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Cosynthesis of monofluoroacetate and 4-fluorothreonine by resting cells of blocked mutants of *Streptomyces cattleya* NRRL8057

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Dedicated to Professor Dr. Kenji Soda on the occasion of his 70th birthday

Abstract

Streptomyces cattleya NRRL 8057 produces monofluoroacetate and 4-fluorothreonine from inorganic fluoride. Mutants blocked in fluorometabolite production were prepared by chemical mutagenesis, and cosynthesis experiments with these blocked mutants were carried out by suspending cells of one blocked mutant in the supernatant broth of another blocked mutant. The harvest age of the cells, pH of the buffer, potassium fluoride concentration and glycerol supplementation were optimized for the monofluoroacetate production by a resting-cell suspension of *S. cattleya*. Successful cosynthesis with pairs of the mutants characterized four distinctive blocked sites in the order N-82, N-44, N-43 and N-47. Additional preparation of blocked mutants by UV irradiation and their cosynthesis assay confirmed that U-303, U-304, U-400 and U-500 were blocked in later steps than N-47. O'Hagan et al. recently proposed that fluoroacetaldehyde, the hypothetical precursor of monofluoroacetate and 4-fluorothreonine, derives from 5′-fluoro-5′-deoxyadenosine, the first fluorinated metabolite synthesized from *S*-adenosyl-L-methionine and inorganic fluoride by the novel enzyme 'fluorinase'. We were able to detect fluorinase activity in crude extracts of wild type and N-47 mutant strains, but not in the other mutant strains whose blocked steps flanked that of N-47.

Keywords: Cosynthesis; *Streptomyces cattleya*; Monofluoroacetate; 4-Fluorothreonine; Fluorinase

1. Introduction

Biosynthesis of fluorinated compounds is interesting, not only at the mechanistic level, but also because of prospects for biotransformation routes to fluorine-substituted compounds through some enzyme reactions. Naturally occurring fluorinated compounds, although limited in number, may indicate the occurrence of biological fluorination. Monofluoroacetate, accumulating in leaves and seeds of a range of tropical and subtropical plants [1–3], has been known to show intense toxicity when metabolized to monofluorocitrate, a potent and specific inhibitor of *cis*-aconitase of the TCA-cycle [4–6]. Although these tropical plants in the form of callus cultures retained the ability to produce monofluoroacetate, the yield was small and the reproducibility was not high enough for the mechanistic study of biological fluorination in plant cells [7,8].

In 1986, Sanada et al. reported that *Streptomyces cattleya* NRRL 8057, a thienamycin producer,

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produces monofluoroacetate and 4-fluorothreonine from inorganic fluoride. Resting cells of *S. cattleya* produced monofluoroacetate and 4-fluorothreonine from inorganic fluoride added to the suspension buffer [9]. The bacterium provided a more convenient system than plants for studying biochemical synthesis of fluorinated compounds, and hypothetical biosynthetic precursors, such as glycerol and β-hydroxypyruvate [10], glycolate [11] and β-fluoropyruvate [12] have been suggested, based on studies of the incorporation of labeled compounds.

Recently, O'Hagan et al. reported a novel enzyme in a crude cell extract of *S. cattleya*, which converted *S*-adenosyl-L-methionine (SAM) and fluoride to 5′-fluoro-5′-deoxyadenosine (5′-FDA) and L-methionine, and named it 'fluorinase' [13] (Fig. 1). The biotransformation of synthetic 5′-FDA by a crude cell extract of *S. cattleya* was also carried out in a ^{19}F NMR spectrometer, giving the signals of 5′-fluoro-5′-deoxyinosine, fluoroacetaldehyde and fluoroethanol besides the signals of monofluoroacetate and 4-fluorothreonine [14]. Unfortunately, any other ^{19}F-signals that may account for the transformation of 5′-FDA to fluoroacetaldehyde were not detected by continuous observation of ^{19}F NMR spectra.

Blocked mutants that accumulate biosynthetic intermediates would serve to identify the '*invisible*' biosynthetic intermediates. For this purpose, we obtained non-producing mutants of *S. cattleya* by chemical mutagenesis and UV irradiation, and characterized these mutants by cosynthesis. Our cosynthesis was carried out in liquid; biosynthetic intermediates that accumulated in the resting-cell broth of *secretor* were separated from the cells by centrifugation and filtration, and then given to the *converter* cells. Monofluoroacetate and 4-fluorothreonine were unambiguously identified on ^{19}F NMR spectra. The mutants obtained and characterized in our study will aid understanding of the biosynthetic relevance of fluorinase activity to fluorometabolite production in *S. cattleya*.

2. Experimental

2.1. Organisms and media

Streptomyces cattleya NRRL 8057 was obtained from Institute of Fermentation Osaka, and used as a parental strain. Mutants N-43, N-44, N-47, N-82, N-101, N-105, and N-127, which produce monofluoroacetate at rates of less than 10% of the parental strain, were prepared by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine treatment of the parent spores [15]. The parental and mutant strains were maintained as spores or mycelia suspended in 20% (w/v) glycerol at −70 °C. Cultivation was initiated by inoculating the suspension into the medium. Medium K contained 2.5% (w/v) soluble starch, 1.5% soybean flour, and 0.2% yeast extract, and normally showed pH 6.3 before and after autoclave sterilization. Potassium fluoride was supplemented as the fluorine source at a concentration of 10 mM in liquid media, or 40 mM in agar plate media.

2.2. Mutation by ultraviolet light irradiation

The spore suspension (10^5 spores/50 μl) was spread on Tryptic Soy Broth agar plates, and allowed to dry in a clean bench for 30 min. The plates were irradiated with UV light (Toshiba GL15 lamp) for 10, 20, 30, 40 and 50 s (3.3×10^{-4} J/cm^2/s), and incubated at 30 °C in the dark for 3 days. Growing colonies were

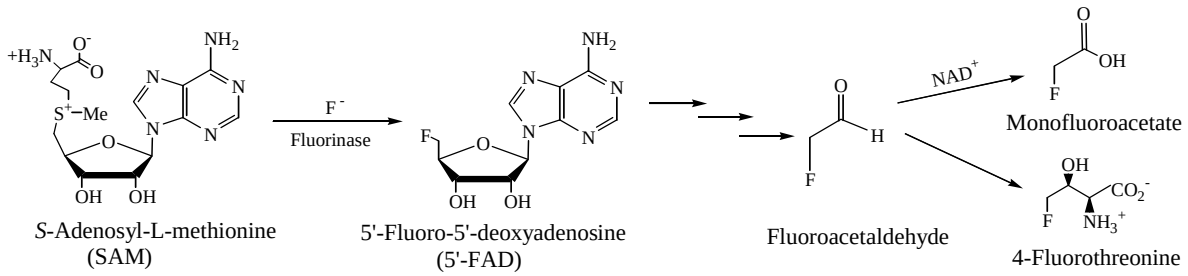

Fig. 1. Proposed pathway of biotransformation of inorganic fluoride to monofluoroacetate and 4-fluorothreonine in *S. cattleya*.

transferred to a pair of agar-plates of minimum media [16] with or without addition of 0.1% (w/v) L-amino acid. L-Arginine, glycine, L-serine, L-histidine and L-threonine were tested. The survival rate and the appearance of amino acid auxotroph mutants were plotted as a function of irradiation time.

2.3. Determination of monofluoroacetate in culture broth and resting-cell broth of S. cattleya

Monofluoroacetate in culture broth and resting-cell broth was derivatized to the methyl ester and determined by capillary gas chromatography with a flame ionization detector installed. Cells were removed from the sample broth by centrifugation, and 1 ml of the supernatant solution was mixed with 50 μl of 10 mM DL-2-hydroxy-*n*-butyric acid solution as an internal standard. The mixture was evaporated to dryness, dissolved in 0.1 ml of 20% (v/v) H_2SO_4, and extracted twice with 500 ml of ethyl ether. The combined ether extracts were mixed with 0.1 ml of methanol, and the ether was allowed to evaporate at ambient temperature. Derivatization with diazomethane and gas chromatographic analysis was carried out as described previously [10]. Methyl monofluoroacetate and methyl DL-2-hydroxy-*n*-butyrate were eluted at 6.5 and 12 min, respectively. The amount of monofluoroacetate was estimated from the peak area of methyl monofluoroacetate and the internal standard.

2.4. Cosynthesis by cell suspension

Cosynthesis is a metabolic complementation test, in which biosynthetic intermediates *secreted* by one blocked mutant are *converted* to end products by another blocked mutant. The complementation can be observed only when the '*secretor*' mutant lacks the enzyme catalyzing a step later than the '*converter*' mutant [17–19]. Accordingly, the mutant strain used as the *secretor* was grown in 100 ml of medium K at 37 °C for 3 days. The cells were washed with 100 ml of 0.7% (w/v) NaCl solution and suspended in 25 ml of 50 mM sodium MES buffer, pH 6.5, supplemented with 50 mM glycerol. The cell suspension was incubated at 37 °C for 3 days under aeration. The cells were completely removed by centrifugation and filtration using a bottle top filter (Corning, 0.45 μm). The filtrate was lyophilized to dryness. The lyophilized powder was dissolved in 4 ml of deionized sterile water, and mixed with 10 mM potassium fluoride. If necessary, the pH was adjusted to 6.5. Cells of *converter*, prepared by growing the other blocked mutant in 8 ml of medium K at 37 °C for 5 days, were suspended with this solution and incubated at 37 °C for 3 days under aeration. The cells were removed by centrifugation, and the supernatant solution was lyophilized to dryness. The dried powder was dissolved in 800 ml of D_2O for ^{19}F NMR analysis. Monofluoroacetate and 4-fluorothreonine were detected at −217 and −232 ppm, respectively, on ^{19}F NMR spectra obtained at 188 MHz with a Varian VXR-200 spectrometer. The *secretor/converter* roles were switched in a parallel experiment to confirm the unilateral complementation between the two mutant strains.

2.5. Fluorinase assay of blocked mutants

Blocked mutants of *S. cattleya* were grown for 6 days in 500 ml medium as previously described [11]. The cells were harvested and washed with Tris buffer (50 mM, pH 7.8) and suspended in the same buffer (0.1 g wet cell/ml). Ultrasonication and centrifugation gave the supernatant solution that was used directly as the crude extract, without dialysis, as previously described [14]. Transformation was initiated by adding 0.4 mM *S*-adenosyl-L-methionine and 10 mM NaF to the crude cell extract, and the reaction mixture was incubated at 28 °C for 24 h. ^{19}F NMR spectra were recorded on a Varian 300 MHz NMR spectrometer at an ambient temperature.

3. Results and discussion

3.1. Mutation

L-Arg was chosen as the indicator of auxotroph mutant of *S. cattleya* for it was a better indicator than any of glycine, L-serine, L-histidine, and L-threonine. Although the survival rate decreased linearly as the function of UV irradiation time from 10 to 50 s, the L-Arg auxotroph mutant appeared mostly at the rates of 12% on 40 s irradiation and 7% on 30 s irradiation, and less than 1% when irradiated for shorter or longer than these time periods. Mutants blocked in

fluorometabolite production, U-303, U304, U-400 and U-500, were obtained under the 30-, 40- and 50 s irradiation.

3.2. Effect of culture age, pH, fluoride concentration, and precursors on monofluoroacetate production by resting cells

For the purpose of establishing a reproducible cosynthesis assay system, monofluoroacetate production by resting cells was optimized. First, monofluoroacetate production was monitored in the culture broth of *S. cattleya* grown at 30 and 37 °C (Fig. 2A). Incubation at 37 °C yielded slightly better production than incubation at 30 °C until 5 days. Although temperature affected monofluoroacetate production more significantly at a later stage, around 18 days, such long-term cell preparation was not employed in the resting-cell study due to its technical inconvenience. Aeration appeared to be another important factor for monofluoroacetate production when *S. cattleya* was grown in various volumes of K medium in a 1 l Sakaguchi flask with aeration at 100 rpm (Fig. 2B). Adequate aeration may be required in some oxidative enzyme reactions during the course of biosynthesis of monofluoroacetate.

Resting-cell suspensions were prepared by harvesting batch cultures grown at 37 °C under optimized aeration. Monofluoroacetate production by cells grown for 3, 5 and 7 days was assessed after the cells were suspended in 50 mM MES buffer containing 10 mM KF and 50 mM glycerol at pH 5.0–7.0 for 72 h (Fig. 3A). Enzyme systems for monofluoroacetate production appeared to be maximally induced by 5 days. Maximal monofluoroacetate production was observed at pH 6.5 but fell sharply at both higher and lower pH. Indeed, the decrease in monofluoroacetate production with increasing pH was so marked that at pH 7.0 the production was only 12% of that at pH 6.5. The buffer pH may not significantly influence the dissociation state of fluoride anion because pK_a value of fluoride anion is 3.2. Perhaps, a narrow optimal pH range for the monofluoroacetate production by cell suspension may reflect the optimal pH of a fluoride uptake system induced in *S. cattleya* [11]. Fluoride concentration also has a narrow optimal range for monofluoroacetate production (Fig. 3B). Obviously, monofluoroacetate production by resting cells depended on the supplementation of inorganic fluoride in the buffer. The optimal KF concentration fell in a narrow range around 10 mM, suggesting that higher concentrations of fluoride can exert adverse effects on the cellular system by inhibiting some enzyme reactions. Accordingly, all the reactions were carried out with 10 mM KF at pH 6.5 in the present study.

Possible metabolic precursors were incubated with the cell suspensions for 72 h in the presence of 10 mM potassium fluoride, and the monofluoroacetate production was compared with that of suspensions incubated without supplementation. With 50 mM glycerol, monofluoroacetate production was 2.8 times that of incubation with fluoride alone (Fig. 3C). Slight decreases in monofluoroacetate production were observed when 10 mM creatine (95%), 10 mM β-hydroxypyruvate (82%), and 10 mM glycine (81%) were replaced with 50 mM glycerol. Complete

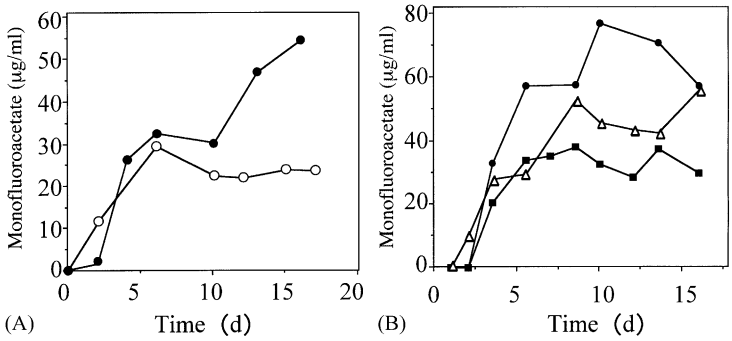

Fig. 2. Effects of temperature and aeration on monofluoroacetate production in culture broth. (A) *S. cattleya* was grown in 50 ml of medium K contained in a 500 ml Sakaguchi flask at 30 °C with aeration at 120 rpm (○) or at 37 °C with aeration at 100 rpm (●). (B) *S. cattleya* was grown in 100 (●), 400 (△) and 800 (■) ml of medium K in 1 l Sakaguchi flask with aeration at 100 rpm at 37 °C.

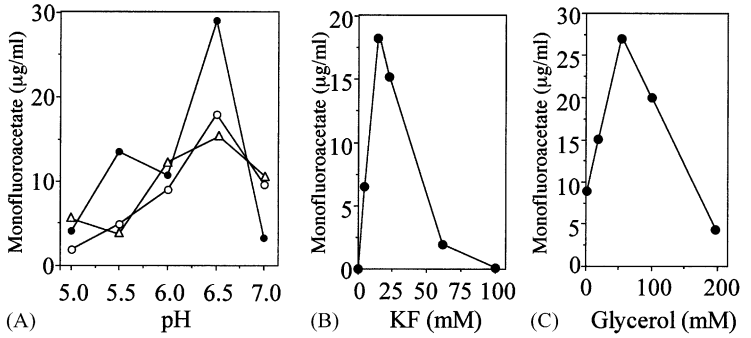

Fig. 3. Effects of culture age and pH (A), KF concentration (B) and glycerol concentration (C) on monofluoroacetate production by cell suspensions of *S. cattleya*. (A) The cell suspension (0.2 g wet cell/ml) prepared from 3-day-old (○), 5-day-old (●) and 7-day-old (△) culture was incubated for 72 h with 10 mM KF in 100 mM MES buffer in a pH range from 5.0 to 7.0. (B) The cell suspension (0.2 g wet cell/ml) prepared from 5-day-old culture was incubated with designated concentrations of KF and 50 mM glycerol for 72 h in 100 mM MES buffer, pH 6.5. (C) The cell suspension (0.2 g wet cell/ml) prepared from the 5-day-old culture was incubated with designated concentrations of glycerol and 10 mM KF for 72 h in 100 mM MES buffer, pH 6.5.

suppression of monofluoroacetate production was observed when the cells were incubated with 50 mM D-glucose, 10 mM sodium glycerate and 10 mM L-serine.

3.3. Cosynthesis

Among the blocked mutants obtained by chemical mutagenesis, N-101, N-105 and N-127 were found to be low-producer strains, they could produce fluorometabolites on their own. Since the low-producers were not appropriate for the cosynthesis experiments, the four remaining mutants, N-43, N-44, N-47 and N-82 were tested in all combinations. The results of cosynthesis are summarized in Fig. 4. In a set of cosynthesis experiments using the pair of N-43 and N-44, ^{19}F NMR signals of monofluoroacetate and 4-fluorothreonine were observed only when N-43 was the secretor. No fluorometabolites were produced when the *secretor*/*converter* roles were switched between the two blocked mutants. The unilateral complementation suggests that N-43 was producing one

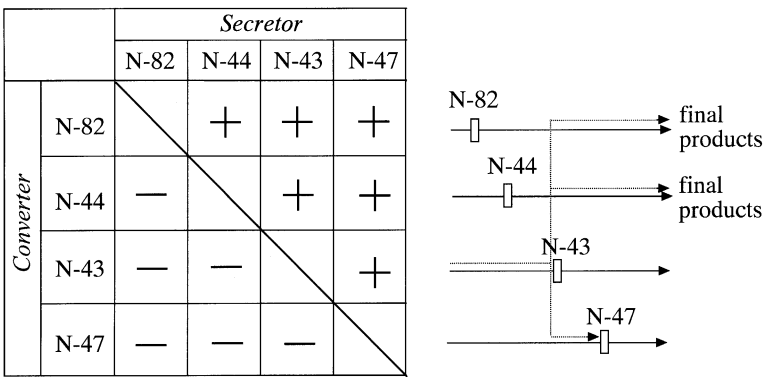

Fig. 4. Cosynthesis of monofluoroacetate and 4-fluorothreonine by blocked mutants N-43, N-44, N-47 and N-82. Left: (+) monofluoroacetate and 4-fluorothreonine clearly detected on 188 MHz ^{19}F NMR; (−) no cosynthesis was observed. Right: an example is illustrated for the mutant N-43 whose biosynthetic intermediate is converted to the final products by N-44 or N-82, but not by N-47. The order of impaired biosynthetic steps was determined as N-82, N-44, N-43 and N-47.

or several intermediates that were converted into the final products by the N-44 strain. Therefore, it was concluded that N-43 and N-44 were blocked at different biosynthetic steps: N-43 being blocked at a later step than N-44. In the combination of blocked mutants N-43 and N-47, fluorometabolites production was observed when N-47 was the secretor, suggesting that N-47 was blocked nearer to the final products than N-43. Thus, it is suggested from the above results that strain N-43 was blocked nearer to fluorometabolites than N-44, but still further than N-47. Accordingly, the relative roles of each mutant as a *converter* or a *secretor* were clearly determined for all combinations of the four stable non-producing mutants, and the order of blocked enzyme reaction was determined as N-82, N-44, N-43 and N-47.

U-303, U-304, U-400 and U-500 were a new series of non-producing mutants, which were obtained for the purpose of identifying the blocked step later than that of the N-47 mutant. All the four new mutants served as a *secretor* when N-47 was the *converter*, and cosynthesis was not observed in the reverse process. These results suggest that these mutants were impaired at biosynthetic steps later than N-47.

3.4. Fluorinase activity

Fluorinase activity was observed in crude extracts of wild type *S. cattleya* and the mutant strain N-47. The characteristic double triplet signals at, 227.4 ppm on ^{19}F NMR spectra were attributed to 5′-fluoro-5′-deoxyadenosine (Fig. 5), but no other ^{19}F signals, such as those of monofluoroacetate and 4-fluorothreonine, were produced in the cell extracts of wild type and N-47 mutant strains. Crude extracts prepared from the blocked mutants, N-43, N-44, N-82, U-303, U-304, U-400, U-500 failed to catalyze the bioconversion of *S*-adenosyl-L-methionine to 5′-FDA. Lack of fluorinase activity in these mutants arouses a question whether fluorinase is really a part of the biosynthetic pathway of monofluoroacetate and 4-fluorothreonine. In fact, the biosynthetic intermediates which connect 5′-FDA and fluoroacetaldehyde have yet to be identified. Although we cannot completely rule out the possibility that fluorinase of these mutant strains requires some precautions when assayed in cell-free system, such as supplementation of metals and cofactors, removal of inhibitory ligands, and protection from thermal and

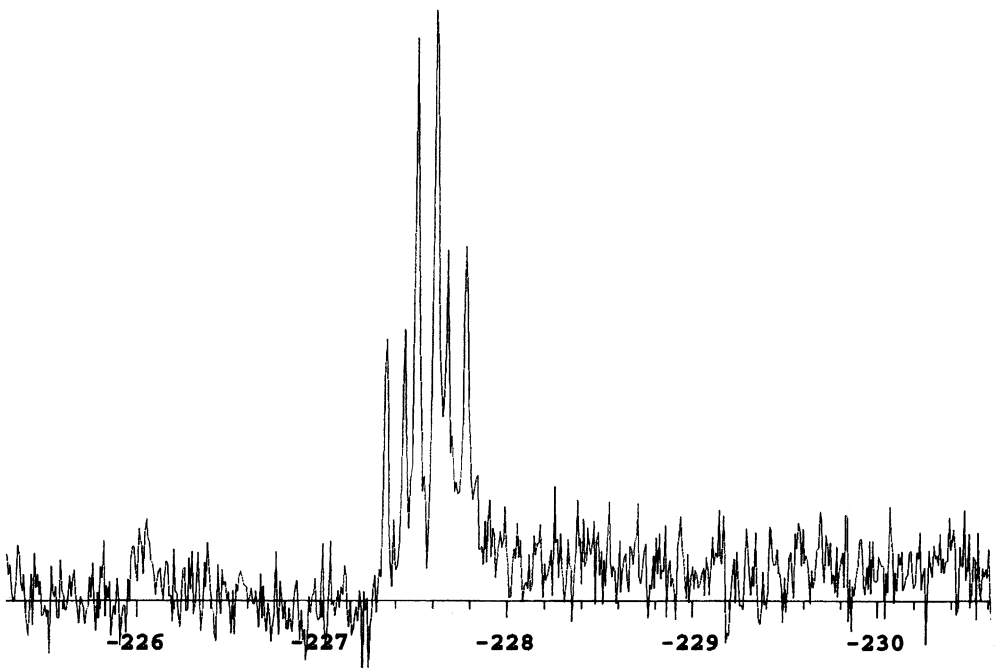

Fig. 5. ^{19}F NMR signal of 5′-FDA produced in crude cell extract of N-47.

oxidative stresses, it is also conceivable from our experiments that the biosynthesis of monofluoroacetate and 4-fluorothreonine may be independent of the formation of 5′-FDA by fluorinase. Blocked mutants obtained and characterized in our study will be useful in elucidating unidentified biosynthetic intermediates, which were '*invisible*' in the continuous observation of crude cell extract on ^{19}F NMR spectroscopy. These blocked mutants have been prepared originally for the purpose of molecular cloning of gene clusters involved in fluorometabolite production in *S. cattleya*. Genetic information may also help us to clarify the biochemical relevance between fluorinase and the fluorine metabolism in *S. cattleya*.

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