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

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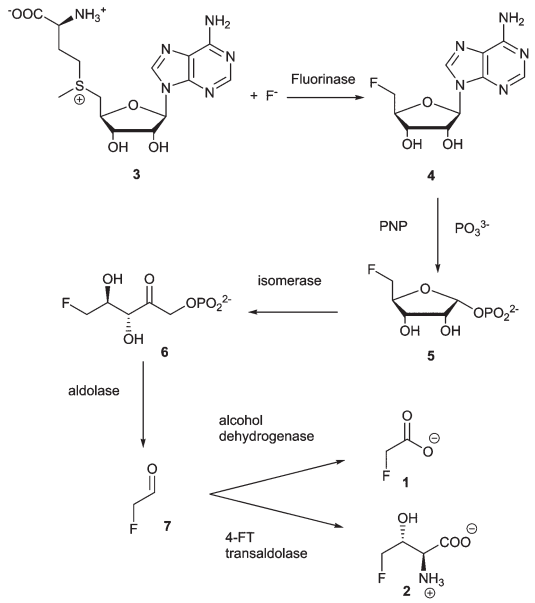
Genome sequencing identified a fluorinase gene in the marine bacterium *Streptomyces xinghaiensis* NRRL B-24674. Fermentation of the organism with inorganic fluoride (2 mM) demonstrated that the organism could biosynthesise fluoroacetate and that fluoroacetate production is sea-salt dependent. This is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



Scheme 1 Biosynthetic pathway to fluoroacetate **1** and 4-fluorothreonine **2** in bacteria.

More than 70% of our planet's surface is covered by oceans. Marine ecosystems differ from terrestrial ones substantially, *e.g.* with high chloride concentrations (~0.6 M or 19 000 ppm).[9]

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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 **1** producer. A fluorinase gene was identified by genome sequencing of the organism. Fluoroacetate **1** 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.[12] The strain produces a novel alkaloid which was named xinghaiamine A.[14] Due to its unique phenotype, it was subjected to genome sequencing in 2011 (accession no. AFRP01000000).[13] Its genome sequence was annotated in the RAST server.[15] 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, Fig. S2 and Table S2†). *In silico* analysis indicated that the fluorinase gene *flA*4 in *S. xinghaiens*is is located immediately adjacent to *flB*4, encoding the second biosynthetic enzyme of the fluoroacetate pathway (Scheme 1), a purine nucleotide phosphorylase (PNP).[16] Unlike the gene arrangement[17] in *S. cattleya*, there is a higher degree of clustering of the genes responsible for fluorometabolite biosynthesis in the more recently identified organisms. For example the genes encoding the 4-fluorothreonine transaldolase (4-FTase) are located very close to their respective *flA* homologues only in these latter cases.[8,21] 4-FTase is a pyridoxal phosphate (PLP) enzyme responsible for the last step in 4-fluorothreonine biosynthesis and it 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.[18] A *flFT* knockout in *S. cattleya* resulted in a mutant able only to produce fluoroacetate **1**, which validated its role in 4-fluorothreonine **2** biosynthesis.[22] In *S. xinghaiensis* there is a truncated *flFT* transaldolase with only 96 amino acids in length lying adjacent to the *flA* gene which shares a very high sequence identity (70%) only with the epimerase motif of the other 4-FTases.[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-fluorothreonine **2**. We are also able to identify three candidate fluoroacetate **1** biosynthetic genes, those encoding a methylthioribose-1-phosphate isomerase, a fructose aldolase and an alcohol dehydrogenase in the genome of *S. xinghaiensis*. They are not located particularly close to *flA*4, however this is also the case in *S. cattleya* and *Streptomyces* sp. MA37.

To investigate further, *S. xinghaiensis* was grown in shake flask culture supplemented with fluoride (2 mM) in fresh water. It did not behave like other *Streptomyces* in typical *Streptomyces* media such as International *Streptomyces* Protocol (ISP) 2 to 7 and Starch 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 (30 g L^{-1}) a healthy growth was established suggesting a sea salt dependency for this marine bacterium. The supernatant§ of a 10-day culture was analysed by $^{19}F\{^{1}H\}$-NMR. The organism produced fluoroacetate **1** (−217.44 ppm, t, $^{2}J_{HF}$ = 47.8 Hz) as a sole fluorometabolite (Fig. 2). The concentration of **1** rose to ~1 mM after 19-d fermentation using a known concentration of an added fluoromethyl containing reference (5′-fluoro-5′deoxyadenosine) to the NMR sample (ESI, Fig. S4†). The ability of *S. xinghaiensis* to elaborate fluoroacetate **1** suggests that the identified biosynthetic cluster plays a similar role to the one in *S. cattleya* and *Streptomyces* sp. MA37. The absence of any 4-fluorothreonine **2** is consistent with the truncated *flFT*4 gene but its role is unclear.

To the left of the *flFT*4 gene (Fig. 1) are four genes encoding putative auxiliary functions, including DNA regulation (*flF*, *G* and *I* homologues), and transporter functions (*flH* homologues), which are also highly conserved in the genes clustered around *flA* in all other fluorinase containing organisms (*S. cattleya*, *Streptomyces* sp. MA37, *N. brasiliensis* and *Actinoplanes* sp.). Interestingly, the translated sequence of the FlF4 transporter is shortened to only 119 amino acids in length compared to the corresponding one in *S. cattleya* of 185 amino acids. To the right of the *flA* gene (Fig. 1) are two genes encoding putative auxiliary functions. In *S. cattleya*, their homologous are *orfA* and *orfB*, which are situated adjacent to *flFT* on a megaplasmid and very remote from the fluorinase gene *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 *Streptomyces* sp. OH-5093.[19]

At one end of the cluster is *flK*4 coding for a fluoroacetyl-CoA thioesterase (70% sequence identity to *flK* in *S. cattleya*). The *flK* gene is thought to confer resistance to fluoroacetate cytotoxicity.[17] The encoded fluoroacetyl-CoA thioesterase FlK efficiently hydrolyse fluoroacetyl-CoA over acetyl CoA, preventing the conversion of fluoroacetyl-CoA to the respiratory toxin fluorocitrate.[20] In the case of *S. xinghaiensis*, the *flK*4 gene is also in close proximity to the corresponding *flA*4 gene, consistent with a toxicity resistance role. To explore a link between *flK*4 and fluoroacetate **1** biosynthesis an in-frame gene deletion of *fl*K4 was conducted using a temperature-dependent suicidal plasmid pKC1139. About two 2-kbp sequences flanking both sides of *fl*K4 gene were amplified and cloned into pKC1139. The construct was introduced into *S. xinghaiensis* through con-

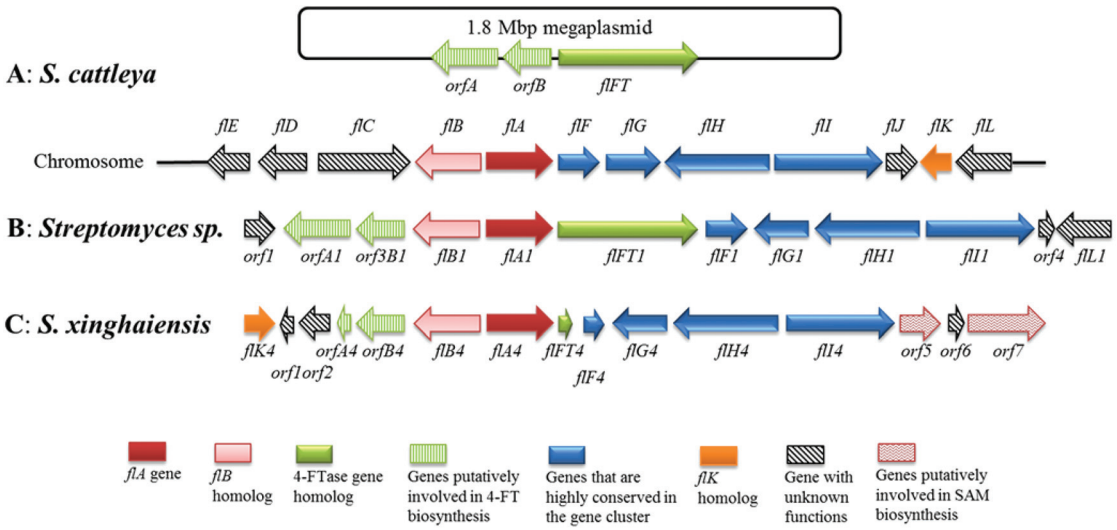

Fig. 1 Organisation of genes around the fluorinase (*flA*) from the bacterial fluorometabolite producers: (A) *S. cattleya* (Spencer cluster); (B) *Streptomyces* sp. MA37; (C) *Streptomyces xinghaiensis*. The homologous genes are colour coded for visual comparison: *flA*, fluorinase; *flB*, purine nucleoside 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.

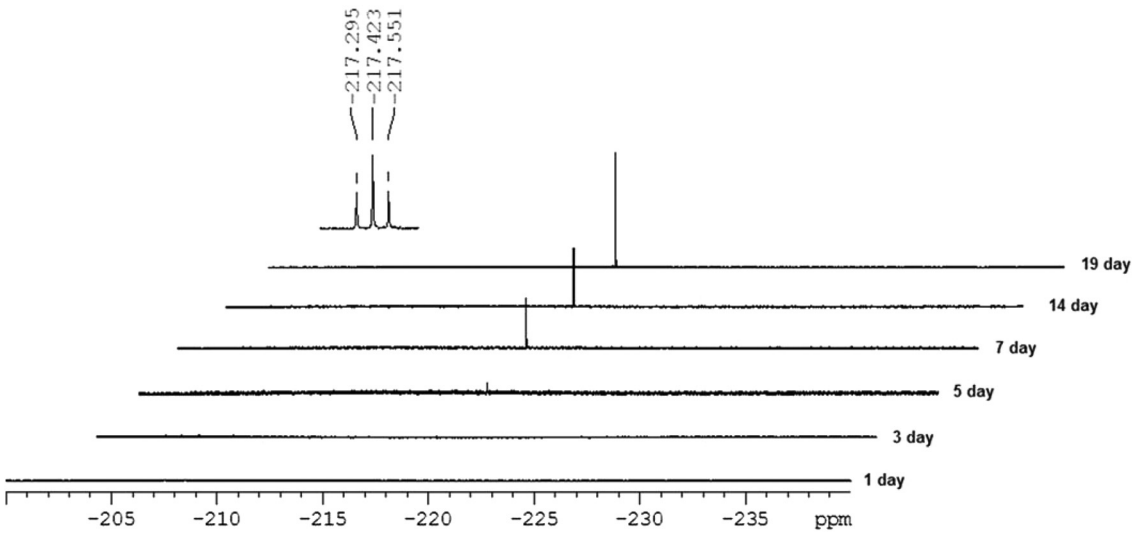

Fig. 2 ^{19}F NMR spectroscopic analysis of fluoroacetate **1** in the supernatant of the culture medium from the marine-derived *S. xinghaiensis* NRRL B24674. Insert: the coupling of fluoroacetate.

jugation, and the double-cross recombination mutant WDY40 was screened out by PCR. ^{19}F NMR analysis of the supernatant of the mutant WDY40 strain demonstrated that the knockout completely abolished the fluoroacetate **1** production (Fig. S5 B†), consistent with the previous report in *S. cattleya*.[22] Complementation of *flK*4 in the mutant WDY40, resulting in the mutant WDY41, restored the production of **1**, suggesting a key role for *fl*K4 in the regulation of fluoroacetate **1** production (Fig. S5 C†), consistent with a putative toxicity resistance role.

Conclusions

In silico analysis has indicated that the marine-derived actinomycete, *Streptomyces xinghaiensis*, contains similar genes to those in *S. cattleya* and *Streptomyces* sp. MA37 for the biosynthesis of fluoroacetate and 4-fluorothreonine. However the cluster in *S. xinghaiensis* had a truncated transaldolase analogous to that involved in the last step of 4-fluorothreonine biosynthesis in the other two organisms. Culturing demonstrated that *S. xinghaiensis* has the capacity to produce only fluoro-

acetate but not 4-fluorothreonine. Production of fluoroacetate is sea-salt dependent. Inactivation of the *flK*4, the putative resistance gene to fluoroacetate toxicity, encoding a fluoroacetyl-CoA thioesterase, resulted in the loss of fluoroacetate 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.

Acknowledgements

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Notes and references

§ The supernatant of *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-500 MHz instrument (^{19}F at 470.3 MHz). The chemical shifts of ^{19}F-NMR were calculated with respect to $CFCl_3$.

The identified gene cluster in *Streptomyces xinghaiensis* has already been deposited in European Nucleotide Archive with accession no HG975299.

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