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Fluoroacetate biosynthesis from the marine-derived bacterium *Streptomyces xinghaiensis* NRRL B-24674

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Genome sequencing identified a fluorinase gene in the marine bacterium *Streptomyces xinghaiensis* NRRL B-24674. Fermentation of the organism with inorganic fluoride (2mM) demonstrated that the organism could biosynthesise fluoroacetate and that fluoroacetate production is sea-salt dependent. This is the first fluorometabolite producing microorganism identified from the marine environment.

Organofluorine compounds have been widely exploited by the pharmaceutical industry.\(^1\) Well over 20% of current drugs in clinical trials contains a fluorine atom. Fluorinated entities have also found extensive use in agrochemicals and in tuning the properties of performance high-value organic materials.\(^2\) In contrast, nature has hardly evolved a biochemistry of fluorine, and fluorinated natural products are extremely rare.\(^3\) Fluoroacetate \(^1\) is the most ubiquitous fluorometabolite found as a toxic component of many tropical and sub-tropical plants.\(^4\) In 1986, a soil bacterium *Streptomyces cattleya* was shown to have the capacity to produce fluoroacetate \(^1\) and the antibiotic, 4-fluorothreonine \(^2\) when grown in the presence of fluoride ion.\(^5\) Subsequently the origin of the fluorometabolites of *S. cattleya* has been studied and the pathway is shown in Scheme 1.\(^6\) Enzymatic C-F bond formation is catalysed by the fluorinase, which converts S-adenosyl-L-methionine \(^3\) to 5'-fluoro-5'-deoxyadenosine \(^4\). The pathway then progresses through fluororibose phosphate \(^5\) and then fluororibulose phosphate \(^6\). An aldolase catalyses a retro-aldol reaction to generate fluoroacetaldehyde \(^7\), which is processed in two directions; oxidation generates fluoroacetate \(^1\), and a PLP-transaldolase enzyme generates 4-fluorothreonine \(^2\).\(^7\) Fluorinase genes remain sparse. In 2014, more than a decade after the first identification, we reported and assayed three new fluorinases from two terrestrial actinomycetes (Streptomyces sp. MA37 and *Actinoplanes sp.* N902-109) and an actinomycete pathogen, *Norcardia brasiliensis*.\(^8\) *Streptomyces sp.* MA37 produces fluoroacetate \(^1\) and 4-fluorothreonine \(^2\) in culture and also several unidentified fluorometabolites. *N. brasiliensis* was unable to produce fluorometabolites under laboratory culture conditions, and the *Actinoplanes sp* strain, although sequenced, is not available in the public domain to culture. To date the plants and bacteria that produce fluorometabolites are from terrestrial organisms.

More than 70% of our planet’s surface is covered by oceans. Marine ecosystems differ from terrestrial ones substantially, eg. with high chloride concentrations (~0.6M or 19,000 ppm)\(^9\). By contrast, fluoride concentrations average only 1.3 ppm in surface water. Consequently chlorinated natural products dominate halogenated marine metabolite isolates.\(^10\) In 2003, a series of 5-fluorouracil derivatives was isolated from extracts of the marine sponge *Phakellia fusca* Schmidt, collected from the South China Sea.\(^11\)

Considering the direct relationship between these derivatives and the widely-used anticancer drug, it is most likely that the sponge accumulated 5-fluorouracil from industrial effluent rather than by a de novo fluorination biosynthesis.
Here we report that the marine bacterium *Streptomyces xinghaiensis* NRRL B-24674 is a fluoroacetate producer. A fluorinase gene was identified by genome sequencing of the organism. Fluoroacetate production was observed in culture and was found to require high salinity.

*S. xinghaiensis* NRRL B-24674 was isolated in 2009 from a marine sediment sample around Xinghai Bay, in Dalian, China. The strain produces a novel alkaloid which was named xinghaiamine A. Due to its unique phenotype, it was subjected to genome sequencing in 2011 (accession no. AFRP01000000). Its genome sequence was annotated in the RAST server. The length of the deposited sequence is approximately 6.79 Mbp with 2312 contigs. Homologue analysis identified a putative fluorinase gene in the contig with the NCBI access No (AFRP01002228.1) and the encoded protein sequence shared high sequence identity (84%) with the other four known fluorinases, including a 21 amino acids loop, a unique signature of the fluorination enzymes identified so far. (ESI, Figure S2 and Table S2).

**Figure 1.** Organisation of genes around the fluorinase (flA) from the bacterial fluorometabolite producers: A) *S. cattleya* (Spencer cluster); B) *Streptomyces sp.* MA37; C) *Streptomyces xinghaiensis*. The homologous genes are colour coded for visual comparison: flA, fluorinase; flB, purine nucleoside phosphorylase; flF and flG, DNA binding proteins; flH, Na⁺/H⁺ antiporter; flI, S-adenosylhomocysteine lyase; flJ and flL, DNA binding proteins; flK, fluoroacetyl-CoA lyase; flFT, 4-FT transaldolase.

In *S. cattleya* and *Streptomyces sp.* MA37, the gene clusters responsible for fluorometabolite biosynthesis are located relatively close to each other. However, in the newly identified organism *S. xinghaiensis*, the genes encoding the 4-fluorothreonine transaldolase (4-FTase) are located very close to their respective flA homologues only in these latter cases. 4-FTase is a pyridoxal phosphate (PLP) enzyme responsible for the last step in 4-fluorothreonine biosynthesis and it

**Figure 2.** ¹⁹F NMR spectroscopic analysis of fluoroacetate 1 in the supernatant of the culture medium from the marine-derived *S. xinghaiensis* NRRL B24674. Insert: the coupling of fluoroacetate.
appears to contain two domains, the larger one most closely related to a PLP-dependent serine hydroxymethyl transferase (SHMT) motif and the smaller to an epimerase, suggesting that the observed transaldolase activity has evolved from a hybrid construction of two historical activities.\textsuperscript{18} A $\text{flK}$ knockout in \textit{S. cattleya} resulted in a mutant able only to produce fluoroacetate 1, which validated its role in 4-fluorothreonine 2 biosynthesis.\textsuperscript{19} In \textit{S. xinghaiensis} there is a truncated $\text{flK}$ transaldolase with only 96 amino acids in length lying adjacent to the $\text{flA}$ gene which shares a very high sequence identity (70\%) only with the epimerase motif of the other 4-\textit{FT}ases.\textsuperscript{18} Two thirds of the gene seems to be missing and it has no SHMT or PLP binding motif so clearly could not carry out the transaldolase reaction to generate 4-fluoroacetone 2. We are also able to identify three candidate fluorolute 1 biosynthetic genes, those encoding a methylthioribose-1-phosphate isomerase, a fructose aldolase and an alcohol dehydrogenase in the genome of \textit{S. xinghaiensis}. They are not located particularly close to $\text{flA}4$, however this is also the case in \textit{S. cattleya} and \textit{Streptomyces sp. MA37}.

To investigate further, \textit{S. xinghaiensis} was grown in shake flask culture supplemented with fluoride (2mM) in fresh water. It did not behave like other \textit{Streptomyces} in typical \textit{Streptomyces} media such as International \textit{Streptomyces} Protocol (ISP) 2 to 7 and \textit{Streptomyces} Casein medium and failed to produce healthy cell mass. No organofluorine signal was observed in $^{19}$F NMR in these samples. However when the medium was supplemented with artificial sea salt (30g/L) a healthy growth was established suggesting a sea salt dependency for this marine bacterium. The supernatant of a 10-day culture was analysed by $^{19}$F($^3$H)-NMR. The organism produced fluoroacetate 1 (217.44 ppm, $^3$J$^{HF} = 47.8$ Hz) as a sole fluorometabolite (Figure 2). The concentration of 1 rose to ~1mM after 19-d fermentation using a known concentration of an added fluoromethyl containing reference (5'-fluoro-5'deoxy-adenosine) to the NMR sample (ESI, Figure S4). The ability of \textit{S. xinghaiensis} to elaborate fluorolute 1 suggests that the identified biosynthetic cluster plays a similar role to the one in \textit{S. cattleya} and \textit{Streptomyces sp. MA37}. The absence of any 4-fluoroacetone 2 is consistent with the truncated $\text{flFT}$4 gene but its role is unclear.

To the left of the $\text{flFT}$4 gene (Figure 1) are four genes encoding putative auxiliary functions, including DNA regulation ($\text{flF}$, $G$ and $I$ homologues), and transporter functions ($\text{flH}$ homologues), which are also highly conserved in the genes clustered around $\text{flA}$ in all other fluorinase containing organisms (\textit{S. cattleya}, \textit{Streptomyces sp MA37}, \textit{N. brasiliensis} and \textit{Actinoplanes sp.})\textsuperscript{,} Interestingly, the translated sequence of the $\text{FIF}$4 transporter is shortened to only 119 amino acids in length compared to the corresponding one in \textit{S. cattleya} of 185 amino acids. To the right of the $\text{flA}$ gene (Figure 1) are two genes encoding putative auxiliary functions. In \textit{S. cattleya}, their homologous are $\text{orfA}$ and $\text{orfB}$, which are situated adjacent to $\text{flFT}$ on a megaplasmid and very remote from the fluorinase gene $\text{flA}$ which is located on the chromosome. OrfA homologues belong to a superfamily of drug metabolite transporter proteins and they share a high sequence identity (47\%) with ORF1 involved in the biosynthesis of 4-chlorothreonine in \textit{Streptomyces sp. OH-5093}.\textsuperscript{19} At one end of the cluster is $\text{flK}$ coding for a fluorocetyl-CoA thioesterase (70\% sequence identity to $\text{flK}$ in \textit{S. cattleya}). The $\text{flK}$ gene is thought to confer resistance to fluoroacetate cytotoxicity.\textsuperscript{17} The encoded fluorocetyl-CoA thioesterase FIK efficiently hydrolyse fluorocetyl-CoA over acetyl CoA, preventing the conversion of fluorocetyl-CoA to the respiratory toxin fluorocitrate.\textsuperscript{20} In the case of \textit{S. xinghaiensis}, the $\text{flK}$ gene is also in close proximity to the corresponding $\text{flA}$4 gene, consistent with a toxicity resistance role. To explore a link between $\text{flK}$4 and fluorolute 1 biosynthesis an in-frame gene deletion of $\text{flK}$4 was conducted using a temperature-dependent suicidal plasmid pKC1139. About two 2-kbp sequences flanking both sides of $\text{flK}$4 gene were amplified and cloned into pKC1139. The construct was introduced into \textit{S. xinghaiensis} through conjugation, and the double-cross recombination mutant WDDY40 was screened out by PCR. $^{19}$F NMR analysis of the supernatant of the mutant WDDY40 strain demonstrated that the knockout completely abolished the fluorolute 1 production (Fig S5 B), consistent with the previous report in \textit{S. cattleya}.\textsuperscript{21} Complementation of $\text{flK}$4 in the mutant WDDY40, resulting in the mutant WDDY41, restored the production of 1, suggesting a key role for $\text{flK}$4 in the regulation of fluorolute 1 production (Fig S5 C), consistent with a putative toxicity resistance role.

\section*{Conclusions}

\textit{In silico} analysis has indicated that the marine-derived actinomycete, \textit{Streptomyces xinghaiensis}, contains similar genes to those in \textit{S. cattleya} and \textit{Streptomyces sp. MA37} for the biosynthesis of fluoracetate and 4-fluorothreonine. However the cluster in \textit{S. xinghaiensis} has a truncated transaldolase analogous to that involved in the last step of 4-fluorothreonine biosynthesis in the other two organisms. Culturing demonstrated that \textit{S. xinghaiensis} has the capacity to produce only fluoracetate but not 4-fluorothreonine. Production of fluoracetate is sea-salt dependent. Inactivation of the $\text{flK}$, the putative resistance gene to fluoracetate toxicity, encoding a fluorocetyl-CoA thioesterase, resulted in the loss of fluoracetate production, and re-insertion of the gene restored its production. This is the first micro-organism from the marine environment shown to produce a fluorometabolite in culture.

\section*{Notes and references}
\begin{itemize}
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\item The supernatant of \textit{Streptomyces xinghaiensis} culture was collected on time course of 1, 3, 5, 7, 14, 19 days. Each sample was subject to $^{19}$F-NMR analysis. $^{19}$F-NMR spectra were recorded with and without proton decoupling on a Bruker AV-500MHz instrument ($^{19}$F at 470.3 MHz). The chemical shifts of $^{19}$F-NMR were calculated with respect to CFCl3. The identified gene cluster in \textit{Streptomyces xinghaiensis} has already been deposited in European Nucleotide Archive with accession no HG975299.
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