

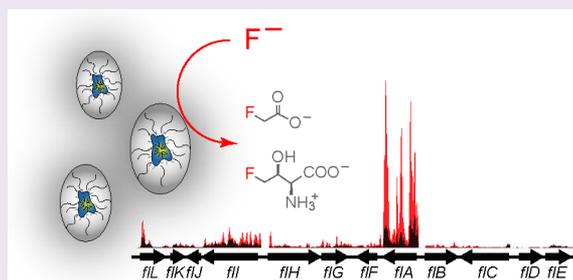
Temporal and Fluoride Control of Secondary Metabolism Regulates Cellular Organofluorine Biosynthesis

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S Supporting Information

ABSTRACT: Elucidating mechanisms of natural organofluorine biosynthesis is essential for a basic understanding of fluorine biochemistry in living systems as well as for expanding biological methods for fluorine incorporation into small molecules of interest. To meet this goal we have combined massively parallel sequencing technologies, genetic knockout, and *in vitro* biochemical approaches to investigate the fluoride response of the only known genetic host of an organofluorine-producing pathway, *Streptomyces cattleya*. Interestingly, we have discovered that the major mode of *S. cattleya*'s resistance to the fluorinated toxin it produces, fluoroacetate, may be due to temporal control of production rather than the ability of the host's metabolic machinery to discriminate between fluorinated and non-fluorinated molecules. Indeed, neither the acetate kinase/phosphotransacetylase acetate assimilation pathway nor the TCA cycle enzymes (citrate synthase and aconitase) exclude fluorinated substrates based on *in vitro* biochemical characterization. Furthermore, disruption of the fluoroacetate resistance gene encoding a fluoroacetyl-CoA thioesterase (FIK) does not appear to lead to an observable growth defect related to organofluorine production. By showing that a switch in central metabolism can mediate and control molecular fluorine incorporation, our findings reveal a new potential strategy toward diversifying simple fluorinated building blocks into more complex products.



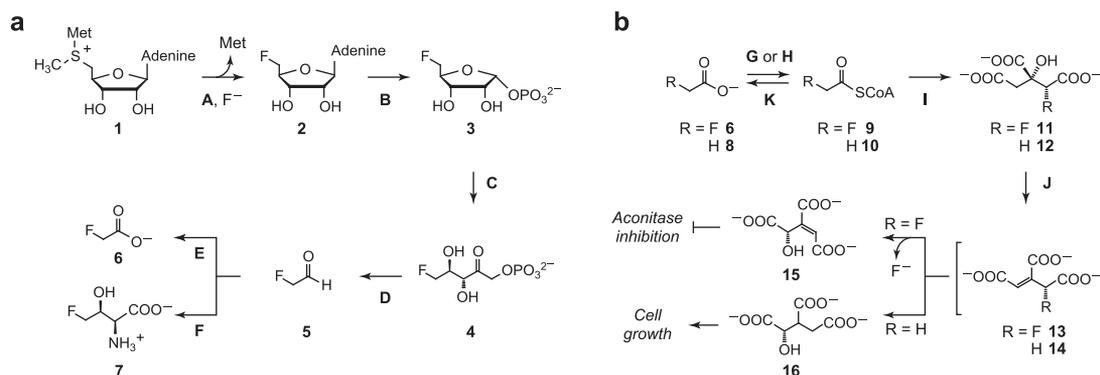
The continuing search for new biodiversity has yielded the discovery of many organisms with unusual chemical behaviors. One interesting chemical phenotype that has been observed in nature is the ability of *Streptomyces cattleya*, a soil-dwelling bacterium, to catalyze the formation of C–F bonds with fluoride as an environmentally green and abundant fluorine donor.^{1,2} Unlike other halogen-containing natural products (X = Cl, Br, I) that number in the thousands,³ organofluorine compounds are quite rare^{2,4,5} and relegated to a small number of compounds mostly derived from the fluoroacetate pathway (Scheme 1A).^{6–8} This dearth of naturally occurring compounds containing fluorine is especially striking given that fluorination has emerged as a particularly powerful tool for improving the efficacy of small-molecule drugs of synthetic origin for activity toward biological targets.^{9–11} Even with the limited examples available, site-specific fluorination is also highly effective in a biological milieu as fluoroacetate is a simple yet highly toxic molecule that is capable of inactivating the tricarboxylic acid cycle (TCA) with the substitution of a single hydrogen substituent for fluorine (Scheme 1B).^{12,13}

Our group is interested in exploring the fluorine physiology of *S. cattleya*, which is the only genetic host characterized to date that encodes a C–F bond-forming enzyme,^{14,15} with the overall goal of elucidating how living systems handle this unique element in a cellular environment. Beyond fundamental studies directed at exploring how new and exotic chemical phenotypes evolve, we also hope to develop an understanding of cellular organofluorine management that could help to

expand the synthetic biology of fluorine by engineering pathways for production of complex organofluorines from these simple building blocks. We therefore set out to study the metabolic machinery that underlies the fluorine physiology of *S. cattleya* using a combination of genomic, genetic, and *in vitro* biochemical methods. From these studies, we find that organofluorine biosynthesis is both fluoride- and growth phase-regulated, which provides a temporal mechanism to avoid endogenous fluoroacetate poisoning as it is only produced when cell growth and the TCA cycle shut down. This pattern of production is consistent with the behavior of other streptomycetes, where secondary metabolism is typically turned on during stationary phase,¹⁶ and supports the hypothesis that organofluorine production constitutes a normal cellular function for *S. cattleya* despite the apparent lack of gene clustering in the downstream steps of the fluoroacetate pathway. Indeed, the fluorinase, which catalyzes the C–F bond-forming reaction, is the sixth most highly transcribed gene in the genome in the presence of fluoride, which is consistent with its slow rate of turnover¹⁴ and further implies that fluorine secondary metabolism plays a major role in the *S. cattleya* life cycle.

Received: April 29, 2012

Accepted: June 27, 2012

Scheme 1. Biological Organofluorine Pathways^a

^a(a) The proposed biosynthetic pathway for fluoroacetate and fluorothreonine in *S. cattleya*. S-adenosylmethionine (1), 5'-fluorodeoxyadenosine (2), 5-fluorodeoxyribose-1-phosphate (3), 5-fluorodeoxyribulose-1-phosphate (4), fluoroacetaldehyde (5), fluoroacetate (6), fluorothreonine (7). Fluorinase (FLA, A), 5'-fluorodeoxyadenosine or methylthioadenosine phosphorylase (FLB, B), methylthioribulose-1-phosphate isomerase (MRI, C), fuculose-1-phosphate aldolase (F1P aldolase, D), fluoroacetaldehyde dehydrogenase (FAIDH, E), fluorothreonine transaldolase (FT transaldolase, F). (b) Lethal synthesis of fluorocitrate and inactivation of aconitase. Acetate (8), fluoroacetyl-CoA (9), acetyl-CoA (10), fluorocitrate (11), citrate (12), fluoroaconitate (13), aconitate (14), 4-hydroxy-transaconitate (15), isocitrate (16). Acetate kinase and phosphotransacetylase (AckA and Pta, G), acetyl-CoA synthetase (ACS, H), citrate synthase (CS, I), aconitase (J), fluoroacetyl-CoA thioesterase (FIK, K).

RESULTS AND DISCUSSION

Assembly of a *de novo* Working Genome Sequence of *S. cattleya* Using Short Reads. In order to gain insight into the organofluorine biosynthetic potential of *S. cattleya*, we decided to utilize next-generation sequencing methods to assemble a *de novo* draft genome sequence as a broad and non-biased approach to characterizing its genetic content (Supplementary Figure S1). The reads were initially assembled using Velvet v. 1.0.14,¹⁷ Image,¹⁸ and CAP3¹⁹ to produce a genome of 8.07 Mb over 154 contigs with an N_{50} of 133 kb (Supplementary Table S2). For comparison, we also tested a newer and more automated short-read assembler, SOAPdenovo63mer v 1.05,²⁰ to produce an assembly of 8.00 Mb over 192 contigs with an N_{50} of 158 kb (Supplementary Table S2).

The completed genome of *S. cattleya* has been recently reported and deposited with the NCBI²¹ and can be used for comparison of the quality of both of the Illumina-based assemblies (Supplementary Figure S2 and S3). NUCMER from the MUMMER package²² was used to map the contigs from the assemblies to the complete genome. The draft genomes obtained from Velvet and SOAPdenovo mapped to the completed genome with overall error rates of 0.06% and 0.02%, respectively. In the Velvet assembly, 34% of the contigs in the draft genome mapped directly to the complete genome with an additional 33% of contigs containing small insertions or deletions (37 base average length). The remaining contigs (31%) align well at the local level with the reference genome but contain an incorrect scaffolding event from the initial Velvet assembly (Supplementary Figure S2) that joins distant parts of the chromosome within the same contig. In comparison, the majority of the SOAPdenovo assembly (95% of contigs) maps directly to the reference with no scaffolding errors and a small number of contigs (4%) with insertions or deletions of moderate size (305 base average length). Regions in the complete *S. cattleya* genome that were absent from either the Velvet (70 regions, 49 kb) or SOAPdenovo (136 regions, 101 kb) assemblies mainly resulted from gaps located in repetitive regions of the genome (rRNA, transposase and polyketide synthase coding sequences; Supplementary Table S3),

demonstrating the known difficulty of assembling redundant sequences using very short reads.

Fluorometabolism Is Regulated Both by Fluoride and the *S. cattleya* Life Cycle. Overall, it appears as if *S. cattleya* contains more copies of the pathway enzymes compared to other sequenced streptomycetes, which could possibly serve as a rich source of enzymes with fluorine specificity (Supplementary Table S4). We hypothesized that expression of genes specifically involved in fluorometabolism could be regulated by the presence of fluoride given its potentially low bioavailability. Indeed, numerous putative transcription factors are found within the fluorinase (*fIA*) gene cluster, although none appear to be homologous to a reported chloride-dependent transcription factor.²³ We consequently cultured *S. cattleya* in GYM media at pH 5 in order to facilitate fluoride uptake and tracked the uptake of fluoride, production of organofluorines, and collected biomass at different stages of metabolism (Figure 1a,b). We initially screened and sequenced cDNA libraries prepared from cultures 0.5, 2, 6, and 44 h after fluoride addition (Supplementary Figure S4) using the Illumina platform in order to find the onset of fluoride-based differential gene expression. The short reads were mapped with BWA²⁴ to both the complete NCBI genome sequence as well as our draft genome sequence to avoid any inconsistencies in ORF identification. We further validated the results from the 48 h time point by sequencing independent 48 h cDNA libraries as well as by comparison to results obtained from a custom microarray designed from our draft genome sequence (Supplementary Figure S5).

On the basis of read-mapping to the *fIA* gene cluster, it is clear that the fluorinase is significantly more highly expressed in the presence of fluoride than other genes in the cluster (Figure 1c,d). Indeed, it appears to be the most highly expressed gene of known function (sixth overall) in the entire genome 48 h after fluoride addition (Figure 1c) and upregulated approximately 2.8-fold in response to fluoride. The expression of *fIA* is also subject to strong temporal control with upregulation tied to the onset of stationary phase, which is often observed in genes involved in secondary metabolite biosynthesis.²⁵ The gene encoding fluoroacetyl-CoA specific thioesterase, FIK, was found to be constitutively expressed at low levels, which is

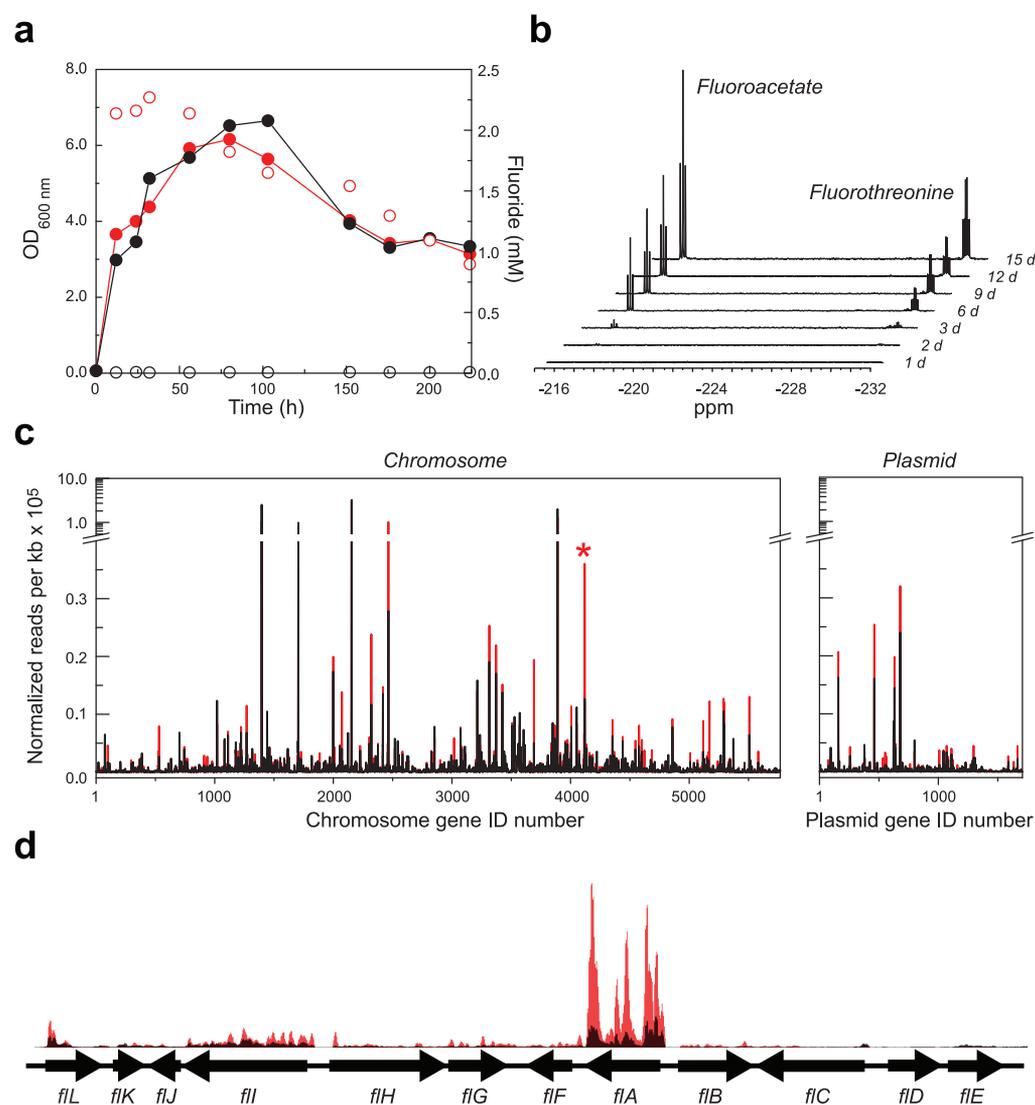


Figure 1. Analysis of the fluoride response in *S. cattleya*. (a) Growth and fluoride uptake in *S. cattleya* cultures in GYM media at pH 5. Growth of cultures measured by $OD_{600\text{nm}}$ (filled circles) and fluoride concentration (open circles) with (red) and without (black) 2 mM sodium fluoride added 24 h after inoculation. (b) Monitoring organofluorine production by ^{19}F NMR in the culture supernatant. (c) Transcriptional landscape of *S. cattleya* 48 h after the addition (red) or no addition (black) of fluoride. Values are quantile normalized reads per kb for predicted protein coding sequences. The fluorinase (*flA*) is indicated by a star. (d) RNA sequencing reads mapped onto the fluorinase gene cluster 48 h after the addition of fluoride (red) compared with no added fluoride (black).

consistent with its role in resistance against fluoroacetate poisoning. Examining putative members of the fluoroacetate/fluorothreonine pathway (Scheme 1a) indicated that the 5'-fluorodeoxyadenosine phosphorylase (FIB), the fluoroacetaldehyde dehydrogenase (FAIDH), and the fluorothreonine (FT) transaldolase shared the same transcriptional response pattern with late-stage upregulation based on fluoride addition (Figure 2a,b). However, none of the paralogs of either the methylthioribose-1-phosphate isomerase (MRI) or the fucose-1-phosphate (F1P) aldolase appear to be transcriptionally activated by fluoride or within the same time frame (Figure 2c–e). Nevertheless, these genes are indeed expressed but may be constitutively active or regulated at a post-transcriptional level.²⁶

Identification and Characterization of an Unusual MRI Fusion Enzyme (MRI2). The most unusual characteristic exhibited by *S. cattleya* revealed by the genome sequence perhaps occurs in the third step of the proposed pathway

involving the MRI (Scheme 1A). While it shares a copy of the MRI conserved in streptomycetes (*mri1*) (Supplementary Figure S7), *S. cattleya* also contains a second unusual MRI (*mri2*) fused to a class II aldolase fold that demonstrates homology to methylthioribulose-1-phosphate (MTRu1P) dehydratases and F1P aldolases and could catalyze the downstream step in either methionine salvage or fluorometabolite biosynthesis, respectively. Although the methionine salvage pathway varies in its distribution across the sequenced streptomycetes, both *S. avermitilis* and *S. scabiei* contain genes encoding separate MRIs and MTRu1P dehydratases that lie on opposite strands. Indeed, the only homologues to the MRI/MTRu1P dehydratase fusion in the non-redundant protein database come from the actinomycete *Nocardia farcinica*, one of which is clustered with genes involved in acyl-CoA metabolism (Supplementary Figure S8). Interestingly, the MRI2 fusion enzyme in *S. cattleya* is directly adjacent to a secondary metabolite cluster encoding the production of a hybrid

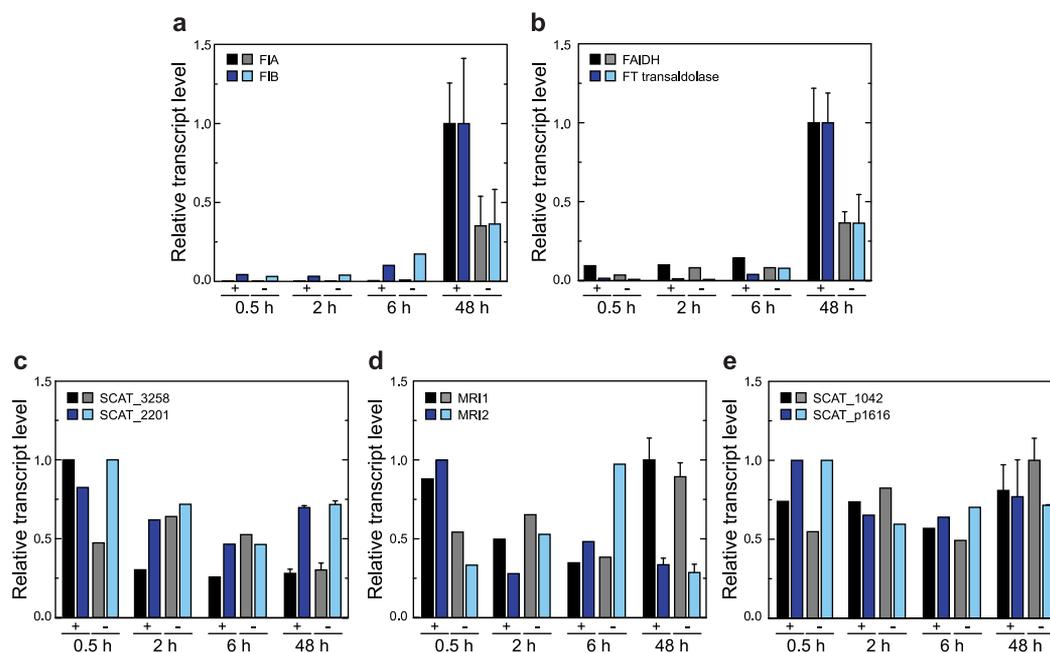


Figure 2. Time- and fluoride-dependent changes in transcript levels of predicted pathway genes and their paralogs. Transcript levels were quantified by RNA sequencing 0.5, 2, 6, and 48 h after the addition of 2 mM sodium fluoride (+) and compared to no addition (–). Values at 48 h are the mean of two replicates, and error bars indicate one SD. Transcription levels of reads per kb were normalized to the maximum value for each predicted open reading frame in parentheses. (a) FIA (36,036 reads/kb) and FIB (1,091 reads/kb), (b) the predicted fluoroacetaldehyde dehydrogenase gene (FAIDH, 2,677 reads/kb) and the fluorothreonine transaldolase gene (FT transaldolase, 975 reads/kb), (c) methylthioadenosine phosphorylase paralogs (SCAT_3258, 432 reads/kb; SCAT_2201, 411 reads/kb), (d) MRI1 (192 reads/kb) and MRI2 (278 reads/kb), and (e) F1P aldolase paralogs (SCAT_1042, 241 reads/kb; SCAT_p1616, 78 reads/kb).

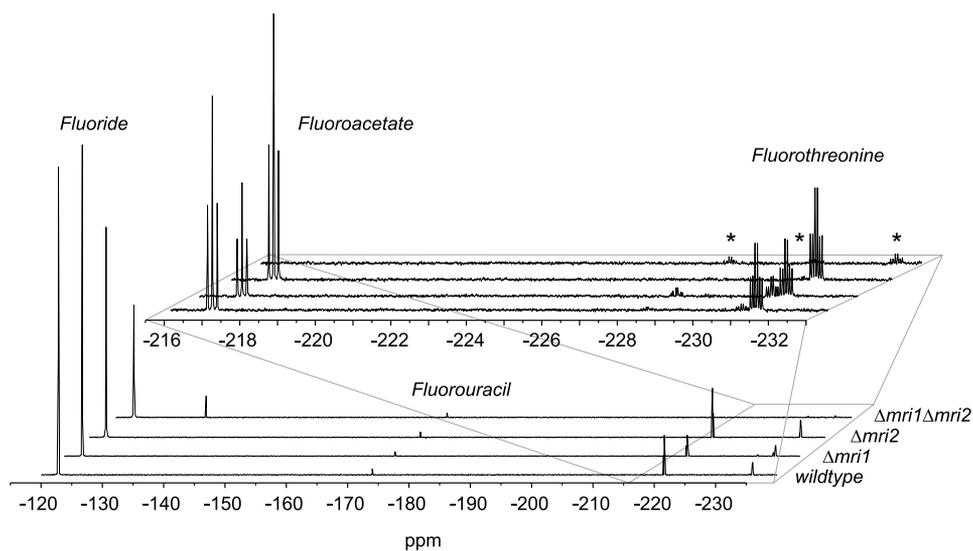


Figure 3. Organofluorine production in *S. cattleya* and MRI knockout strains. ^{19}F NMR analysis of culture supernatants from wildtype, $\Delta mri1$, $\Delta mri2$, and $\Delta mri1\Delta mri2$ strains of *S. cattleya* 6 d after the addition of sodium fluoride (2 mM).

polyketide/nonribosomal peptide (Supplementary Figures S9 and S10), which could potentially implicate the use of an unknown fluorinated subunit as a vehicle for fluorine introduction into a complex natural product in a manner analogous to chlorine in salinosporamide A.²⁷

The shared MRI (MRI1) had previously been shown to accommodate fluorine as a substituent in place of the thiomethyl group.⁸ We therefore set out to biochemically characterize the MRI fusion enzyme (MRI2). *In vitro* studies indicate that MRI2 can utilize fluorinated substrates and that the function of the C-terminal fusion is the MTRu1P

dehydratase reaction utilized in methionine salvage (Supplementary Figure S11). Interestingly, no MTRu1P intermediate was observed with the thiomethyl-substituted substrate, possibly implicating substrate channeling or local concentration effects resulting from the fusion of the two activities that could distinguish the two MRI-dependent pathways *in vivo*.

In order to further assess the role of MRI1 and MRI2 in fluoroacetate (6) and fluorothreonine (7) production, we disrupted the genes in the *S. cattleya* chromosome *via* a stable double-crossover. *S. cattleya* $\Delta mri1$ continues to exhibit an organofluorine profile similar to that of wildtype *S. cattleya*

Table 1. Kinetic Parameters for Acetate Assimilation Enzymes^a

			k_{cat} (s^{-1})	K_{M} (μM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{M}^{-1} \text{s}^{-1}$)
ACS	<i>S. cattleya</i>	acetate	40 ± 1	9 ± 1	(4.4 ± 0.5) × 10 ⁶
		fluoroacetate	2.03 ± 0.08	(5.2 ± 0.9) × 10 ⁴	(3.9 ± 0.7) × 10 ¹
	<i>E. coli</i>	acetate	1.04 ± 0.03	6 ± 1	(1.7 ± 0.3) × 10 ⁵
		fluoroacetate	0.14 ± 0.01	(8 ± 3) × 10 ⁴	(1.8 ± 0.7) × 10 ⁰
AckA	<i>S. cattleya</i>	acetate	410 ± 20	(1.7 ± 0.2) × 10 ⁴	(2.4 ± 0.3) × 10 ⁴
		fluoroacetate	1.04 ± 0.01	(5.7 ± 0.4) × 10 ³	(1.8 ± 0.1) × 10 ²
	<i>E. coli</i>	acetate	(1.34 ± 0.03) × 10 ³	(2.7 ± 0.2) × 10 ⁴	(5.0 ± 0.4) × 10 ⁴
		fluoroacetate	6.7 ± 0.2	(3.3 ± 0.4) × 10 ⁴	(2.0 ± 0.3) × 10 ²
PTA	<i>S. cattleya</i> ^b	acetyl-CoA	4.0 ± 0.2	200 ± 20	(2.0 ± 0.2) × 10 ⁴
		fluoroacetyl-CoA	160 ± 8	670 ± 70	(2.4 ± 0.3) × 10 ⁵
	<i>E. coli</i> ^c	acetyl-CoA	37 ± 1	160 ± 20	(2.3 ± 0.3) × 10 ⁵
		fluoroacetyl-CoA	430 ± 20	160 ± 30	(2.7 ± 0.5) × 10 ⁶

^aInitial rates for ACS and AckA were determined with respect to acetate (8) and fluoroacetate (6), whereas initial rates for PTA were determined with respect to acetyl-CoA (10) and fluoroacetyl-CoA (9). Kinetic parameters were obtained by nonlinear fitting of the dose–response curves to the Michaelis–Menten equation. All data are ±SD ($n = 3$). ^bRate in the acyl-CoA forming direction: acetyl-CoA 3.5 ± 0.1 $\mu\text{M s}^{-1} \mu\text{M PTA}^{-1}$; fluoroacetyl-CoA 3.4 ± 0.1 $\mu\text{M s}^{-1} \mu\text{M PTA}^{-1}$. ^cRate in the acyl-CoA forming direction: acetyl-CoA 32 ± 3 $\mu\text{M s}^{-1} \mu\text{M PTA}^{-1}$; fluoroacetyl-CoA 80 ± 10 $\mu\text{M s}^{-1} \mu\text{M PTA}^{-1}$.

(with accelerated fluoride uptake), while the $\Delta mri2$ strain appears to have lost the ability to produce unidentified and low-abundance organofluorines observed in the wildtype strain (Figure 3 and Supplementary Figure S12). Although the disappearance of the unidentified organofluorines in $\Delta mri2$ profile is interesting with regard to the production of novel fluorinated natural products, these results indicate that neither MRI1 nor MRI2 appear to be specifically dedicated to fluoroacetate (6) and fluorothreonine (7) biosynthesis. In contrast, the $\Delta mri1\Delta mri2$ double-knockout strain biosynthesizes only low levels of unidentified organofluorines that are different than the wildtype, $\Delta mri1$, and $\Delta mri2$ strains under the same growth conditions without producing fluoroacetate (6) or fluorothreonine (7) (Figure 3). These observations support the model that an MRI-catalyzed step is indeed on pathway to fluoroacetate and fluorothreonine production as previously proposed²⁸ and that disruption of either *mri1* or *mri2* can be compensated by the second copy. However, it is difficult to conclude whether the change in organofluorine profile is related to direct disruption of the biosynthetic pathway or by regulatory changes tied to the *S. cattleya* growth and metabolic cycle since the $\Delta mri1\Delta mri2$ strain maintains a deficiency in fluoride uptake as well as growth differences in liquid and on solid media (in the presence and absence of fluoride) that may be related to those reported for gene disruption and overexpression in the *mri1* locus for related actinomycetes (Supplementary Figure S12).^{29,30}

FIK Is Not Absolutely Required for Fluoroacetate Resistance. A major question that we set out to explore is how *S. cattleya* is able to manage the toxic intracellular production of fluoroacetate (6), given its high effectiveness as an inhibitor of the TCA cycle (Scheme 1b). The fluoroacetyl-CoA (9) specific thioesterase, FIK, has been proposed to be involved in resistance³¹ as it can selectively reverse the formation of fluoroacetyl-CoA (9) with a 10⁶-fold selectivity over acetyl-CoA (10) and has been shown to confer fluoroacetate (6) resistance to *E. coli* (Scheme 1b).³² However, FIK may not be the sole source of fluoroacetate (6) resistance, which could also involve acetate (8) assimilation, fluorocitrate (11) production, or aconitase reactivity with fluorine-based enzyme selectivity. To test this possibility, we first disrupted the *flk* gene in the *S. cattleya* chromosome by homologous recombination and

characterized the resulting Δflk strain with respect to fluoroacetate (6) resistance. Although the Δflk strain retains a slight growth defect, it does not demonstrate any appreciable change in organofluorine production, growth rate, or final optical density resulting from fluoride or fluoroacetate (6) addition under organofluorine production conditions (Supplementary Figure S13). Thus, any FIK-independent resistance mechanisms identified in this study could provide a roadmap for constructing a fluoroacetate-resistant host that retains the ability to generate and work with the fluoroacetyl-CoA (9) building block for downstream production of complex organofluorines.

***S. cattleya* Has a Lowered Capacity for Acetate Assimilation.** Analysis of the *S. cattleya* genome shows that it contains a single copy of a malate synthase but is missing an isocitrate lyase that would be required to constitute a functional glyoxylate shunt, which is canonically utilized for cell growth on acetate (8). The absence of a complete glyoxylate shunt may indicate that *S. cattleya* may be able to reduce fluoroacetate (6) entry into the TCA cycle and the resulting toxicity by avoiding utilization of acetate (8) for cell growth. Indeed, our initial studies show that *S. cattleya* does not grow on acetate (8) as a sole carbon source under conditions in which *S. coelicolor* can. Other pathways for acetate (8) uptake and activation to acetyl-CoA (10) include the acetate kinase (AckA)/phosphotransacetylase (PTA) and the acetyl-CoA synthetase (ACS) pathways (Scheme 1b). Similar to five streptomycetes with completely sequenced and annotated genomes (*S. coelicolor*, *S. avermitilis*, *S. scabiei*, *S. bingchengensis*, and *S. griseus*), *S. cattleya* contains one copy of each of these genes.

These enzymes were cloned from *S. cattleya* genomic DNA and heterologously expressed in *E. coli* (Supplementary Figure S14). The steady-state kinetic parameters for fluorinated and nonfluorinated substrate congeners were then measured and compared to orthologous enzymes from *E. coli*, a bacterium known to be susceptible to fluoroacetate (6) poisoning via the AckA/PTA pathway.³³ Studies on the *S. cattleya* ACS suggest that *S. cattleya* is unable to assimilate fluoroacetate (6) through its ACS and are in agreement with those from the *E. coli* ACS, implying that fluoroacetate (6) is not imported through this pathway (Table 1). Examination of the kinetic parameters for AckA and PTA individually from *S. cattleya* and *E. coli* suggest

Table 2. Kinetic Parameters for Selected Citrate Synthases^a

			k_{cat} (s^{-1})	$K_{0.5}$ (μM)	n	$k_{\text{cat}}/K_{0.5}$ ($\text{M}^{-1} \text{s}^{-1}$)
<i>S. cattleya</i>	Cit1	acetyl-CoA	8.0 ± 0.3	15 ± 2	1.0 ± 0.1	$(5.3 \pm 0.7) \times 10^5$
		fluoroacetyl-CoA	3.3 ± 0.2	16 ± 4	0.8 ± 0.1	$(2.2 \pm 0.9) \times 10^5$
		oxaloacetate	8.0 ± 0.6	130 ± 20	1.2 ± 0.1	$(6 \pm 1) \times 10^4$
	Cit2	acetyl-CoA	34 ± 1	4.9 ± 0.4	1.7 ± 0.2	$(6.9 \pm 0.6) \times 10^6$
		fluoroacetyl-CoA	1.12 ± 0.02	0.98 ± 0.05	2.0 ± 0.3	$(1.14 \pm 0.06) \times 10^6$
oxaloacetate		24.9 ± 0.6	3.8 ± 0.4	1.3 ± 0.2	$(6.6 \pm 0.7) \times 10^6$	
<i>S. coelicolor</i>	CitA	acetyl-CoA	17.9 ± 0.4	47 ± 2	1.9 ± 0.1	$(3.8 \pm 0.1) \times 10^5$
		fluoroacetyl-CoA	1.94 ± 0.06	15.4 ± 0.8	1.7 ± 0.2	$(1.26 \pm 0.08) \times 10^5$
<i>E. coli</i>	GltA	acetyl-CoA	80 ± 10	170 ± 60	0.8 ± 0.1	$(5 \pm 2) \times 10^5$
		fluoroacetyl-CoA	1.22 ± 0.03	5.1 ± 0.3	1.9 ± 0.3	$(2.4 \pm 0.2) \times 10^5$

^aInitial rates for all the citrate synthases were determined with respect to acetyl-CoA (10) and fluoroacetyl-CoA (9). Additionally, initial rates for the *S. cattleya* citrate synthases were determined with respect to oxaloacetate. Kinetic parameters were obtained by nonlinear fitting of the dose–response curve to the Hill equation. All data are mean \pm SD ($n = 3$).

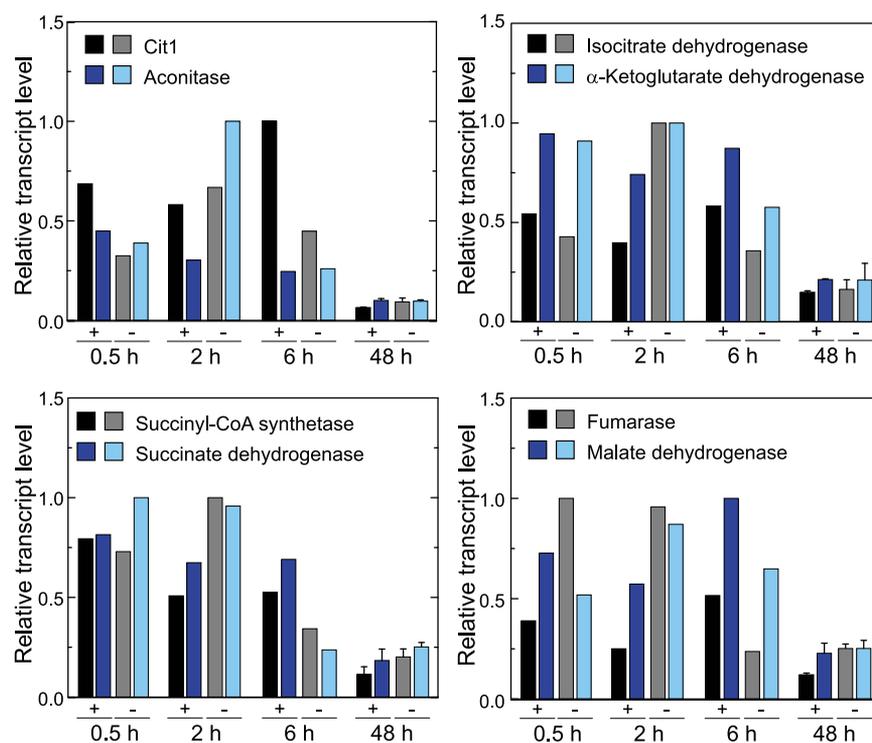


Figure 4. Transcription patterns for TCA cycle genes with respect to fluoride and time. Transcript levels were quantified by RNA sequencing 0.5, 2, 6, and 48 h after the addition of 2 mM sodium fluoride (+) and compared to no addition (–). Values at 48 h are the mean of two replicates, and error bars indicate one SD. Transcription levels of reads per kb were normalized to the maximum value for each predicted open reading frame in parentheses with a representative subunit given for multi-subunit enzymes. Time courses are shown for Cit1 (7,130 reads/kb), aconitase (4,238 reads/kb), isocitrate dehydrogenase (3,495 reads/kb), α -ketoglutarate dehydrogenase (3,094 reads/kb), succinyl-CoA synthetase (5,383 reads/kb), succinate dehydrogenase (2,050 reads/kb), fumarase (2,141 reads/kb), and malate dehydrogenase (6,207 reads/kb).

S. cattleya's enzymes are capable of assimilating acetate (8) and fluoroacetate (6) with a lower overall catalytic efficiency than those of *E. coli*, but with no additional selectivity with respect to fluorine (Table 1).

***S. cattleya* TCA Cycle Enzymes Do Not Exclude Fluorinated Substrates.** The most transferable fluoroacetate (6) resistance mechanism would either be a citrate synthase (CS) that excludes fluoroacetyl-CoA (9) as a substrate or an aconitase that is resistant to fluorocitrate (11) poisoning because either enzyme could be used to replace a synthetic host's native enzymes and allow downstream fluoroacetyl-CoA (9) utilization without toxicity. *S. cattleya* contains four predicted CS paralogs (Cit1–4), with Cit1 as the primary CS (Supplementary Figure S15) and Cit2 as the next most highly

transcribed CS. We cloned and expressed Cit1, Cit2, CitA, and GltA from *S. cattleya*, *S. coelicolor*, and *E. coli* (Supplementary Figure S16) and determined their steady-state kinetic parameters with respect to acetyl-CoA (10) and fluoroacetyl-CoA (9) as well as oxaloacetate for the *S. cattleya* citrate synthases (Table 2, Supplementary Figure S17). Despite its high sequence identity to CitA,³⁴ Cit1 exhibits lower overall catalytic activity and lacks positive cooperativity based on its Hill coefficient. A multiple sequence alignment of Cit1 with 10 orthologs and GltA show that Cit1 contains several mutations of highly conserved residues at the predicted regulatory interfaces³⁵ despite its very high overall similarity (Supplementary Figure S18). Therefore, Cit1 may still have specialized properties with regard to regulation despite its similarity to

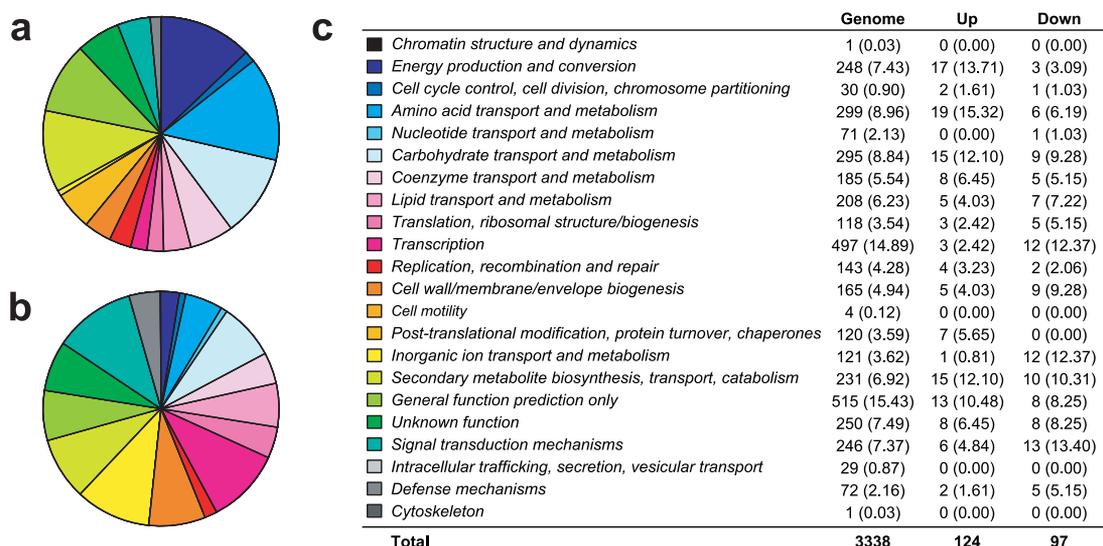


Figure 5. COG categories for genes differentially expressed with respect to fluoride. COG categories were identified by the IMG-ER annotation pipeline. COG categories represented by genes that are (a) upregulated and (b) downregulated in the presence of fluoride at 48 h. (c) Comparison of COG category representation in the differentially expressed genes with that of the entire genome. The number of open reading frames represented by each COG is given, and the percentage of total genes with COG categories is in parentheses.

CitA and GltA under *in vitro* assay conditions. In comparison, a secondary CS from *S. cattleya*, Cit2, displays a k_{cat}/K_M that is an order of magnitude higher compared to the other characterized CSs but is not highly transcribed under these conditions. Further downstream, it appears as if the sole *S. cattleya* aconitase is susceptible to fluorocitrate (11) inhibition based on assays of cell lysates prepared from cultures grown both in the presence and absence of fluoride to control for possible fluoride activation of an auxiliary resistance gene (Supplementary Figure S20). Taken together, it seems as if the *S. cattleya* TCA cycle enzymes remain competent for the lethal synthesis of fluorocitrate (11) and the resulting shutdown of the aconitase-catalyzed step.

Temporal Control of Fluoroacetate Toxicity and Fluoride-Dependent Transcriptional Changes in the TCA Cycle. Since our biochemical studies point to the conclusion that mechanisms based on enzymatic fluorine selectivity do not seem to be the major contributor to *S. cattleya* organofluorine resistance, we then sought to examine changes at the transcriptional level that could also provide resistance by temporal coordination between fluorometabolism and central metabolism. In comparing the expression level of TCA cycle genes over time and with the addition of fluoride, it appears as if they are highly responsive to growth phase but not to fluoride (Figure 4 and Supplementary Figure S21). It therefore appears as if organofluorine biosynthesis is controlled by the cell cycle rather than the converse. Interestingly, an apparent operon of α -ketoglutarate dehydrogenase genes was upregulated in response to fluoride, which may imply that carbon enters the TCA cycle through glutamate or glutamine in order to bypass the CS- and aconitase-dependent steps (Figure 4 and Supplementary Figure S21). Thus, *S. cattleya* likely only turns on the fluorometabolite gene cluster and other related genes once the TCA cycle flux decreases as cells exit exponential growth and biomass accumulation. However, the fluoride dependence of *flA* expression indicates that there are several layers to the regulatory network involving fluoroacetate (6) and fluorothreonine (7) production.

Overall Transcriptional Response to Fluoride. Other transcriptional responses to the presence of fluoride are notable 48 h after the addition of fluoride. In particular, there appears to be a change in 5- and 3-carbon metabolism, which are substrates and products of the fluoroacetate pathway, respectively. A copy of the glyceraldehyde-3-phosphate dehydrogenase, distinct from the glycolytic copy, is the third most highly upregulated protein (27-fold) in the presence of fluoride. Additionally, glycerol-3-phosphate dehydrogenase and glycerol kinase are also upregulated (5-fold each). Changes in 5-carbon metabolism are also observed with upregulation of xylose isomerase (2.9-fold) and xylulose kinase (2.6-fold) and downregulation of L-ribulose-5-phosphate-4-epimerase (2.5-fold), L-ribulokinase (2.5-fold), and L-arabinose isomerase (2.4-fold) in the presence of fluoride. These changes in transcription may be involved with organofluorine biosynthesis as 5-carbon sugar related transcriptional responses could be an alternative approach to controlling pools of the C_5 organofluorine intermediates in the cell.

BiNGO³⁶ was used to examine functional enrichment of differentially expressed genes in the presence of fluoride based on clusters of orthologous groups (COG) categories provided by the IMG-ER annotation (Figure 5). The energy production and conversion COG appears to be overrepresented in genes upregulated in the presence of fluoride, with the majority of these enzymes predicted to be involved in redox reactions (dehydrogenases, reductases, and oxidases). Another enriched COG is amino acid transport and metabolism, among which are aminotransferases, proteases, and amino acid transporters. The final significantly upregulated COG is secondary metabolite biosynthesis, transport, and catabolism, consisting mostly of polyketide synthases and their accessory proteins. For downregulated categories, the signal transduction and inorganic ion transport and metabolism COGs are overrepresented, with the latter resulting from genes predicted to be involved in iron transport and siderophore related processes.

Conclusions. Our group is interested in understanding the underlying design principles that allow an organism to manage an unusual element like fluorine with the long-term goal of

developing a template for expanding the scope of enzymatic fluorination chemistry in living systems. Toward this effort, we have used a combination of genomic, cell profiling, genetic, and biochemical approaches to explore and elucidate the systems-level behavior of *S. cattleya* with respect to fluoride and organofluorine resistance. In this regard, organofluorine biosynthesis appears to be transcriptionally regulated both by fluoride as well as by growth phase. Both the genes encoding the putative FAIDH and the FT transaldolase share this behavior, which is consistent with a biosynthetic role in fluoroacetate (6) and fluorothreonine (7) production, whereas none of the MRI or FIP aldolase paralogs follow the same transcriptional pattern.

Using biochemical methods to assess the fluorine specificity of the acetate activation systems (AckA/Pta and ACS), CS, and aconitase, we have shown that the enzymes from *S. cattleya* do not appear to exclude the fluorinated metabolites as the major mechanism of resistance. Instead, the management of organofluorine toxicity appears to be highly controlled at the transcriptional level where organofluorine production is initiated in stationary phase only after the TCA cycle is shut down. In contrast to organofluorine biosynthesis, we observe no fluoride dependence to the transcription of TCA cycle enzymes, implying that organofluorine production does not control the growth phase of *S. cattleya*. However, the primary CS, Cit1, does appear to have different biochemical and regulatory properties compared to closely related orthologs that may lead to differences in TCA cycle behavior at the post-translational level. Other global shifts in metabolic flux could result from re-routing of carbon flux through glutamate-based anaplerotic reactions based on the upregulation of a secondary α -ketoglutarate dehydrogenase operon as well as changes in 3- and 5-carbon metabolism. These results suggest a metabolic framework to begin exploring the engineered biosynthesis of complex organofluorines from fluoroacetate while avoiding cellular toxicity from the fluorinated building block.

While growth phase-controlled production of secondary metabolites in streptomycetes by either global or pathway-specific transcription factors²⁵ is common, the additional layer of response to fluoride could be interesting with regard to identifying new mechanisms of fluoride-based gene regulation. As there are no fluoride-dependent protein transcription factors known at this time, it is also possible that a constitutively produced organofluorine could be responsible for amplifying expression of pathway genes. There are several predicted transcription factors found within the *fIA* gene cluster, including one (*fIG*) that is upregulated 2.7-fold in the presence of fluoride. However, analysis of the upstream regions of fluoride-regulated pathway genes did not yield any clear consensus sequence, indicating that their transcription may not be controlled by a single specific regulator. Indeed, several predicted transcriptional regulators are differentially expressed in the presence of fluoride, including a member of the *whiB* family thought to be involved in cellular differentiation,³⁷ which interestingly appears not to be conserved in other sequenced streptomycetes. Another possibility is that fluoride-sensing mechanisms based on structural RNAs could be used to control the expression level of a key regulator. In this regard, *S. cattleya* appears to contain a member of the recently reported *crcB* family of fluoride riboswitches²⁶ (Supplementary Figures S22 and S23). However, the known consensus sequences are not found within the *fIA* gene cluster and may suggest a different mechanism or RNA sequence motif.

In addition to elucidating how organofluorine synthesis may be controlled *in vivo*, we are also interested in exploring the question of whether complex organofluorines already exist in *S. cattleya*. Although neither MRI1 nor the MRI2 fusion enzyme appears to be dedicated to fluoroacetate (6) or fluorothreonine (7) biosynthesis, the disruption of both genes leads to the loss of organofluorine production, which may result either from direct involvement of both enzymes in organofluorine production or loss of growth phase activation of fluorometabolite biosynthesis. The disappearance of low abundance organofluorines from the wildtype *S. cattleya* profile upon disruption of *mri2* is also interesting with regard to the production of uncharacterized fluorinated natural products. Biochemical characterization of the MRI1 fusion enzyme indicates that it is competent to react with fluorinated substrates to produce 2,3-diketo-5-fluoropentyl-1-phosphate, which could be on a pathway to generate a fluorinated amino acid *via* the methionine salvage pathway. Like other actinomycetes, *S. cattleya* demonstrates a high potential for production of secondary metabolites from acetate- and amino acid-derived building blocks, some of which may possibly accept alternative building blocks (Supplementary Tables S5 and S6) and is an interesting area for further exploration.

METHODS

De novo Assembly and Annotation of the *S. cattleya* NRRL 8057 Genome from Short Reads. *S. cattleya* ATCC 35852 genomic DNA was isolated using a modified salting-out protocol³⁸ and assayed for purity by amplification and sequencing of the 16S rRNA genes. Four paired-end libraries (Supplementary Figure S1) were sequenced on an Illumina Genome Analyzer to generate 45-base forward and reverse reads (1.5 Gb) and 61-base forward and 45-base reverse reads (2.7 Gb). The short reads were assembled using Velvet v. 1.0.14¹⁷ and Image¹⁸ or SOAP v. 1.05 and GapCloser.²⁰ Putative open reading frames as well as structural RNAs were detected and annotated by submission to the IMG Expert Review pipeline at the Joint Genome Institute.³⁹

Preparation of mRNA for Characterization of the *S. cattleya* Fluoride-Dependent Transcriptional Response. *S. cattleya* was grown in GYM media (pH 5.0) with glass beads (5 mm) to disperse growth. Sodium fluoride (2 mM) was added to the appropriate cultures 24 h after inoculation. Biomass was collected 0.5, 2, 6, 24, and 48 h after the addition of fluoride. Total RNA was isolated using Qiagen RNeasy Mini Kit, and rRNA was depleted in two rounds using the MICROBExpress Kit (Ambion).

RNA Sequencing. mRNA was fragmented before synthesizing cDNA for library preparation. Each lane of RNA-seq reads was mapped to the completed genome of *S. cattleya*²¹ using BWA.²⁴ Reads per feature were then extracted from the alignment file using HTseq-count.⁴⁰ The counts per feature for each condition and time point were quantile normalized using the preprocessCore R package.⁴¹ For differential expression analysis, Multiexperiment Viewer (MeV) was used to perform statistical analysis using edgeR.⁴²

DNA Microarrays. Custom DNA microarrays (Agilent Technologies) were designed using eArray for a total of 15,744 60-mer probes. One sample *t*-test was applied to log₂ fold changes to determine differentially expressed genes.

Construction of Vectors for Protein Expression and Isolation of His-Tagged Proteins. Expression plasmids were constructed by amplification of target genes (Supplementary Table S1) from the appropriate genomic DNA and insertion into the pET16 or 1b (Addgene 29653) vectors. His-tagged proteins were expressed in *E. coli* BL21(de3) at 16 °C and isolated using standard methods. pRARE2 was co-transformed for the expression of *Streptomyces* spp. and *Bacillus subtilis* proteins.

Enzyme Assays. Acetyl-CoA synthetase,⁴³ acetate kinase,⁴³ phosphotransacetylase,⁴⁴ and citrate synthase⁴⁴ were assayed using

literature methods. Phosphotransacetylase activity was also determined in the acyl-CoA forming direction using a discontinuous HPLC assay. MRI2 was assayed using methylthioribose-1-phosphate prepared according a modified literature protocol⁴⁵ and monitored by LC–MS.

Measurement of Aconitase Activity in *S. cattleya* Cell Lysates. *S. cattleya* cell lysates were prepared from cultures using lysozyme (1 mg mL⁻¹) treatment followed by passage through a French pressure cell (25,000 psi). Aconitase was recharged with iron and assayed using a modified literature method to monitor the production of isocitrate.⁴⁶

Construction of *S. cattleya* $\Delta mri1::Hm^R$, $\Delta mri2::Am^R$, $\Delta mri1::Hm^R\Delta mri2::Am^R$, and $\Delta flk::Am^R$ strains. Plasmids for gene disruption contained a cassette consisting of an apramycin (Am) or hygromycin (Hm) resistance marker and an origin of transfer (*oriT*) flanked by *S. cattleya* genomic DNA sequence derived from the upstream and downstream regions (2 kb) of the open reading frame to be disrupted. Plasmids were transferred into *S. cattleya* by conjugation with *E. coli* GM272 pUZ8002 using a modified literature protocol.³⁸ Disruption of the target gene was verified by sequencing following isolation of the exconjugants.

Organofluorine Production. Single colonies of *S. cattleya* wildtype, $\Delta mri1::Hm^R$, $\Delta mri2::Am^R$, and $\Delta mri1::Hm^R\Delta mri2::Am^R$ strains were grown in GYM pH 5 with glass beads. After 24 h, sodium fluoride (2 mM) was added to the cultures and aliquots were removed at 1, 2, 3, 6, 9, 12, and 15 d after fluoride addition. Fluoride concentration in the culture supernatant was measured using a fluoride ion selective electrode (Mettler Toledo) and organofluorine production was monitored by ¹⁹F NMR after lyophilizing the supernatant with a fluorouracil internal standard.

Complete Materials and Methods. Detailed procedures can be found in the Supporting Information.

■ ASSOCIATED CONTENT

■ Supporting Information

Full description of materials and methods, sequences of oligonucleotides, genome assembly statistics, gene cluster diagrams, RNaseq and DNA microarray data, SDS-PAGE, phylogenetic and biochemical data for enzymes, and data for knockout strains. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank B. Thuronyi for cloning the *E. coli* *ackA* and *pta* genes and J. Gomez-Escribano and M. Bibb (John Innes Centre) for materials and assistance in developing conjugation protocols for *S. cattleya*. We would also like to acknowledge B. Curry (Agilent Technologies) and C. Canlas (College of Chemistry NMR Facility) for assistance in analysis of custom microarrays and ¹⁹F NMR, respectively. This work was funded by the generous support of the Arnold and Mabel Beckman Foundation, the Hellman Family Fund, and a National Institutes of Health New Innovator Award (1 DP2 OD008696). M.C.W. and A.M.W. also acknowledge the support of a National Institutes of Health NRSA Training Grant (1 T32 GMO66698) and a National Science Foundation Graduate Research Fellowship (to A.M.W.). The College of Chemistry NMR Facility at the University of California, Berkeley is supported in part by the National Institutes of Health (1S10RR023679-01 and S10 RR16634-01).

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