COMMUNICATION TO THE EDITOR

Fosfomycin Monophosphate and Fosfomycin Diphosphate, Two Inactivated Fosfomycin Derivatives Formed by Gene Products of *fomA* and *fomB* from a Fosfomycin Producing Organism *Streptomyces wedmorensis*

Sir:

Fosfomycin (FM, Fig. 1) is a medically important antibiotic produced by various species of *Streptomyces*[1) as well as *Pseudomonas syringae*[2) and *Pseudomonas viridiflava*[3). FM is characterized by unique structural functions such as a C–P bond and an epoxide[4). Acting as an analogue of phosphoenolpyruvate, it irreversibly inhibits phosphoenolpyruvate UDP-*N*-acetylglucosamine-3-*O*-enolpyruvyltransferase (enolpyruvyltransferase), the enzyme which catalyzes the first step of peptidoglycan biosynthesis, and shows almost no toxicity to humans[5).

Many studies have been undertaken to examine the mechanisms that are responsible for FM resistance in pathogenic bacteria. *Serratia marcescens*, for example, has *fosA* as a resistance gene against FM, whose product (FosA) catalyzes the addition of glutathione to FM[6). Another FM-resistance gene (*fosB*) was found in *Staphylococcus epidermidis*[7). The deduced amino acid sequences of these gene products exhibit 36.6% identity to each other. However, the molecular basis of FM resistance in the FM-producing *Streptomyces* has not been reported.

As reported previously, we have elucidated the biosynthetic pathway of FM consisting of at least four steps and cloned the relevant gene cluster from *S. wedmorensis* using FM non-producing mutants[8,9). DNA sequencing analysis revealed the presence of six open reading frames with unknown functions in the sequenced fragment, in addition to the four genes necessary to FM biosynthesis. So far studied, most antibiotic-producing *Streptomyces* possessed resistance genes and regulatory genes in their antibiotic production gene cluster. Antibiotic resistance genes have been cloned from various organisms using antibiotic sensitive hosts, *Streptomyces lividans* or *Escherichia coli*.

We investigated the FM gene cluster to identify a gene(s) which confers FM resistance to *E. coli*. Deletion analysis of various fragments in the FM biosynthetic gene cluster revealed that both *fomA* and *fomB*, formerly called as *orfA* and *orfB*[9), respectively, and characterized as FM-resistance genes, were required for complete resistance of *E. coli* to FM. In addition, we found that FM was converted to inactive forms and then reactivated again upon incubation with cell-free extracts prepared from *E. coli* harboring *fomA* and *fomB* genes in the presence of ATP (data not shown). In this paper, we describe the structures of FM diphosphate (**1**) and FM monophosphate (**2**), two inactivation products of FM formed by the action of gene products of *fomA* and *fomB* expressed in *E. coli*.

A plasmid that inserted *fomA* and *fomB* just downstream from the pUC119 *lacZ* promoter in the same transcriptional direction was constructed and introduced into *E. coli*. A cell-free extract of this transformant and 5.4 mM FM were incubated in the presence of ATP at a final concentration of 16 mM at 30°C for 4 hours. Analysis by thin layer chromatography revealed that three major compounds **1**, **2** and **3** existed in the reaction mixture. The reaction products were purified by Dowex-1 and Sephadex G-10 chromatography. The pure fractions containing each compound were freeze-dried to give powders of **1**, **2** and **3** as sodium salts.

The molecular formulas of **1**, **2** and **3** were determined to be $C_3H_9O_{10}P_3$, $C_3H_8O_7P_2$ and $C_3H_8O_7P_2$, respectively, by HR-FABMS [*m/z* 364.8952 $(M-2H+3Na)^+$, $+\Delta 0.7$ mmu and *m/z* 386.8793 $(M-3H+4Na)^+$, $+\Delta 2.8$ mmu for **1**, *m/z* 262.9477 $(M-H+2Na)^+$, $+\Delta 1.5$ mmu and *m/z* 284.9315 $(M-2H+3Na)^+$, $+\Delta 3.3$ mmu for **2**, and *m/z* 238.9515 $(M+Na-2H)^-$, $+\Delta 2.9$ mmu for **3**].

The ^1H and ^{31}P NMR data of these compounds are summarized in Table 1. The close similarities of the ^1H NMR spectral features of **1** and **2** with those of FM proved the presence of the epoxide moiety in these compounds. However, **1** showed two additional ^{31}P NMR signals at -22.33 and -8.08 ppm which are very similar to β- and γ-phosphorus signals of ATP. **2** showed only one additional ^{31}P NMR signal at -10.64 ppm which can be compared with the β-phosphorus signal of ADP. These spectral data determined the structures of **1** and **2** as FM diphosphate and FM monophosphate

Fig. 1. Structures of FM and FM derivatives inactivated by gene products of *fomA* and *fomB*.

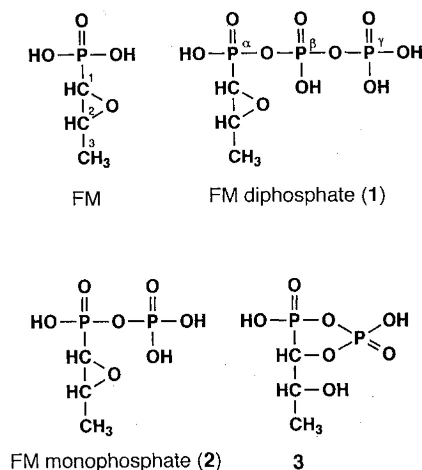

Table 1. ^{1}H and ^{31}P NMR spectral data of FM and **1**, **2** and **3** in D_2O.

		FM	1	2	3
^{1}H NMR	H-1	2.83 (dd, J=5.0, 19.0)	3.23 (dd, J=4.5, 24.4)	3.16 (dd, J=4.9, 24.4)	4.05 (m)
	H-2	3.27 (m)	3.37 (m)	3.36 (m)	4.12 (m)
	H-3	1.47 (d, J=5.5)	1.50 (d, J=5.2)	1.50 (d, J=5.9)	1.30 (d, J=6.7)
^{31}P NMR	α	10.14 (s)	5.26 (d, J=23.8)	3.76 (d, J=23.9)	15.34 (d, J=23.3)
	β		−22.33 (dd, J=20.2, 23.8)	−10.64 (d, J=23.9)	−2.09 (d, J=23.3)
	γ		−8.08 (d, J=20.2)		

The ^{1}H chemical shifts are expressed in ppm downfield from internal 3-(trimethylsilyl)-propanesulfonic acid sodium salt. The ^{31}P chemical shifts is relative to phosphoric acid as external standard at 0 ppm.

derivatives, respectively, as shown in Fig. 1.

In the ^{1}H NMR spectrum of **3** which possesses the same molecular formula as **2**, two oxymethine protons appeared at 4.05 and 4.12 ppm in place of two epoxide methine signals observed in **2**. In addition, two ^{31}P NMR signals at 15.34 and −2.09 ppm coupled each other showed large downfield shifts as compared with the corresponding ^{31}P NMR signals in **2**. These characteristic spectral changes are assigned to the formation of a cyclic phosphate system indicating the structure of **3** as shown in Fig. 1.

As expected from their structures, **1** and **2** but not **3** were transformed to FM by bacterial alkaline phosphatase (Takara Shuzo Co., Ltd.). Since **1** and **2** were gradually converted to **3** at room temperature, **3** is believed to be an artifact of **1** and **2**.

As mentioned above, FM was converted to inactive forms and then activated again *in vitro* during the inactivation reaction. This phenomenon could be explained by assuming that FM was phosphorylated by the gene products of *fomA* and/or *fomB* in the presence of ATP and then, presumably after consumption of ATP, dephosphorylated by endogenous phosphatases of *E. coli*. Further studies are necessary to define the individual function of *fomA* and *fomB*.

Independently of our results, GALCIA *et al.* reported that cell extracts prepared from *E. coli* harboring the *fosC* gene, which had been cloned from the FM-producing organism *P. syringae* PB-5123, inactivated FM in the presence of ATP.[10] They also showed that when FM was incubated as just mentioned and subsequently treated with alkaline phosphatase, the activity of FM remained unchanged. However, the inactivated products were not well characterized. In view of these results, we propose the inactivated FM derivatives formed by the *fosC* gene product may be **1** and/or **2**. The deduced amino acid sequences of these gene products, however, do not show significant homology each other. This fact may suggest that the two FM-producing organisms being taxonomically quite different from each other, independently acquired the self-resistance mechanism against FM during evolution.

Our findings suggested that the self-resistance mechanism of the FM-producing *Streptomyces* is phosphorylation and pyrophosphorylation of the phosphonate function in the FM molecule. Although phosphorylation of antibiotics is well-known as a resistance mechanism against aminoglycoside antibiotics[11], to the best of our knowledge, pyrophosphorylation has never been reported as resistance mechanisms to antibiotics. This new resistance mechanism may be found in pathogenic bacteria as well as antibiotic producing organisms in future.

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