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PFM-Like Enzymes Are a Novel Family of Subclass B2 Metallo-β-Lactamases from *Pseudomonas synxantha* Belonging to the *Pseudomonas fluorescens* Complex

Laurent Poirel,[a,b,c] Mattia Palmieri,[a,d] Michael Brilhante,[e] Amandine Masseron,[a] Vincent Perreten,[e] Patrice Nordmann[a,b,c,f]

[a]Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland
[b]INSERM European Unit (IAME, France), University of Fribourg, Fribourg, Switzerland
[c]Swiss National Reference Center for Emerging Antibiotic Resistance (NARA), University of Fribourg, Fribourg, Switzerland
[d]bioMérieux, Data Analytics Unit, La Balme Les Grottes, France
[e]Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Bern, Switzerland
[f]Institute for Microbiology, University of Lausanne and University Hospital Centre, Lausanne, Switzerland

ABSTRACT A carbapenem-resistant *Pseudomonas synxantha* isolate recovered from chicken meat produced the novel carbapenemase PFM-1. That subclass B2 metallo-β-lactamase shared 71% amino acid identity with β-lactamase Sfh-1 from *Serratia fonticola*. The bla_{PFM-1} gene was chromosomally located and likely acquired. Variants of PFM-1 sharing 90% to 92% amino acid identity were identified in bacterial species belonging to the *Pseudomonas fluorescens* complex, including *Pseudomonas libanensis* (PFM-2) and *Pseudomonas fluorescens* (PFM-3), highlighting that these species constitute reservoirs of PFM-like encoding genes.

KEYWORDS PFM-1, *Pseudomonas fluorescens*, metallo-beta-lactamase

Metallo-β-lactamases (MBLs) are zinc-dependent enzymes that can catalyze the hydrolysis of virtually all β-lactam antibiotics (including carbapenems) except for monobactams and that are resistant to the β-lactamase inhibitors clavulanate, tazobactam, and avibactam (1). They constitute a highly diverse family of enzymes and can be categorized into three subclasses, namely, B1, B2, and B3 (2). The subclass B1 enzymes are the most clinically important since they comprise MBLs such as IMP-1, NDM-1, SPM-1, KHM-1, VIM-1, and VIM-2 (3), widely identified in *Enterobacteriaceae*, *Acinetobacter* spp., and *Pseudomonas* spp. Subclass B2 includes CphA (4, 5), ImiS (6, 7), and AsbM1 (8), which are intrinsic enzymes in *Aeromonas* spp., and Sfh-I (9) from the occasionally pathogenic species *Serratia fonticola*. These carbapenemases are monozinc enzymes that usually shown much higher hydrolysis rates against carbapenem substrates than the other β-lactams (9).

Address correspondence to Laurent Poirel, laurent.poirel@unifr.ch.

Production of MBLs in the *Pseudomonas* genus is frequently observed, with acquired MBL-encoding genes (bla_{IMP}, bla_{VIM}, bla_{SPM}) being reported worldwide mainly in *Pseudomonas aeruginosa* and, to a lesser extent, in *Pseudomonas fluorescens* (10, 11). In addition, intrinsic MBL genes encoding subclass B3 POM-1-like and PAM-1-like enzymes have been identified in *Pseudomonas otitidis* and *Pseudomonas alcaligenes*, respectively (12–14).

P. fluorescens and related species belonging to a same complex are rarely associated with infections in human medicine (15). Nevertheless, *P. fluorescens* can cause bloodstream infections in humans, and most reported cases have been iatrogenic (16). Few studies have focused on the β-lactamase gene content of the *P. fluorescens* complex. While *P. fluorescens* possesses a chromosomally located and inducible Ambler class C β-lactamase gene (17), the acquired but chromosomally located bla_{BIC-1} gene encoding

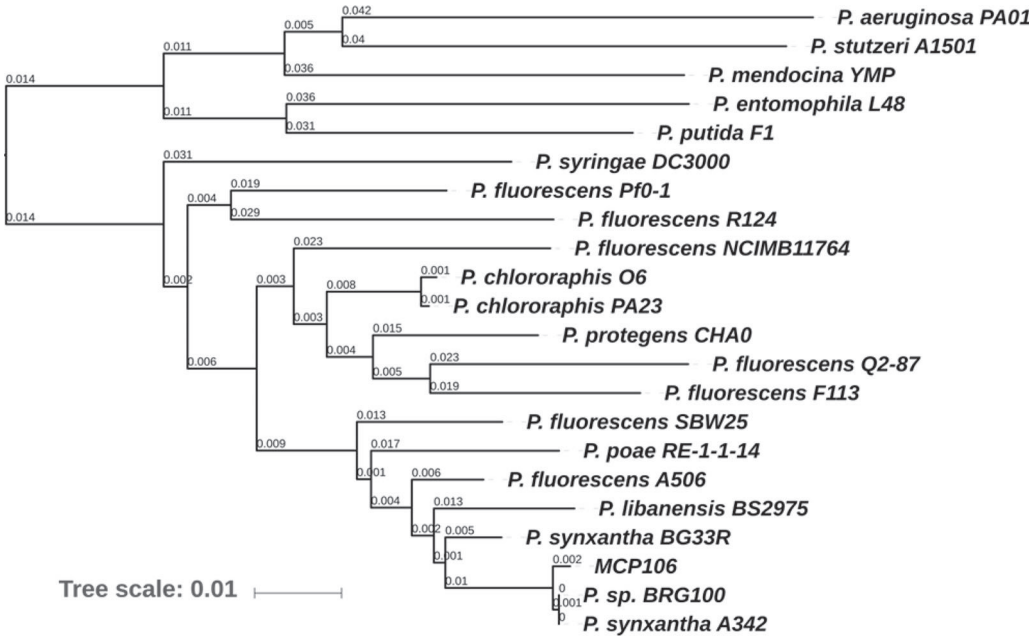

FIG 1 Dendrogram performed by using the seven genes from the multilocus sequence typing (MLST) analysis in comparison with representative genes from other *Pseudomonas* species, in particular, the most closely related ones, which are *Pseudomonas fluorescens* and *Pseudomonas synxantha*. The alignment used for the tree calculation was performed with the Clustal Omega program.

an Ambler class A carbapenemase was previously identified as a source of carbapenem resistance in *P. fluorescens* isolates recovered from the Seine River, Paris (18).

Here, we analyzed a carbapenem-resistant *Pseudomonas* sp. isolate that had been recovered during a survey aimed to study the spread of multidrug-resistant Gram-negative organisms among food varieties and food-producing animals in Switzerland in 2018. Isolate MCP-106 was isolated from chicken meat after an 18-h preenrichment in LB broth and subsequent selection on ChromID CarbaSmart (bioMérieux, La Balme-les-Grottes, France). Carbapenemase production was tested using the Rapid Carba NP test (19). Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis assigned the strain to the *Pseudomonas synxantha* species, and that assignment was further confirmed by analysis of the *rpoB* and *rpoD* gene sequences (Fig. 1). *P. synxantha*, which belongs to the *P. fluorescens* complex (20), is an environmental species that reduces and accumulates the heavy metal chromium (21, 22) that is pathogenic to nematode eggs and may therefore be used as a nematicidal agent (23).

Susceptibility testing performed for β-lactams by disk diffusion showed that *P. synxantha* strain MCP-106 was resistant to amino- and carboxypenicillins, broad-spectrum cephalosporins, aztreonam, and carbapenems. Whole-genome sequencing was performed using an Illumina MiSeq platform (2 $\times$ 150-bp paired ends) to assess the genetic determinants of carbapenem resistance. The obtained reads were trimmed using trimmomatic 0.36, assembled with SPAdes version 3.11.1 (24), and annotated with PROKKA version 1.12. TBLASTN analysis of the DNA contigs using VIM as a reference revealed a chromosomally located MBL protein that was named PFM-1 (*Pseudomonas fluorescens* metallo-β-lactamase). PFM-1 (encoded by the $bla_{\text{PFM-1}}$ gene) consisted of a β-lactamase with 253 amino acids and a relative molecular mass of 28.5 kDa.

A BLASTN analysis against the NCBI database revealed the presence of a bla_{PFM}-like gene (named $bla_{\text{PFM-2}}$, with PFM-2 sharing 92% amino acid identity with PFM-1) in *Pseudomonas libanensis* strain CIP105460 (GenBank accession no. GCA_001439685.1) (25) which actually belongs to the *Pseudomonas fluorescens* sp. complex. In addition, genes encoding PFM-like products were also identified in the genomes of a single *P.*

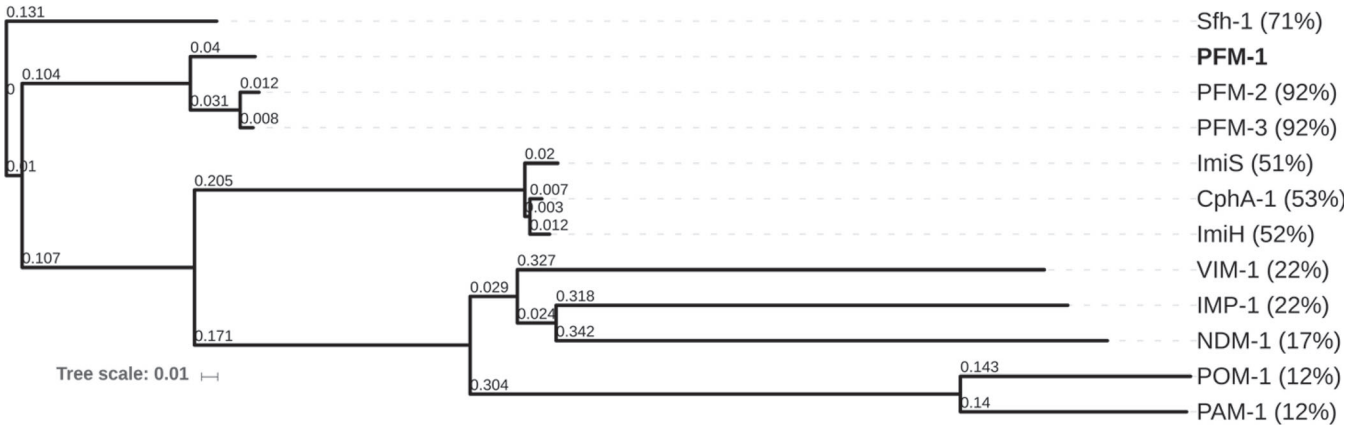

FIG 2 Dendrogram of PFM-1, PFM-2, and PFM-3 in comparison with representative class B β-lactamases subjected to neighbor-joining analysis. The alignment used for the tree calculation was performed with the Clustal Omega program. Numbers in parentheses indicate percentages of amino acid identity with PFM-1. The β-lactamases used for the comparisons (GenBank accession numbers) were Sfh-1 (NZ_AUZV01000091.1), CphA-1 (X57102), ImiS (Y10415), ImiH (AJ548797), VIM-1 (AJ278514), IMP-1 (EF027105), NDM-1 (KJ018857), POM-1 (EU315252), and PAM-1 (AB858498). Percentages of amino acid identities compared to PFM-1 are indicated.

fluorescens strain (WP_050516231.1) and two *Pseudomonas brenneri* strains, sharing 90% amino acid identity with PFM-1 (WP_128593843.1 and OAE14554.1). Furthermore, a gene encoding a more distantly related enzyme (75% amino acid identity) was found in the genome of a *Pseudomonas chlororaphis* strain (WP_038635452.1). However, no other bla_{PFM}-like gene was identified in any other *P. fluorescens* genomes (or in any genomes of species belonging to the same complex), despite numerous genomes of strains belonging to the *P. fluorescens* complex ($n = 145$) having been fully sequenced.

We then screened 10 *P. fluorescens* strains from our laboratory collection, all of which had been recovered from human, animal, or environmental samples. A PCR-based approach using primer pair PFM-1-Fw (5′-GTTACGCCTGATGGACTTTG-3′) and PFM-1-Rv (5′-CTTAGAAGCATGTCAGTGCG-3′) for bla_{PFM-1} and primer pair PFM-2-Fw (5′-CTGATCAGAAAATGTGGGGC-3′) and PFM-2-Rw (5′-GACACGCCGTGTTTCTATATC-3′) for bla_{PFM-2} was employed. A single strain gave a positive result, and Sanger sequencing identified a bla_{PFM}-like gene (bla_{PFM-3}) encoding a protein sharing 91% amino acid identity with PFM-1. The bla_{PFM-3} gene was identified from *P. fluorescens* PF1, an isolate recovered from a water sample from the Seine River in Paris, France, and also producing the Ambler class A carbapenemase BIC-1 (18). PFM-2 and PFM-3 differed by five amino acids.

Pairwise alignment of the sequences of the PFM-like amino acid sequences with those of other MBLs revealed that these newly identified enzymes were most closely related to the subclass B2 MBL enzymes. PFM-1 shares 71% amino acid identity with Sfh-1, originally identified in *Serratia fonticola* strain UTAD54 (9), and 53% identity with CphA-1 from *Aeromonas hydrophila* (26). It shared very low identity with subclass B1 MBLs such as NDM-1 (17%) and VIM-1 and IMP-1 (22%) (Fig. 2). Protein alignments of the β-lactamase PFM-1 with representative subclass B2 MBLs revealed the presence of conserved amino acid residues known to be involved in binding to zinc of class B β-lactamase (BBL) (27) (Fig. 3). The motif Asn-Tyr-His-Thr-Asp (positions 116 to 120 [BBL nomenclature]), being a distinctive feature of subclass B2 MBLs and presumably involved in the coordination of the two zinc ions found in the active site of these enzymes, was identified in PFM-like enzymes. Amino acids Asp120, Cys-221, and His-263, presumably involved in the binding of the second zinc ion in subclass B2 MBLs, were also conserved in the PFM-like proteins.

In order to gain insight into the β-lactam resistance phenotype conferred by the corresponding proteins, the bla_{PFM-1}, bla_{PFM-2}, and bla_{PFM-3} genes of *P. synxantha* strain MCP-106, *P. libanensis* strain CIP105460, and *P. fluorescens* PF1 were cloned into plasmid pTOPO (Invitrogen, Illkirch, France) and expressed in *Escherichia coli*. Cloning experi-

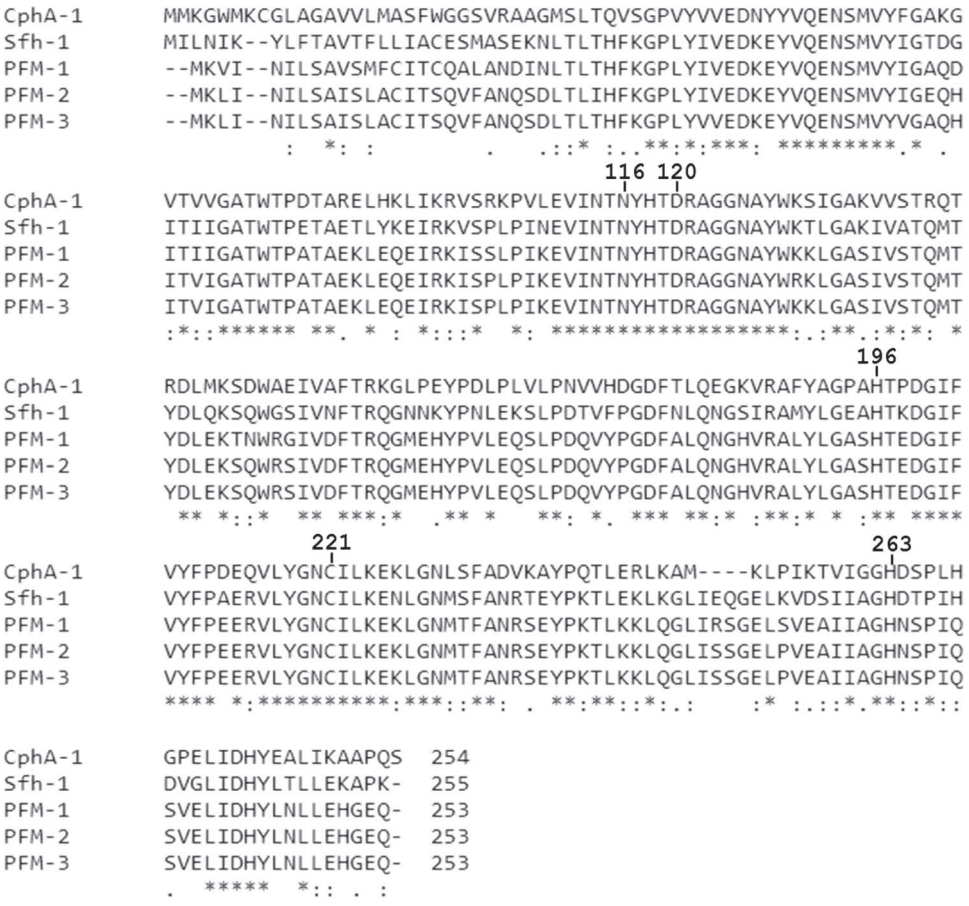

FIG 3 Alignment of the amino acid sequences of subclass B2 MBLs. Residues conserved in the enzymes are indicated by asterisks; colons indicate conservation between groups with strongly similar properties; dots indicate conservation between groups with weakly similar properties. The BBL numbering scheme (in bold) is used for residues conserved in MBLs.

ments were performed using the pCR-blunt TOPO cloning kit (Invitrogen, Illkirch, France) after amplification of the genes with primers PFM-1-Fw and PFM-1-Rv for bla_{PFM-1} and with primers PFM-2-Fw and PFM-2-Rw for bla_{PFM-2} and bla_{PFM-3}. The resulting recombinant plasmids were transformed into chemically competent *E. coli* TOP10 strains. Once expressed in *E. coli* TOP10, similar resistance phenotypes were observed with the different PFM variants, with reduced susceptibility to carbapenems seen (Table 1) but paradoxically no effect on the other β-lactams tested such as amoxicillin, ticarcillin, cefoxitin, cefotaxime, and ceftazidime (data not shown). MICs of carbapenems were determined by Etest and showed values for the PFM-3-producing recombinant strain that were higher than those obtained with the PFM-1-producing and PFM-2-producing recombinant strains, particularly for imipenem (Table 1).

Purification of the PFM-1 enzyme was performed using a four-liter LB broth culture of *E. coli* TOP10 (pTOPO-bla_{PFM-1}) recombinant strain supplemented with kanamycin (50 μg/ml) and inoculated for 24 h at 37°C under shaking conditions. The bacterial culture was centrifuged, and the pellet was resuspended in Tris-HCl buffer (50 mM Tris-HCl, 100 μM $ZnCl_2$, pH 8.5) and sonicated using a Vibra-Cell 75186 sonicator (Thermo Fisher Scientific). After filtration using a 0.22-μm-pore-size nitrocellulose filter, the crude extract was loaded in a Q-Sepharose column connected to an ÄKTAprime chromatography system (GE Healthcare, Glattbrugg, Switzerland) and eluted with a linear NaCl gradient. The presence of the β-lactamase was monitored using the Rapid Carba NP test (19), and the fractions showing the highest β-lactamase activity were pooled and dialyzed against 100 mM phosphate buffer (pH 7.0), prior to 10-fold

TABLE 1 MICs of carbapenems for *E. coli* TOP10 recipient strain with and without the bla_{PFM} genes and for *Pseudomonas* isolates

Carbapenem	MIC (μg/ml)						
	P. fluorescens MCP-106 (PFM-1)	*P. libanensis* CIP105460[a] (PFM-2)	*P. fluorescens* PF1[b] (PFM-3)	*E. coli* TOP10 (pPFM-1)	*E. coli* TOP10 (pPFM-2)	*E. coli* TOP10 (pPFM-3)	*E. coli* TOP10 empty vector
Imipenem	12	12	>32	1	4	16	<0.06
Meropenem	6	3	>32	1	1	2	<0.06
Ertapenem	>32	>32	>32	0.5	1	4	<0.06
Amoxicillin-clavulanic acid[c]	>32	>32	>32	0.12	0.25	0.06	<0.06
Piperacillin	2	0.25	0.5	0.06	0.12	<0.06	<0.06
Piperacillin-tazobactam[d]	1	0.5	0.5	<0.06	0.06	<0.06	<0.06
Ceftazidime	8	1	2	<0.06	<0.06	<0.06	<0.06
Cefotaxime	32	8	0.5	<0.06	<0.06	<0.06	<0.06
Cefoxitin	>32	>32	>32	<0.06	<0.06	<0.06	<0.06
Cefepime	4	0.25	0.5	<0.06	<0.06	<0.06	<0.06

[a]CIP105460 was originally described by Dabboussi et al. (25).
[b]PF1 was originally described by Girlich et al. (18).
[c]Clavulanic acid was used at a concentration of 2 μg/ml.
[d]Tazobactam was used at a concentration of 4 μg/ml.

concentration performed with a Vivaspin 20 concentrator (GE Healthcare). The purified β-lactamase extract was immediately used for enzymatic determinations.

The protein concentrations were measured using Bradford reagent (Sigma-Aldrich, Buchs, Switzerland), and the purity of the enzyme was estimated by SDS-PAGE analysis (GenScript, NJ, USA). The purity of PFM-1 was estimated to be >95%, with a single dominant band visible on the SDS-polyacrylamide gel. Kinetic measurements were performed at room temperature using phosphate-buffered saline (PBS) buffer (0.1 M, pH 7) supplemented with $ZnSO_4$ (5 μM) using a UV/visible Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, Buckinghamshire, United Kingdom). This kinetic analysis confirmed that PFM-1 hydrolyzed carbapenems; however, the catalytic efficiency was slightly lower than that seen with the previously described subclass B2 MBLs (Table 2). In contrast, hydrolysis of other β-lactam substrates such as benzylpenicillin or cefotaxime was not detected (k_{cat} value $< 0.01\ s^{-1}$). This study therefore characterized a novel family of subclass B2 MBLs with substantial carbapenemase activity. Compared to other subclass B2 MBLs, PFM-1 hydrolysis is limited to carbapenems, and the catalytic efficiency is lower.

The levels of G+C content of bla_{PFM-1} (50%) and $bla_{PFM-2/-3}$ (52%) differed from the expected range of the G+C content of *Pseudomonas* genes (ca. 60%); in addition, the fact that no other bla_{PFM}-like genes were identified in several fully sequenced genomes of *P. fluorescens* strains available in the GenBank databases further suggests a non-*Pseudomonas* origin. However, no obvious genetic element that could have been involved in the acquisition of that gene was observed in its nearby genetic environment. Similarly, no mobile genetic elements were identified in their upstream vicinity by analyzing the genes showing significant identities with bla_{PFM-1} in the GenBank database. It may be speculated that those genes have been acquired by transformation since *P. fluorescens* strains, as with many other Gram-negative nonfermenters, are spontaneously transformable at high frequency (28). However, a discrepancy was

TABLE 2 Kinetic parameters of purified β-lactamase PFM-1 and comparison with other B2 MBLs[a]

Substrate	K_{cat} (s^{-1})					K_m (μM)					K_{cat}/K_m ($M^{-1}\ s^{-1}$)				
	PFM-1	Sfh-I	CphA	AsbM1	ImiS	PFM-1	Sfh-I	CphA	AsbM1	ImiS	PFM-1	Sfh-I	CphA	AsbM1	ImiS
Imipenem	22	50	1,200	70	350	950	80	340	230	100	2.3×10^4	6.4×10^5	3.5×10^6	3×10^5	9×10^5
Meropenem	15	110	3,100	220	300	530	210	1,340	630	308	2.8×10^4	5×10^5	2.3×10^6	3.5×10^5	3×10^6
Ertapenem	10	NR	NR	NR	NR	310	NR	NR	NR	NR	3.1×10^4	NR	NR	NR	NR

[a]Kinetic data are displayed for Sfh-1, CphA, and AsbM1 as reported previously by Fonseca et al. (30), Vanhove et al. (31), and Yang and Bush (8), respectively. ImiS kinetic values are presented for imipenem and meropenem as reported by Sharma et al. (32) and Crawford et al. (6), respectively. NR, not reported.

always noticed between all of the putative MBL-encoding genes (including bla_{PFM-1}) and the surrounding chromosomal sequences in term of GC content (ca. 50% versus ca 60%), suggesting a foreign origin (data not shown).

This work underlines that *P. fluorescens*-like species may possess class B β-lactamase genes that are, however, not systematically present in their genomes. Although strains belonging to the *P. fluorescens* complex are rarely involved in human infections, they are widely disseminated in the environment and parts of the human microbiota and can also be found in chicken meat (16). Those bacterial species may therefore constitute reservoirs of antimicrobial resistance genes (29).

Data availability. The sequences of PFM-1, PFM-2, and PFM-3 have deposited in the NCBI database under GenBank accession numbers MN065826 (PFM-1), MN080496 (PFM-2), and MN080497 (PFM-3). The sequence of the whole genome of *P. synxantha* strain MCP-106 has been deposited under GenBank accession number VSRO00000000.1, BioProject accession no. PRJNA561277, and BioSample accession no. SAMN12612925.

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