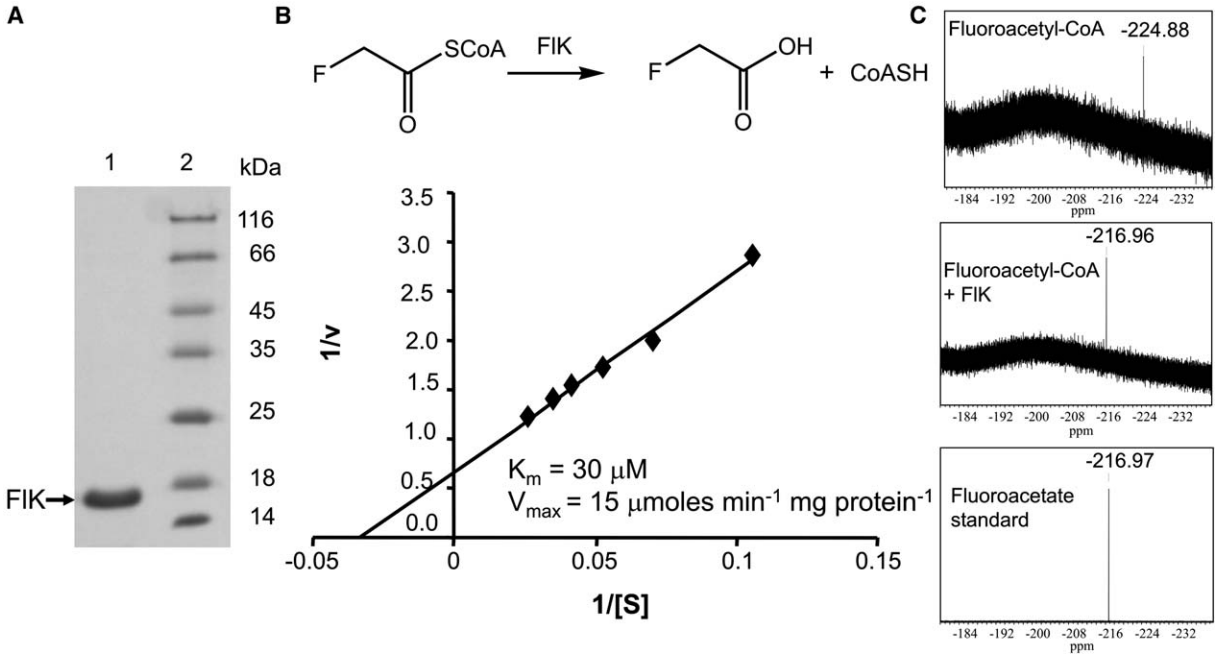

Figure 6. Characterization of Purified FlK

(A) SDS-PAGE of FlK. Lane 1: purified recombinant FlK. Lane 2: protein molecular weight markers (MBI Fermentas).

(B) Lineweaver-Burk plot of fluoroacetyl-CoA hydrolysis catalyzed by FlK. v, initial rate of CoASH release (μM/min); [S], fluoroacetyl-CoA concentration (μM).

(C) ^{19}F-NMR monitoring of the formation of fluoroacetate by FlK catalyzing the hydrolysis of fluoroacetyl-CoA.

exhibited a K_m of 48 μM and a V_{max} of 2.7 μmol/min/mg protein [29]. Long-chain fatty acid CoAs are the substrate of most of the characterized acyl-CoA hydrolases deposited in the enzyme database BRENDA. These hydrolases exhibit K_m values in a range from 1 μM to 50 μM and a V_{max} less than 5 μmol/min/mg protein.

Fluoroacetyl-CoA hydrolase-like activity has been observed in a crude mitochondrial enzyme extract from the plant *Dichapetalum cymosum* [27]. It has been shown that fluoroacetyl-CoA is a substrate for citrate synthase and reacts with oxalacetate to produce 2-fluorocitrate [30–32]. Lauble et al. presented experimental evidence indicating that the (−)-*erythro* diastereomer of 2-fluorocitrate can be further converted to 4-hydroxy-*trans*-aconitate, a strong competitive inhibitor of aconitase, thereby blocking the tricarboxylic acid cycle [33]. Both in vivo and in vitro synthesis of fluoroacetyl-CoA from fluoroacetate by acetyl-CoA synthetase has been reported [34, 35]. The presence of a fluoroacetyl-CoA-specific thioesterase in fluoroacetate-producing *S. cattleya* is significant in that it provides an effective self-defense mechanism to prevent any fluoroacetyl-CoA formed from entering the tricarboxylic acid cycle.

Regulatory and Other Genes

flI encodes a putative protein of 489 amino acids and shows a very high overall sequence homology to *S*-adenosyl-L-homocysteine hydrolase (AdoHcyase), which catalyzes reversible hydrolysis of *S*-adenosyl-L-homocysteine (AdoHcy) into homocysteine and adenosine (Figure 7B). We heterologously expressed and purified FlI (Figure 7A) in order to investigate this hypothesis. The enzyme does indeed hydrolyze AdoHcy (forward reaction) and is also capable of catalyzing AdoHcy formation if supplied with homocysteine and adenosine (reverse reaction; Figure 7).

It has been previously demonstrated that AdoHcy is a competitive inhibitor of FlA [36]. In our study, the production of 5′-FDA from SAM catalyzed by purified FlA decreased more than 85% in the presence of AdoHcy at a 5 to 1 molar ratio of substrate to inhibitor (Supplemental Data). FlI might therefore promote 5′-FDA (2) biosynthesis by limiting the concentration of this inhibitor in the cells. K_m and V_{max} for FlI-catalyzed hydrolysis of AdoHcy have been determined to be 174.5 μM and 189 μmol/min/mg protein, respectively (Supplemental Data). These values are much higher than those for similar enzymes from other sources. We speculate that at a normal physiological concentration of AdoHcy, FlI may work at its minimum velocity without disturbing primary metabolism and only functions efficiently when there is extra AdoHcy present. Additionally, FlI-catalyzed AdoHcy hydrolysis was inhibited significantly (more than 95%) by SAM at an AdoHcy:SAM molar ratio of 67:1 (Supplemental Data). Intermediate inhibition of AdoHcy hydrolase activity by SAM has been previously reported [37]. The crossinhibition of FlA and FlI by each other's substrates indicates a possible role played by both SAM and AdoHcy in fine-tuning of the activities of these enzymes.

The predicted product of *flJ* is similar to a family of proteins of unknown function. A possible regulatory role for this gene is suggested by the presence of the rare TTA codon encoding leucine at amino acid position 43. A+T-rich codons are rarely used in high-G+C-containing *Streptomyces* DNA. TTA codons have been

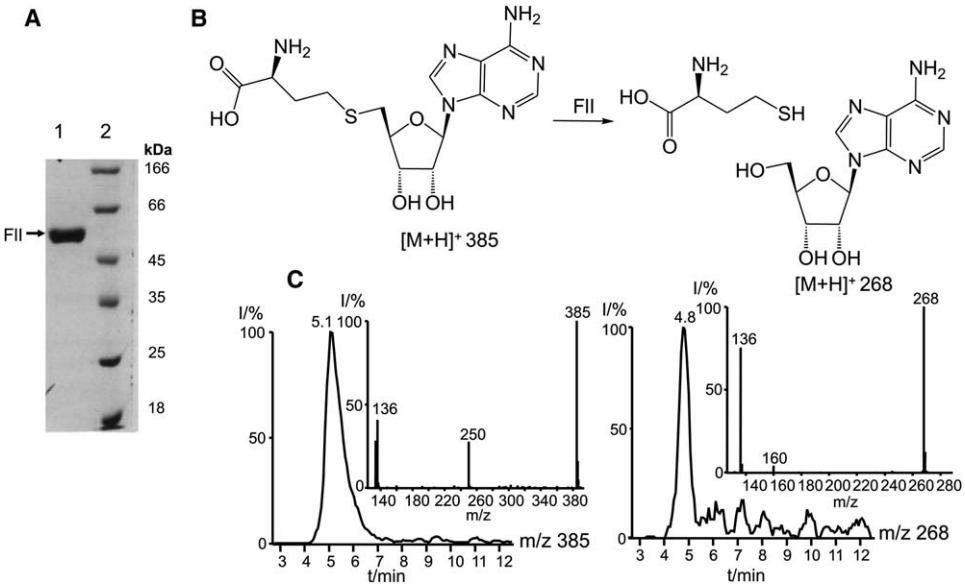

Figure 7. Characterization of Purified FII

(A) SDS-PAGE of FII. Lane 1: purified recombinant FII. Lane 2: protein molecular weight marker (MBI Fermentas).

(B) Reaction catalyzed by the *S*-adenosylhomocysteine hydrolase FII.

(C) LC-MS ion traces and ESI-MS of *S*-adenosylhomocysteine and adenosine generated by FII.

found in many *Streptomyces* genes expressed in late growth phase but not in genes known to be required for primary metabolism. These late-expressing genes include antibiotic pathway-specific genes, antibiotic resistance and biosynthesis genes, sporulation genes, and many genes with unknown function [38]. Studies on *Streptomyces coelicolor* mutants deficient in the formation of aerial mycelium have identified several unlinked *bld* genes. Mutants specifically deficient in the *bldA* gene fail to produce aerial mycelium and antibiotics. The product of the *bldA* locus is a tRNA molecule which recognizes and translates the rare TTA codon for leucine [39]. The accumulation of the mature *bldA* tRNA has also been shown to be growth phase dependent and reaches maximal level late in growth. This may facilitate integrated control of differentiation and secondary metabolism by ensuring that key genes contain such a codon and are expressed only at later stages of the life cycle.

The *fl* locus contains four genes predicted to encode helix-turn-helix DNA binding proteins [40]: *flE* (222 amino acids), *flF* (185 amino acids), *flG* (234 amino acids), and *flL* (225 amino acids). They are likely to be involved in transcriptional control of open reading frames of the *fl* locus.

As mentioned earlier, the activities responsible for the late steps in fluorometabolite biosynthesis, such as the conversion of fluoroacetaldehyde (**4**) to 4-fluorothreonine (**6**) and its oxidation to fluoroacetate (**5**), have previously been identified in partially purified enzyme preparations of *S. cattleya* [11, 13, 14, 41]. The genes encoding these enzymes, however, do not appear to be present within the *fl* cluster. Further experiments are needed to investigate whether there is a second locus that contains these missing genes or whether enzymes catalyzing similar reactions in primary metabolism may accept fluorinated substrates.

Significance

The cloning and analysis of the first gene cluster involved in organofluoro compound formation (*fl*) are presented. Twelve open reading frames with possible roles in biosynthesis of fluorometabolites have been identified around the previously characterized fluorinase gene (*flA*) of *Streptomyces cattleya*. One open reading frame designated *flB* has been shown to encode the nucleoside phosphorylase activity that converts 5′-fluoro-5′-deoxyadenosine, the first fluorinated intermediate produced by FlA, into 5-fluoro-5-deoxy-*D*-ribose-1-phosphate. FII has been determined to be an *S*-adenosylhomocysteine hydrolase, suggesting that this enzyme is necessary for the fluorinase to be fully active, as adenosylhomocysteine has been shown to be a potent inhibitor of this enzyme. Of particular interest is the presence of the *flK* gene, shown to encode a fluoroacetyl-CoA-specific thioesterase. The expression of FlK ensures that when fluoroacetyl-CoA is formed by the producing strain it is then immediately degraded before it can be further metabolized to produce 4-hydroxy-*trans*-aconitate, a lethal inhibitor of the tricarboxylic acid cycle.

Experimental Procedures

Strains, Culture Conditions, and Plasmids

Streptomyces cattleya was propagated in a defined medium for *S. cattleya* [10] at 30ºC. SuperCos 1 (Stratagene) was used as the vector for construction of the genomic DNA library. Library transfections were performed in *Escherichia coli* XL-1 Blue MR (Stratagene). Recombinant cosmids were subcloned in pSHG397 [42] and recombinants were selected by chloramphenicol. Plasmids were manipulated in *E. coli* DH10B (GIBCO-BRL) by using standard protocols [43]. pET28a(+) was used as the vector for protein expression in *E. coli* BL21 (DE3) (Novagen). All procedures were carried out according to manufacturers' recommendations.

Preparation and Screening of *S. cattleya* Genomic Library

Total DNA was isolated from *S. cattleya* by procedure B of Kieser et al. [44]. For the generation of the cosmid library, total DNA was partially digested with BamHI, dephosphorylated with shrimp intestinal alkaline phosphatase, and ligated directly into SuperCos 1 and packaged with Gigapack Gold packaging extract (Stratagene) without size fractionation. All procedures were done in accordance with the manufacturer's recommendations (Stratagene).

Screening of the *S. cattleya* library was performed by a PCR-based methodology. Briefly, 960 colonies of the *S. cattleya* cosmid library were grown in 1 ml twice-strength LB (2× LB containing 50 μg kamamycin and 100 μg ampicillin) cultures on 96-well plates for 24 hr at 37ºC. Fifty microliters of each culture was transferred to a 96-well microtiter plate by a multichannel pipette and stored at −80ºC with an equal volume of 80% glycerol (40% final). Small-scale DNA preparations (High Yield Protocol 1, Plasmid Minipreparation with MultiScreen, Millipore) were made from the remaining 900 μl of each culture and resuspended in 100 μl sterile water. This provided ten 96-well plates with 100 μl suspended DNA in each well that could be easily crossreferenced to an equivalent sample of cells stored in ten corresponding 96-well plates at −80ºC. To simplify the PCR screen, 1 μl DNA was transferred from each corresponding position on the 96-well plate to a single master plate, so that position A1 of the master plate contained 1 μl DNA from position A1 of each of the ten microtiter plates. This plate was then screened by PCR with oligonucleotides designed internal to the published sequence of the *flA* gene, designated pSCF2 5′-GAG GAG CAC GGC TAC CTG GAG GCG TAC GAG GTC ACC TCG-3′, pSCF5 5′-GCC AAG GGC GGT GCC CGC GGC CAG TGG GCG-3′, and pSCF10 5′-TGG TCG ATG GCG GAG ACC ACG CCC ACC AGC-3′. PCR reactions were performed in 50 μl final volumes with a final concentration of 2% DMSO and Biomix Red PCR premix (Bioline). PCR conditions were preheating at 94ºC for 10 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 60ºC for 1 min and extension at 72ºC for 1 min, with a final 10 min infilling at 72ºC. Expected PCR product sizes of 239 bp and 350 bp were observed for three wells. All ten samples that had contributed DNA to these three wells were then re-screened by the above protocol. Three cosmids were thus identified as giving an appropriate PCR product size of 350 bp, namely SC5H2, SC8E11, and SC8H9. The stored replica deep plates were used to inoculate cultures with each of the above cosmid clones. Cosmid DNA for sequencing was prepared by using the Qiagen Midi-prep DNA purification kit from liquid cultures (50 ml) in 2× LB medium with appropriate antibiotic selection. The presence of the *flA* gene sequence in all three cosmids was further confirmed by direct sequencing of the cosmid DNA with pSCF5 and pSCF10 primers that identified the expected *flA* sequences.

One cosmid SC8E11 was sequenced in its entirety. Sequence data were obtained from assembly of overlapping Sau3AI fragments acquired from partial digestion of the parent cosmid. The 2–5 kbp fraction of the digest was eluted from an agarose gel by using the QIAEX II gel extraction kit (Qiagen) and subcloned into pSHG397 [42]. The entire sequence of the SC8E11 clone was determined from forward and reverse primary sequences of 192 templates with gaps filled by oligonucleotide-directed sequencing.

DNA Sequencing

All sequencing was performed in the Department of Biochemistry DNA Sequencing Facility, University of Cambridge. An Applied Biosystems 800 molecular biology CATALYST robot was used to apply Taq dideoxy terminator sequencing reactions (Big Dye Terminator kit, Applied Biosystems) to an ABI 373A sequencer according to the manufacturer's protocol. End sequencing of cosmids used T3 and T7 primers. Plasmid sequencing used pUC forward and reverse primers. SeqEd v 1.0.3 was used for sequence editing. Database searches used the BLAST algorithm. Sequence assembly employed the GAP (genome assembly program), version 4.2 [45].

Expression and Purification of FIB, FIK, and FII

The *flB*, *flK*, and *flI* genes were amplified by PCR utilizing cosmid SC8E11 as a template. The following oligonucleotides were used as primers in the PCR reactions: pSCphos1 5′-CCG GAG GTG CGC ATA TGC GGG CAC GGA AAT-3′ and pSCphos2 5′-GGG TAC CGG AGG GAT CCG GGC CCC GCG CTG-3′ for *flB*, pflK-3 5′-AGT GAG GAG TGG CAT ATG AAG GAC GGC ATG-3′ and pflK-2 5′-GCG GAT CCC GCC GGA GAA CCT GAT CGT GCT-3′ for *flK*, and pfll-1 5′-GCA GTC GAA GGC GCT AGC ATG GCA GTC GTA-3′ and pfll-2 5′-GCT TCC ATC TCG AGC GTG CCC CCT CAG TAT-3′ for *fll*. To facilitate cloning, restriction sites were engineered at the 5′ primer/3′ primer: NdeI/XhoI for *flB*, NdeI/BamHI for *flK*, and NheI/XhoI for *fll*, as indicated by the underlining. PCR was carried out with *Pfu* DNA polymerase with 25 cycles of denaturing at 94ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 1 min plus a final extension at 72ºC for 10 min. The PCR products were digested with appropriate restriction enzymes, purified by gel extraction (Qiagen), and inserted into a pET28a(+) plasmid. The inserts of the recombinant plasmids were verified by DNA sequencing. The recombinant plasmids were introduced into *E. coli* Rosetta (DE3)pLysS-competent cells (Novagen). An overnight culture (10 ml) from a single colony of the transformants was used for inoculation of 1 liter of fresh LB medium containing kanamycin (50 μg/ml). The culture was incubated at 37ºC until OD_{600} = 0.5–0.8 was reached. Overexpression of the proteins was induced by isopropylthiogalactoside (0.2 mM) overnight at 16ºC with shaking at 220 rpm. Cells were harvested by centrifugation and the overexpressed recombinant protein was released by sonication for 4 min (1 s on, 10 s off). The supernatant of the cell lysate was allowed to pass through a column of Co^{2+} ion-charged His-Bind metal chelating resin (Novagen). Nonspecific proteins were removed by washing the column with washing buffer (0.5 M NaCl, 5–60 mM imidazole, 20 mM Tris-HCl [pH 7.9]) and the recombinant protein was eluted with elution buffer (0.5 M NaCl, 200 mM imidazole, 20 mM Tris-HCl [pH 7.9]). The purified proteins were stored at 4ºC in a buffer containing 200 mM KCl and 20 mM Tris-HCl (pH 7.5). Enzymes used for kinetic studies were further purified by gel filtration on an ÄKTA Explorer FPLC system with a HiLoad 16/60 Superdex 200 Prep Grade column and a mobile phase containing 200 mM KCl and 20 mM Tris-HCl (pH 7.5). The protein concentrations were determined by using Bradford protein dye reagent (Sigma).

Assay of FIB

A typical reaction for FIB activity assay contained 5′-FDA (**2**) (1 mM [17], KH_2PO_4 [2.5 mM, adjusted to pH 7.5 with 5 M KOH]), purified FIB (40 μg) in 100 μl HEPES buffer (50 mM, pH 7.5). Control reactions were carried out in the absence of either FIB or 5′-FDA (**2**) or KH_2PO_4. The reaction mixture was incubated at 37ºC for 60 min and terminated by the addition of chloroform (100 μl). Protein precipitate was removed by centrifugation. The supernatant was analyzed by HPLC, LC-MS, and ^{1}H- and ^{19}F-NMR.

For the LC-MS experiments, a ThermoFinnigan LCQ fitted with an ESI source connected to an Agilent HP 1100 HPLC system was used. One hundred microliter samples were injected onto a Phenomenex Synergy polar RP18 column (125 mm × 2 mm, 4 μm; Phenomenex, Macclesfield Cheshire, UK) and separated by gradient elution. HPLC conditions: 100% A for 5 min, gradient to 100% B in 35 min, 100% B for 5 min; A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA; flow rate 0.3 ml/min. The ESI-MS and retention time of the product of the FIB reaction adenine was verified with an authentic standard.

5′-fluoro-5′-deoxyadenosine (5′-FDA, **2**) ESI-MS: $[M+H]^+$ 270 (100), 136 (38); retention time: 9.7 min.
Adenosine ESI-MS: $[M+H]^+$ 268 (100), 136 (63); retention time: 4.3 min.
Adenine ESI-MS: $[M+H]^+$ 136 (100); retention time: 3.7 min.

For the ^{19}F-NMR experiments, the assay was upscaled by using 5′-FDA (1 mM), KH_2PO_4 (2.5 mM, adjusted to pH 7.5 with 5 M KOH), purified FIB (500 μg) in 500 μl HEPES buffer (50 mM, pH 7.5). After 1 hr reaction time, ^{19}F-NMR experiments were performed directly after addition of 100 μl D_2O to the aqueous reaction mixture on a Bruker Avance 400 MHz NMR spectrometer by using CCl_3F for calibration.

5′-fluoro-5′-deoxyadenosine (5′-FDA) (**2**): ^{19}F-NMR (380 MHz, D_2O/H_2O, ppm): δ −230.78.
5-fluoro-5-deoxy-ribose-1-phosphate (**3**): ^{19}F-NMR (380 MHz, D_2O/H_2O, ppm): δ −231.02.

For the ^{1}H-NMR experiments, we used the following assay conditions: 5′-FDA or adenosine (1 mM), KH_2PO_4 (2.5 mM, adjusted to pH 7.5 with 5 M KOH), purified FlB (500 μg) in 3 ml 20 mM Tris (pH 7.2). After incubation for 1 hr, the reaction was quenched by precipitation of the enzyme. One tenth of a milliliter of chloroform was added, and the mixture was vortexed for 1 min and centrifuged for 2 min. The supernatant was collected and the precipitation procedure was repeated twice. The aqueous supernatant was freeze dried. The sample was dissolved in 500 μl D_2O and analyzed by using a Bruker Avance 500 MHz spectrometer fitted with a TCI cryoprobe. Chemical shifts of ^{1}H-NMR spectra are given in ppm (δ) based on the solvent signal: D_2O 4.67 ppm.

α-5-fluoro-5-deoxy-ribose-1-phosphate (3): ^{1}H-NMR (500 MHz, D_2O, ppm): δ 4.10–4.13 (m, 1H, H-C2), 4.14–4.16 (m, 1H, H-C3), 4.31–4.38 (m, 1H, H-C4), 5.69 (dd, *J* = 6.7, *J* = 3.8, 1H, H-C1).
Adenine: ^{1}H-NMR (500 MHz, D_2O/HEPES buffer, ppm): 8.20 (s, 1H), 8.24 (s, 1H).
Ribose-1-phosphate: ^{1}H-NMR (500 MHz, D_2O/HEPES buffer, ppm): δ 3.57–3.74 (m, 2H, H-C5), 4.05 (dd, *J* = 6.3, *J* = 4.1, 1H, H-C3), 4.10–4.13 (m, 1H, H-C2), 4.22 (dd, *J* = 8.3, *J* = 3.4, 1H, H-C4), 5.62 (dd, *J* = 6.4, *J* = 4.1, 1H, H-C1). The generation of ribose-1-phosphate was compared to an authentic reference.

For the kinetic study of FlB activity, 40 ng FlB was incubated in 500 μl Tris-HCl (50 mM, pH 7.5) buffer at 30ºC with 5′-FDA at concentrations of 40, 50, and 60 μM. A 100 μl aliquot of each reaction was taken out after 1, 2, 3, 4, and 5 min incubation and vortexed immediately with an equal volume of chloroform to precipitate the enzyme. The reactions were analyzed by HPLC with an XTerra RP18 column (4.6 × 250 mm, 5 μm) with a mobile phase of 0%–15% acetonitrile over 25 min on an Agilent HP 1100 HPLC system. The abundance of adenine formed was quantified based on the absorbance of adenine at 254 nm wavelength. Data points are the mean value of three replicates. An authentic adenine standard (Sigma) was used for the standard curve.

Synthesis of Fluoroacetyl-CoA

To 100 mg (1 mmol) fluoroacetate sodium salt in 2 ml dry THF, 100 μl DMF and 1 mmol oxalylchloride (2 M CH_2Cl_2 solution) were added dropwise. The solution was stirred under reflux for 6 hr. After cooling, one quarter (0.25 mmol) of the solution was added quickly under rigorous stirring to 50 mg (0.06 mmol) coenzyme A lithium salt dissolved in 1 ml 10% $NaHCO_3$. After 5 min of stirring, the reaction was completed. THF was removed in a gentle stream of nitrogen and the crude fluoroacetyl-CoA was immediately purified by RP18 HPLC (A: H_2O, B: MeOH; 100% A to 100% B in 30 min; flow rate 10 ml/min; Phenomenex Luna 250 × 20 mm, 10 μm) in order to prevent degradation of fluoroacetyl-CoA. Fractions containing fluoroacetyl-CoA were combined after verification by ESI-MS injection. MeCN was removed in vacuo and the sample was lyophilized.

Yield: 35 mg, 71%.
^{1}H-NMR (500 MHz, D_2O, ppm): δ 0.69 (s, 3H), 0.79 (s, 3H), 2.31 (t, *J* = 6.4, 2H), 2.95 (t, *J* = 6.36, 2H), 3.23 (t, *J* = 6.3, 2H), 3.32 (t, *J* = 6.8, 2H), 3.51 (dd, *J* = 9.67, *J* = 4.22, 1H), 3.73 (dd, *J* = 9.67, *J* = 4.22, 1H), 3.87 (s, 1H), 4.11–4.20 (m, 2H), 4.44–4.49 (m, 1H), 4.72 (t, *J* = 5.90, 1H), 4.75–4.78 (m, 1H), 4.89 (d, *J* = 46.32, 2H), 6.05 (d, *J* = 5.84, 1H), 8.29 (s, 1H), 8.49 (s, 1H).
^{19}F-NMR (380 MHz, D_2O/H_2O, ppm): δ −225.10.
HR-ESI-MS: $[M+H]^+$ $C_{23}H_{38}FN_7O_{17}P_3S$ observed: 828.1280; calculated: 828.1242.

Assay of FlK

The thioesterase activity of FlK was routinely assayed by monitoring spectrophotometrically the increase in absorbance at 412 nm due to the reaction of released CoASH with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in a CARY 100 Bio UV-vis spectrophotometer (Varian) by using a previously described method [27] with modification. All assays were performed at 25ºC in a total volume of 0.5 ml in a spectrophotometer cuvette with a 1 cm light path containing the following components (final concentrations): Tris-HCl (100 mM, pH 8.0), fluoroacetyl-CoA (40, 30, 25, 20, 15, and 10 μM) or acetyl-CoA (1000, 700, 300, 250, 200, 150, 100, and 70 μM), DTNB (0.5 mM), and purified FlK (5 ng for assays with fluoroacetyl-CoA as substrate and 5–50 ng for acetyl-CoA experiments). Reactions without FlK or substrates (fluoroacetyl-CoA or acetyl-CoA) were performed as controls. Data points are the mean value of three replicates. The standard curve of CoASH (0–50 μM) was prepared by using commercially available CoASH (Sigma) in Tris-HCl (100 mM, pH 8.0) in the presence of DTNB (0.5 mM). A Lineweaver-Burk plot was used to determine the K_m and V_{max} of FlK-catalyzed fluoroacetyl-CoA hydrolysis. In addition, the formation of fluoroacetate from fluoroacetyl-CoA by FlK was verified by ^{19}F-NMR.

Fluoroacetyl-CoA: ^{19}F-NMR (380 MHz, D_2O/H_2O/50 mM Tris-HCl [pH 8.0], ppm): δ −224.88.
Fluoroacetate: ^{19}F-NMR (380 MHz, D_2O/H_2O/50 mM Tris-HCl [pH 8.0], ppm): δ −216.97.
DTNB-CoA: ESI-MS: $[M+H]^+$ 965 (100), 767 (4), 583 (8), 483 (4), 458 (10), 437 (8).

Assay of FlI

A typical FlI reaction contained 100 μg FlI and either AdoHcy (1.0 mM) (forward reaction) or homocysteine (1.0 mM) and adenosine (1.0 mM) (reverse reaction) in 100 μl Tris-HCl buffer (50 mM, pH 7.5). Control reactions were carried out in the absence of FlI or substrate(s). The reaction mixture was incubated at 37ºC for 60 min and quenched by the addition of chloroform (100 μl) and vortexed for 1 min. The protein precipitate was removed by centrifugation. The supernatant was analyzed by LC-MS (ThermoFinnigan LCQ fitted with an ESI source connected to an Agilent HP 1100 HPLC system). Fifty to one hundred microliter samples were injected onto a Grom-Sil ODS-4HE HPLC column (125 mm × 2 mm, 2 μm; Alltech Grom GmbH, Rottenburg-Hailfingen, Germany) and separated by gradient elution. HPLC conditions: 100% A for 15 min, gradient to 100% B in 10 min, 100% B for 5 min; A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA; flow rate 0.2 ml/min. The retention times and the MS spectra of adenosine and *S*-adenosylhomocysteine were compared to authentic standards. The homocysteine released was derivatized with DTNB and detected by LC-MS. Commercially available homocysteine (Sigma) served as standard.

Adenosine: ESI-MS: $[M+H]^+$ 268 (100), 160 (4), 136 (76); ESI-MS/MS: 268 (8), 136 (100); retention time: 5.1 min.
DTNB-homocysteine: ESI-MS: $[M+H]^+$ 333 (100); retention time: 21.9 min.
S-adenosylhomocysteine: ESI-MS: $[M+H]^+$ 385 (100), 250 (30), 136 (44); ESI-MS/MS of 385: 250 (100), 136 (42); retention time: 4.8 min.

For kinetic studies of FlI activity, in 500 μl 50 mM Tris-HCl (pH 7.5) buffer, 120 ng purified FlI was incubated at 30ºC with AdoHcy at concentrations of 120, 160, and 200 μM. A 100 μl aliquot of each reaction was taken out after 2, 4, 6, 8, and 10 min incubation and vortexed immediately with an equal volume of chloroform to precipitate the enzyme. The reactions were analyzed by LC-MS as described above. The abundance of adenosine formed was quantified based on the area integration of the adenosine peak. The K_m and V_{max} of FlI-catalyzed hydrolysis of AdoHcy was determined by a Lineweaver-Burk plot. Authentic adenosine (Sigma) was used for the standard curve.

Supplemental Data

Supplemental Data include Experimental Procedures and are available at http://www.chembiol.com/cgi/content/full/13/5/475/DC1/.

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Accession Number

The genes designated *fl* gene locus have been deposited in the EMBL nucleotide sequence database under accession number AM055586.