J. Microbiol. Biotechnol. (2009), **19**(1), 51–54
doi: 10.4014/jmb.0711.775
First published online 13 November 2008

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

LC-MS/MS Profiling-Based Secondary Metabolite Screening of *Myxococcus xanthus*

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Received: November 19, 2007 / Accepted: July 31, 2008

Myxobacteria, Gram-negative soil bacteria, are a well-known producer of bioactive secondary metabolites. Therefore, this study presents a methodological approach for the high-throughput screening of secondary metabolites from 4 wild-type *Myxococcus xanthus* strains. First, electrospray ionization mass spectrometry (ESI-MS) was performed using extracellular crude extracts. As a result, 22 metabolite peaks were detected, and the metabolite profiling was then conducted using the *m/z* value, retention time, and MS/MS fragmentation pattern analyses. Among the peaks, one unknown compound peak was identified as analogous to the myxalamid A, B, and C series. An analysis of the tandem mass spectrometric fragmentation patterns and HR-MS identified myxalamid K as a new compound derived from *M. xanthus*. In conclusion, LC-MS/MS-based chemical screening of diverse secondary metabolites would appear to be an effective approach for discovering unknown microbial secondary metabolites.

Keywords: Metabolite profiling, LC-MS/MS, *Myxococcus xanthus*, myxalamid

Microorganisms are an abundant source of new useful natural products [10], and myxobacteria have been shown to be prolific producers of a variety of bioactive secondary metabolites, including antibacterial and antifungal compounds [6, 13].

Recently, the metabolomics approach has been used to classify metabolites based on metabolite profiling studies, allowing rapid analyses of complex data and the determination of novel compounds [15, 17]. The increased interest in metabolite profiling has also arisen from the potential for more comprehensive metabolite analyses using liquid chromatography mass spectrometry (LC-MS) technology [1]. The most important advantages of LC-MS are its high sensitivity and high-throughput in combination with the possibility to confirm the identity of the components present in complex biological samples, along with the detection and identification of unknown and unexpected compounds [7, 16].

Accordingly, to screen diverse secondary metabolites, wild-type *Myxococcus* strains were collected from the MicroBank (http://www.microbank.re.kr) of the Korea Research Institute of Bioscience and Biotechnology (KRIBB) [11]. The 4 selected strains were cultivated on CY agar plates for 3–4 days at 30°C [14]. The basal medium for the metabolite profiling was cultivated in 100 ml of CY media for 7 days at 30°C on a rotary shaker at 150 rpm. Amberlite XAD-16 resin (2%, v/v) was added to promote the productivity of the active substance after 3 days of cultivation [2, 12]. At the end of the cultivation period, the XAD-16 adsorber resin was separated from the culture broth, followed by washing with water for 20 min at 30°C and elution twice with 20 ml of methanol. The methanol extracts were then evaporated under the simultaneous addition of water to a final volume of 50 ml. Thereafter, the water phase was extracted with 10 ml of ethyl acetate, and the organic phase concentrated. Finally, the residues were dissolved with 80% methanol (3 mg/ml) and analyzed by LC-MS. In this study, the LC-MS was performed using a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron Co., U.S.A.) equipped with an electrospray ionization (ESI) source. The HPLC separation was performed using a Finnigan Surveyor Modular HPLC System (Thermo Electron Co., U.S.A.) with a YMC-Pack Pro C18 column (2.1×50 mm, 5 μm), where the system was operated using Xcalibur software (version 1.3 SP2, Thermo Electron). The mobile phases A and B were water and acetonitrile with 0.1% formic acid, respectively, and the gradient elution at a flow rate of 0.2 ml/min was performed as

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follows: 0–30 min for 5–100% B (linear gradient), 30–40 min for 100% B (isocratic), followed by 40–50 min of 5% B (isocratic). The full-scan mass spectra were obtained within a range of *m/z* 50–1,500 using three microscans. Utilizing the capacity of the LCQ mass spectrometer to provide continuous polarity switching, data acquisition was conducted in positive and negative modes. The data-dependent tandem mass spectrometry (MS/MS) experiments were controlled using the menu-driven software provided with the Xcalibur system. All the experiments were performed under automatic gain control conditions. The high-resolution mass spectrometer (HR-MS) was measured in methanol with a PEG400 on a JMS-700 Mstation (JEOL Ltd.) consisting of a high-resolution two-sector mass spectrometer using the FAB ionization method from the Korea Basic Science Institute (KBSI). The ^{1}H NMR 500 MHz was measured in CD_3OD on a Varian UI500 with equipment analysis support from KBSI.

The LC-MS analytical data were optimized using a background subtraction technique of chromatography with the metabolite ID 2.0 software system. The principle of this method is to reduce the background, such as noise and fault peaks, to subtract the medium components from the sample extracts (Fig. 1). In the subsequent optimization for the chromatography and spectrum analyses, data containing more real secondary metabolites were observed, along with more ions present in the processed mass spectra. After exporting the process, the LC-MS data were manually sorted to list such information as the retention time, *m/z* values for $[M+H]^+$, MS/MS fragmentation pattern, and UV spectra from base peak chromatograms (Table 1). Each compound was then identified from reference secondary metabolites already known to be produced by *M. xanthus*.

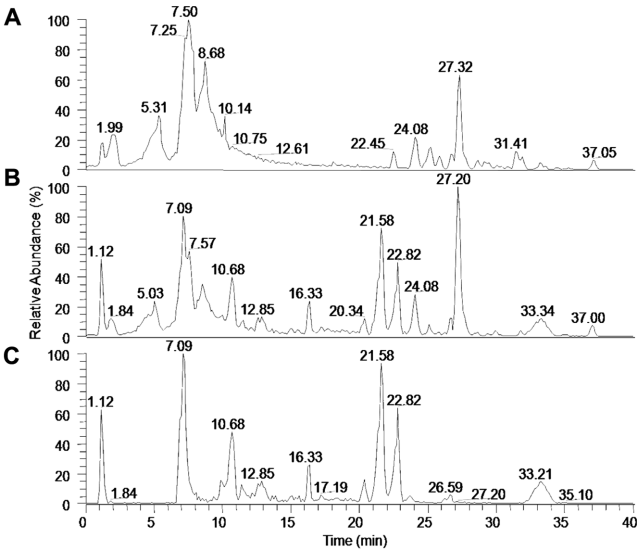

Fig. 1. Background and medium subtracted positive ion chromatogram of *M. xanthus* KYC1427.
A. Chromatogram of medium; **B**. Culture extracts of KYC1427; **C**. Subtracted (**B**)-(**A**) using Metabolite ID 2.0.

Table 1. Screening of secondary metabolites found in crude extracts of *Myxococcus* strains.

No.	Metabolites	Rt (min)	$[M+H]^+$ (*m/z*)	Fragmented ion in MS2 spectra (*m/z*)	ATCC25232	KYC1288	KYC1427	KYC1461	Ref.
1	Myxochelin B	7.09	404	387,251	*	*	*	*	[8]
2	Cittilin A	8.63	631	614,603,586,558,518,490,462,445				*	[13]
3	Myxochelin A	10.59	405	387,269	*	*			[8]
4	Unknown	10.68	446	428,386,310			*	*	
5	Unknown	11.27	460	442,386,324			*		
6	Unknown	11.79	419	401,283	*				
7	DKxanthene-534	12.08	535	518,500,444,416,399,268,251		*			[9]
8	DKxanthene-518	12.85	519	502,400,383,320,268,251	*	*	*	*	[9]
9	Unknown	13.73	545	528,426,408,294		*	*	*	
10	Unknown	14.01	552	534,416	*				
11	Unknown	14.85	536	518,503,426,400,382			*		
12	Unknown	15.23	488	470,401,352	*				
13	Unknown	16.33	404	387,267,281			*		
14	Unknown	16.35	502	484,366	*				
15	Unknown	17.19	516	498,472,442,366	*		*		
16	Unknown	19.77	578	503,489,152,419,416	*				
17	Myxalamid C	20.34	388	370,313,295,290			*		[4]
18	Myxovirescin Bor N1	21.21	622	594,341,286		*			[3]
19	Myxovirescin A2 or T1	21.81	624	579,473		*			[3]
20	Myxalamid B	21.58	402	384,327,309,290,267,215	*		*		[4]
21	Myxalamid A	22.32	416	398,341,323,290,267,215	*	*	*	*	[4]
22	Myxalamid K (New)	23.72	430	412,355,337,304,267,215	*	*	*	*	

Searchable MS/MS spectra libraries based on the results of the liquid chromatography coupled with electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS) with data-dependent acquisition on an ion trap mass spectrometer were compiled with regard to the identification and confirmation of the secondary metabolites from the cultivated *Myxococcus* sp. As a result, the LC-MS/MS analysis of the *M. xanthus* strains identified 10 secondary metabolites peaks as known structures, including myxochelins, DKxanthenes, myxovirescin, and myxalamids, whereas another 12 peaks were determined to be unknown metabolites.

The structure of myxalamid B (peak number **20** in Table 1) was confirmed by LC-MS/MS, HR FAB-MS, and by comparing the ^{1}H NMR spectra (500 MHz, CD_3OD) with those reported by Jansen *et al.* [5]. As such, myxalamid B gave the molecular formula $C_{25}H_{40}NO_3$, as determined by HR FAB-MS at *m/z* 402.2987 $[M+H]^+$ (calcd for 402.3008). Meanwhile, the structure of peak number **22** in Table 1 was expected to be a variant of myxalamid based on the tandem mass spectrometric fragmentation pattern. The myxalamid series exhibited a gap of 14 mass units between myxalamid C (MW 387), myxalamid B (MW 401), and myxalamid A (MW 415), corresponding to CH_2 (methylene group) at the end site. Mass frontier software (version 4.0) was used to simulate and study the fragmentation behavior of the described compounds, and ESI in the positive ion mode was chosen for a detailed study of the fragmentation pattern of the myxalamid series with multiple fragmentation steps. The fragmentation pattern of myxalamid K (*m/z* 430 $[M+H]^+$) was identical to that of myxalamid C (*m/z* 388 $[M+H]^+$), myxalamid B (*m/z* 402 $[M+H]^+$), and myxalamid A (*m/z* 416 $[M+H]^+$) (Fig. 2). Starting from this fragment at *m/z* 430 $[M+H]^+$, the product ion *m/z* 412 $[M+H]^+$ was the result of the loss of water, the product ion at *m/z* 355 $[M+H]^+$ was the result of a combination with the loss of the 57u moiety, and the product ion at *m/z* 337 $[M+H]^+$ was also the result of the loss of water (Fig. 3). HR FAB-MS

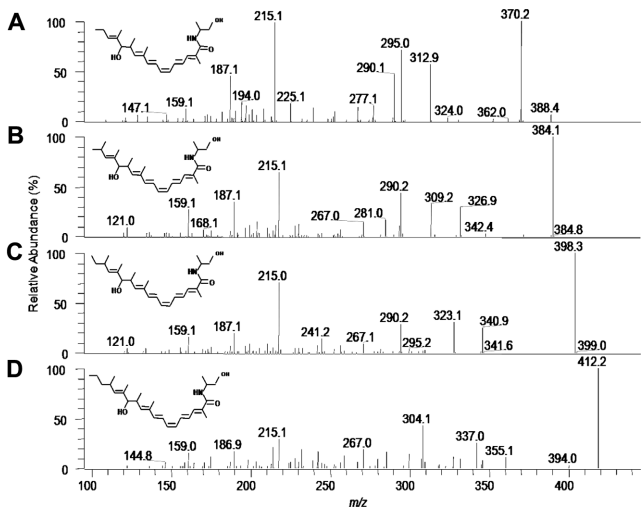

Fig. 2. MS/MS spectra showing similar fragmentation patterns for precursor ions of myxalamid series.
A. Myxalamid C, *m/z* 388 $[M+H]^+$; **B**. Myxalamid B, *m/z* 402 $[M+H]^+$; **C**. Myxalamid A, *m/z* 416 $[M+H]^+$; **D**. Myxalamid K, *m/z* 430 $[M+H]^+$.

for myxalamid K showed *m/z* 429.3254 $[M]^+$ (calcd for 429.3254), which was consistent with the molecular formula $C_{27}H_{43}NO_3$. Therefore, according to these data, myxalamid K was clearly shown to be a new myxalamid variant. Myxalamids, an antibiotic complex, were first isolated from *Myxococcus xanthus* Mx x12 (GBF) in 1983 [5] and showed antibiotic activity against yeast, molds, and some Gram-positive bacteria [12]. Although originally isolated from *M. xanthus,* myxalamids have since been found in many other myxobacteria, including *Cystobacter velatus, Corallococcus coralloides*, *Myxococcus stipitatus*, *Myxococcus virescens*, *Stigmatella aurantiaca*, and *Stigmatella erecta* [12].

To study the metabolic profiles of *M. xanthus*, individual real peaks should be determined from raw LC-MS data

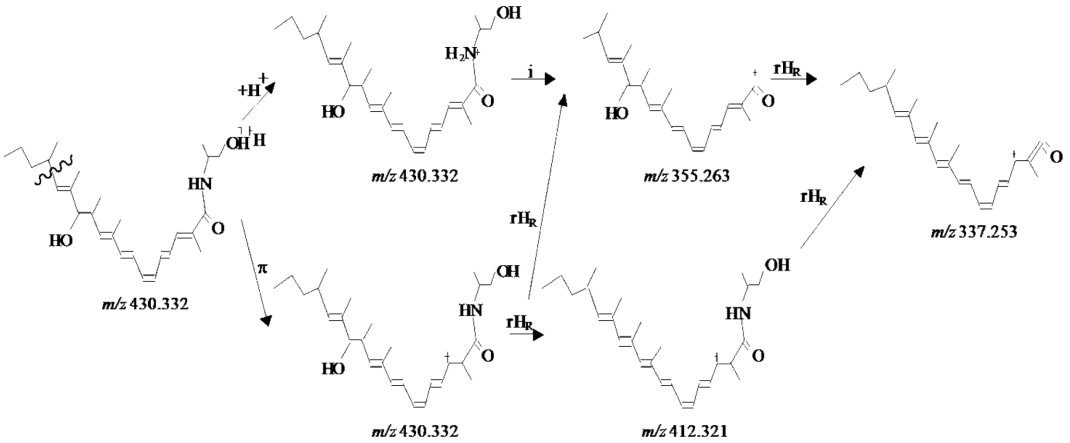

Fig. 3. Proposed fragmentation pathway of myxalamid K *m/z* 430 $[M+H]^+$ using Mass Frontier software.

files. As shown in the present study, the LC-MS/MS-based metabolite profiling of *M. xanthus* secondary metabolites would appear to be a useful technique for distinguishing between known and unknown compounds, and for screening novel compounds without large amounts of cultures. Furthermore, the method saves time and minimizes the repeated discovery of known metabolites.

Acknowledgments

This work was supported by the Korea Science and Engineering Foundation (No. R01-2007-000-20231-0) and Microbial Genomics and Application Center funded by the Korea government (MEST).

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