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Estimation on metabolic pathway of *Pseudomonas* sp. SMIC-3 for 1-methyl-2-pyrrolidinone based on physiological and biochemical analyses

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Abstract–*Pseudomonas* sp. SMIC-3 grown on NMP was physiologically differentiated from that on glucose. Growth of SMIC-3 in an NMP-defined medium was approximately three times lower than that in a glucose-defined medium. Methylamine and 1-methyl succinimide were detected in culture fluid of SMIC-3 grown in an NMP-defined medium. Methylamine content in the culture fluid was very similar to NMP consumed by SMIC-3, but 1-methyl succinimide content was much less than the consumed NMP. Crude enzyme isolated from SMIC-3 grown on NMP catalyzed production of methylamine, 1-methyl succinimide, and succinate from NMP but that on glucose did not. Crude enzyme isolated from SMIC-3 grown on glucose and NMP commonly catalyzed dehydrogenation of pyruvate, isocitrate, and malate coupled to reduction of NAD^+ to NADH. 2D-SDS-PAGE pattern of total soluble proteins isolated from SMIC-3 grown on glucose was significantly different from that on NMP. Physiological function of SMIC-3 for catabolizing NMP may be selectively induced and activated by NMP.

Keywords: *Pseudomonas* sp., 1-Methyl-2-pyrrolidinone, 1-Methyl Succinimide, Methylamine, 2D-SDS-PAGE

INTRODUCTION

1-Methyl-2-pyrrolidinone (NMP) has been employed as an extractive distillation solvent to recover pure hydrocarbons in petrochemical processes and for synthesis of agrochemicals, pharmaceuticals, plasticizers, and stabilizers because of its low toxicity [1]. However, it has been identified as a reproductive toxicant. NMP belongs to recalcitrant organic compounds that are difficult to degrade by the bacterial community in the natural environment. The biochemical ability of bacteria to catabolize NMP can be induced by adaptation to the contaminated environments with a specific organic compound [2]. *Pseudomonas* sp. SMIC-3 was isolated from an enrichment culture containing mixture of xenobiotics that are benzene, toluene, ethyl benzene, xylene, and crude petroleum [3].

Some bacteria grown in sewage contaminated with NMP catabolize NMP by metabolic adaptation [4]. Certain members of the genus *Pseudomonas* catabolize polycyclic aromatic hydrocarbons, toluene, cyanide, carbazole, and carbon tetrachloride by metabolic adaptation [5-10]. Bacteria that acquire metabolic ability for catabolizing a specific xenobiotic may maintain or lose the intrinsic function for catabolizing sugars during adaptation to a specific xenobiotic [11]. Strain SMIC-3 was adapted to catabolize NMP but did not lose the metabolic function for glucose catabolism, which is a useful feature to study the physiological and biochemical differences of strain SMIC-3 according to the difference of substrates. Metabolism of strain SMIC-3 for NMP may not be significantly different from that for glucose except the initial oxidative degradation of NMP. Genus *Pseudomonas* partially oxidizes glucose through glycolysis and completely oxidizes the metabolic intermediates through TCA cycle [12], from which it can be predicted that the metabolic intermediates generated from NMP through the unknown pathway of SMIC-3 may be completely oxidized through TCA cycle.

To estimate the pathway of *Pseudomonas* sp. SMIC-3 for metabolism of NMP, we compared the metabolites produced by SMIC-3 grown on glucose and NMP, the products from NMP by catalysis of crude enzyme, the dehydrogenation activity of crude enzyme for pyruvate, isocitrate, and malate, and 2D-SDS-PAGE patterns of total soluble proteins isolated from SMIC-3 grown on glucose and NMP.

MATERIALS AND METHODS

1. Cultivation and Metabolites of Strain SMIC-3

Pseudomonas sp. SMIC-3 (GenBank accession code, FJ877154) was cultivated in a glucose-defined and an NMP-defined medium at 30 °C under aerobic atmosphere for 36-72 hr. The defined medium was composed of 2 g/L of NH_4Cl, 2 g/L of KH_2PO_4, 2 g/L of $NaNO_3$, 100 mM NMP, and 2 ml/L of trace mineral stock solution. The trace mineral stock solution was composed of 0.02 g/L of $CuSO_4$, 0.02 g/L of $MnSO_4$, 0.02 g/L of $MgSO_4$, 0.02 g/L of $CaCl_2$, 0.02 g/L of $FeSO_4$, 0.002 g/L of $NiCl_2$, 0.002 g/L of $CoCl_2$, 0.002 g/L of $ZnSO_4$, 0.002 g/L of $Al_2(SO_4)_3$, 0.002 g/L of $MoSO_4$, and 10 mM EDTA. NMP separately sterilized from other medium ingredients by membrane-filtering was added to the medium after autoclaving. Cell-free culture fluid was separated from 72 hr-cultivated SMIC-3 by centrifugation at 4 °C and 5,000×g for 30 min and used as a sample for analysis and determination of metabolites. The harvested bacterial cells were lyophilized and precisely weighed to determine growth yield. Glucose and succinate contained in the culture fluid was analyzed by using HPLC, and NMP and unknown organic compounds

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metabolically generated from NMP were analyzed and identified by using GC/GC-MS.

2. Preparation of Crude Enzyme

SMIC-3 cultivated in the glucose- and NMP-defined medium for 36 hr was harvested by centrifugation at 4 °C and 5,000×g for 30 min. The harvested cells were washed with and suspended in 25 mM phosphate buffer (pH 7), and disrupted by bead beater (Fast-Prep-24, MP Biomedicals, USA) at 3,000 strokes and 4 °C for 30 min. Cell debris was discarded by centrifugation at 10,000×g and 4 °C for 60 min, and cell-free extract was used as a crude enzyme. Protein content of crude enzyme was determined using Bradford reagent (Bio-Rad, USA) as a coloring agent and bovine serum albumin as a standard protein (Bio-Rad, USA).

3. Oxidative Degradation of NMP by Catalysis of Crude Enzyme

A reaction mixture composed of crude enzyme (20 mg/ml of protein), 5 mM NAD^+, 10 mM NADH, 1 mM thiothreitol, 25 mM Tris-HCl (pH 7.5), and 50 mM NMP was incubated at 30 °C and 120 strokes per min for 3 hr. After that, the reaction mixture was centrifuged at 4 °C and 20,000×g for 40 min and the supernatant was sampled for analysis and determination of products. Succinate was analyzed by using HPLC, and other organic compounds contained in the supernatant were analyzed and identified by using GC/GC-MS.

4. Dehydrogenation Activity of Crude Enzyme

Pyruvate, isocitrate, and malate dehydrogenases were assayed using spectral absorbance of NADH regenerated coupled with substrate oxidation. A reaction mixture composed of crude enzyme (20 mg/mL of protein), 25 mM Tri-HCl buffer (pH 7.5), 5 mM NAD^+, 1 mM thiothreitol, and 10 mM pyruvate (isocitrate or malate) was incubated at 30 °C and 120 strokes for 30 min. Spectrophotometric absorbance of the reaction mixture was measured at 340 nm to determine NADH concentration, calculated using extinction coefficient (ε=6,220 $M^{-1}cm^{-1}$) and absorbance at 340 nm based on $Ab=\varepsilon lc$ (ε, extinction coefficient; l, length of light path; c, concentration).

5. HPLC

High performance liquid chromatography (Young-Lin, Korea) was adopted for the analysis of metabolites produced by growing cells and products generated by catalysis of crude enzyme. A small aliquot of the centrifuged and filtrated enzyme reactant (20 μl) was directly injected into the injector of a high performance liquid chromatograph (Gold apparatus, Beckman, Coulter, Brea, CA, USA) equipped with a Shodex Rspak KC-811 ion exclusion column (Showa Denko, Tokyo, Japan) and a model RI-101 refractive index detector (Showa Denko). Succinic acid was quantitatively and qualitatively determined based on the retention time and peak area of standard material.

6. GC/GC-MS

Organic compounds contained in culture fluid of SMIC-3 grown in NMP and products generated from NMP by catalysis of crude enzyme were qualitatively analyzed via GC-MS with modified procedures, adapted from the technique developed by Shin and Jung [13]. One hundred ml of the bacterial culture containing NMP was placed in a 250-ml separating funnel. Approximately 30 g of NaCl and 100 μL of ethylene glycol butyl ether internal standard solution (1,000 mg/L) were added to this solution, and the samples were extracted with 20 mL of methylene chloride by 20 min of mechanical shaking. The solvent layer was then recovered in a flask. One thousand μL of the solvent phase was transferred to a GC vial. At the appropriate time, a 1 μL sample of the solution was analyzed via GC. GC-MS analysis was conducted with a Perkin Elmer Clarus 600 gas chromatograph (GC) interfaced with a mass spectrometer detector (Perkin Elmer Clarus 600T mass spectrometer). GC separation was conducted with Agilent DB-WAXETR capillary column (30 m by 0.32 mm; film thickness, 1 μm) using He as the carrier gas (flow rate, 1.0 ml/min). The injector temperature was adjusted to 250 °C, initial oven temperature was adjusted to 80 °C, and the oven temperature was increased at the rate of 10 °C min^{-1}. The oven temperature was increased to 250 °C and held for 2 min to clean the column. The samples were injected in a splitless injection mode initially and the injector was switched to split mode 2 min after the sample was injected.

7. 2D-SDS-PAGE of Total Soluble Protein

Two-dimensional gel electrophoresis (2D-SDS-PAGE) was performed according to the procedures and methods used by Wilkins et al. [14] with the reagents, kits and apparatuses provided by Bio-Rad (Hercules, USA). SMIC-3 cultivated on glucose and NMP for 36 hr was harvested and washed twice with a 50 mM Tris-HCl (pH 7.5), and disrupted by a bead beater cell disruptor. Cell debris was discarded by centrifugation at 10,000×g and 4 °C for 60 min and protein concentration in soluble extract was determined with Bradford reagent. Proteins in soluble extract were first separated based on isoelectric point using an IPG strip (Readstrip, 170 mm, pH 3-10, Bio-Rad) and an isoelectric focusing system (Protean IEF Cell, Bio-Rad). The isoelectrically focused proteins were separated based on molecular weight by SDS-PAGE. SDS-PAGE was carried out using a precast gel (8-14% gradient, Bio-Rad) and an electrophoresis system (Protean II XL cell). Protein spots on 2D-SDS-PAGE gel were visualized by silver staining (Silver staining kit, Bio-Rad).

8. In-gel Digestion

Protein spots of interest were manually excised from the gel and placed in Ependorf tubes. Gel pieces were destained in a 1 : 1 mixture of 30 ml potassium ferricyanide and 100 mM sodium thiosulfate, washed with 50% acetonitrile (ACN)/25 mM ammonium bicarbonate at pH 7.8, and incubated in 50% ACN for 5 min. Gel pieces were dehydrated in a vacuum centrifugal concentrator and incubated in 10 μL of trypsin (0.02 μg/μL) solution on ice for 45 min. After replacing with 20 mM ammonium carbonate, gel pieces were digested at 37 °C overnight. The following day, 0.5% (v/v) trifluoroacetic acid (TFA) in 50% ACN was added, and the extraction was conducted twice in an ultrasonic water bath. Peptides were extracted in 0.1% formic acid in 2% ACN for further MALDI-TOF MS analysis.

9. MALDI-TOF MS Analysis

Mass analysis was performed on a PerSeptive Biosystem Voyager-DE STR™ MALDI-TOF MS (Applied Biosystems, CA, USA) in a reflector mode for positive ion detection. Peptide extracts were dispensed to a MALDI sample plate, along with matrix solution consisting of 10 mg/ml α-cyano-4-hydroxycinnamic acid, 0.1% TFA and 50% ACN. External peptide calibrants, angiotensin 1 (monoisotopic mass, 1296.6853), rennin substrate (1758.9331) and adrenocorticotropic hormone (2465.1898) were employed for mass calibration. Spectra were internally calibrated using autolytic fragments from trypsin. Proteins were identified by peptide mass fingerprinting with the search engine program, MS-FZI supplemented with

the option for Bacteria in the NCBI database. The criteria for positive identification of proteins were set as follows: (i) at least four matching peptide masses, (ii) 50 ppm or better mass accuracy, and (iii) matching of MW and PI of identified proteins with the values estimated from image analysis.

RESULTS

1. Growth of SMIC-3 on Glucose and NMP

Theoretically, the reducing power generated from glucose ($C_6H_{12}O_6$: O/R=−12+12=0) and NMP (C_5H_9NO: O/R=−9+2=7) is 24H and 27H, respectively, on the basis of oxidation-reduction (O/R) balance (O=+2, H=−1) in the general condition that glucose and NMP are completely oxidized to CO_2. Accordingly, the growth of SMIC-3 should be higher in NMP-defined medium than glucose-defined medium because free energy is produced in proportion to reducing power generated from metabolic oxidation of carbon source. However, growth of SMIC-3 in the NMP-defined medium was approximately three times lower than that in the glucose-defined medium as shown in Fig. 1, which corresponds to dry cell mass and growth yield of SMIC-3 cultivated in glucose- and NMP-defined medium as shown in Table 1.

2. Metabolites by Growing Cells of SMIC-3

SMIC-3 grown on NMP consumed approximately 90 mM of NMP and produced 89.3 mM of methylamine, 3.3 mM of 1-methyl succinimide, and trace amount of succinate for 72 hr as shown in Table 2. Methyl amine contained in bacterial culture fluid was increased in proportion to consumption of NMP, but 1-methyl succinimide was lower than 4 mM and a trace amount of succinate was consistently detected for 72 hr as shown in Fig. 2.

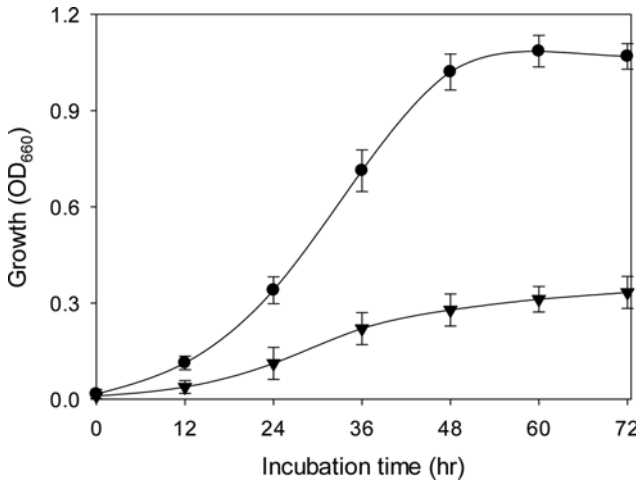

Fig. 1. Growth of *Pseudomonas* sp. SMIC-3 cultivated in the glucose-defined medium (●) and the NMP-defined medium (▼).

Table 1. Growth yield of *Pseudomonas* sp. SMIC-3 grown in glucose- and NMP-defined medium for 72 hr, which was determined based on dry cell mass and substrate consumption

Criteria for evaluation	Cultivated on glucose	Cultivated on NMP
Dry cell mass (g/L)	0.32	0.11
Substrate consumption (mM)	88	92
Growth yield (g cell mass/M substrate)	3.63	1.20

Table 2. Organic compounds contained in culture fluid of SMIC-3 grown in glucose-defined and NMP-defined medium for 72 hr

Glucose-defined medium	NMP-defined medium
Not detected	Methyl amine (89.3 ± 3.3 mM) 1-Methyl succinimide (3.3 ± 0.6 mM) 1-Methyl-2-pyrrolidinone (NMP) (8.2 ± 1.1 mM) Succinate (trace)

3. Oxidative Degradation of NMP by Crude Enzyme

Crude enzyme isolated from SMIC-3 grown on NMP catalyzed the oxidative degradation of NMP but that on glucose did not. Ap-

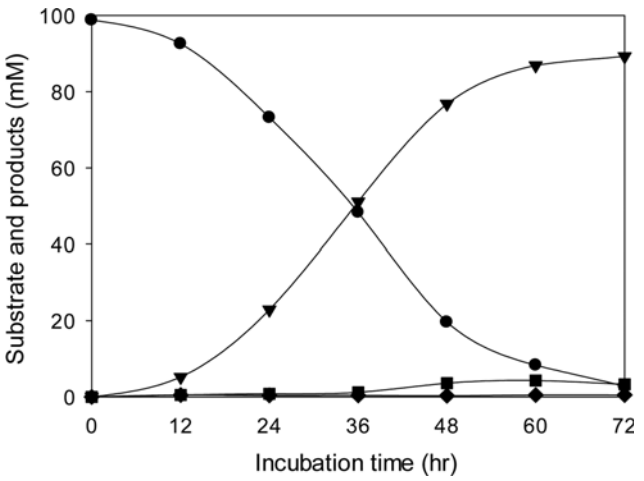

Fig. 2. Time-coursed consumption of NMP (●) and production of methyl amine (▼), 1-methyl succinimide (■), and succinate (◆) by *Pseudomonas* sp. SMIC-3 cultivated in the NMP-defined medium.

Table 3. Products generated from NMP by catalysis of crude enzyme (20 mg/ml of protein) isolated from SMIC-3 grown in glucose-defined and NMP-defined medium for 36 hr

Glucose-defined medium	NMP-defined medium
Not detected	Methyl amine (13.8±0.7 mM) 1-Methyl succinimide (15.1±0.8 mM) 1-Methyl-2-pyrrolidinone (NMP) (31.2±2.1 mM) Succinate (11.2±1.3 mM)

Table 4. Dehydrogenation activity of crude enzyme (protein 20 mg/ml) isolated from SMIC-3 cultivated in NMP-defined and glucose-defined medium for 36 hr. Enzyme activity was determined by NADH regenerated in coupling with reactions of crude enzyme and pyruvate, isocitrate, or malate

Substrates	NADH regeneration (μM min^{-1} $mg\ protein^{-1}$)	
	Glucose-defined medium	NMP-defined medium
Pyruvate NAD^+	2.4±0.3	0.6±0.1
Isocitrate NAD^+	3.6±0.5	1.9±0.2
Malate NAD^+	3.2±0.3	2.7±0.2

proximately 14 mM of methylamine, 15 mM of 1-methyl succinimide, and 11 mM of succinate were generated from NMP by catalysis of the crude enzyme as shown in Table 3. Concentration of 1-methyl succinimide and succinate in enzyme reactant was not very much higher and lower than methyl amine (Table 2), which is definitely different from the concentration of metabolites produced by growing cells of SMIC-3 (Table 2 and Fig. 2).

4. Dehydrogenation Activity of Crude Enzyme

Dehydrogenase catalyzes reduction of NAD^+ to NADH (NADH regeneration) coupled with oxidation of specific metabolic intermediates. Crude enzymes were isolated from SMIC-3 grown on glucose and NMP commonly catalyzed NADH regeneration by reaction with pyruvate, isocitrate, and malate. However, activity for dehydrogenation was significantly different depending on growth condition as shown in Table 4. Generally, the activity of dehydrogenases was proportional to growth rates and growth yield (Table 1 and Fig. 1).

5. 2D-SDS-PAGE and MALDI-TOF MS

Differences of growth and enzyme activities may be caused by the physiological function of SMIC-3 that are differently controlled depending to substrates. As shown in Fig. 3, 2D-SDS-PAGE patterns of total soluble proteins isolated from SMIC-3 grown on glucose and NMP were significantly different and some proteins were not expressed. The differently or selectively expressed proteins were isolated from 2D-gel and analyzed by MALDI-TOF MS. However,

Table 5. Identification of possible proteins by MALD-TOF MS

Spots	MW (approximate)	PI (approximate)	Homologous protein
g1	34,000	6.8	All of proteins separated by 2D-SDS-PAGE were not identified with MALDI-TOF MS database released in GenBank database system. Identification was performed on the basis of isoelectric point, molecular weight, and bacterial species.
g2	55,000	9.1	
g3	43,000	9.3	
g4	37,000	9.0	
g5	32,000	9.3	
g6	30,000	9.3	
g7	24,000	9.4	
n1	99,000	3.8	
n2	87,000	4.8	
n3	77,000	4.5	
n4	74,000	4.6	
n5	52,000	5.2	
n6	47,000	3.8	
n7	31,000	5.8	
n8	66,000	9.2	
n9	43,000	9.2	

all of the numbered proteins on the 2D-gel were not totally identified with the MALD-TOF MS database released in GenBank database system when compared based on the matching peptide mass (MW), pI, protein sources, and bacterial species as shown in Table 5.

DISCUSSION

The catabolizing ability of *Pseudomonas* sp. SMIC-3 for NMP was obtained by long-term cultivation in a defined mineral medium saturated with vapor of various hydrocarbons and crude oil [15]. However, the long-term enrichment culture did not induce the strain SMIC-3 to lose its physiological activity for catabolism of glucose, and the growth rate of SMIC-3 on glucose was three times higher than that on NMP. Relatively high growth of SMIC-3 on glucose is advantageous to produce bacterial cells without using the harmful and

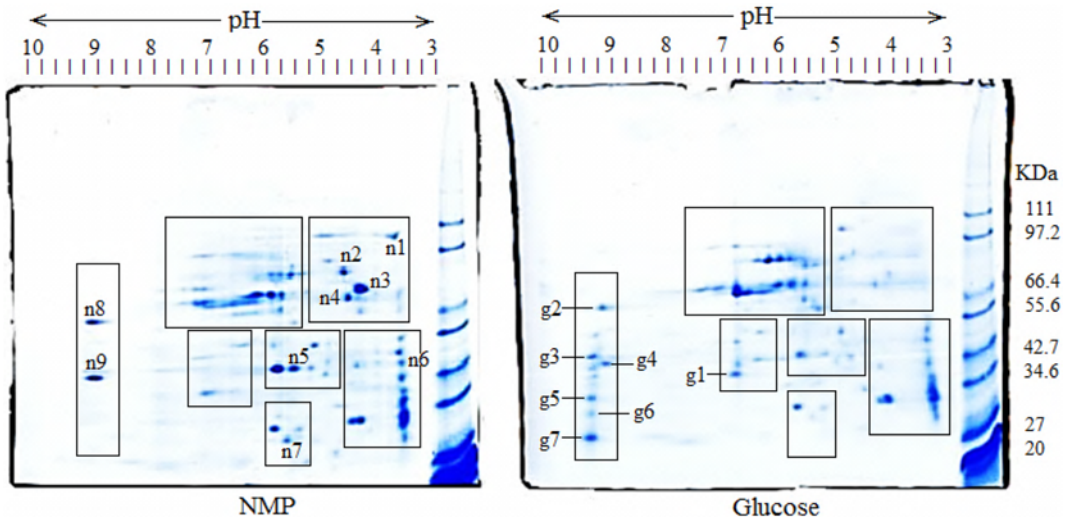

Fig. 3. 2-D-SDS-PAGE of proteins isolated from *Pseudomonas* sp. SMIC-3 cultivated in NMP-defined medium (left) and glucose-defined medium (right) for 48 hr.

expensive compound (methyl amine). SMIC-3 grown on glucose is useful to prepare biocatalyst-immobilizing carrier that can biochemically oxidize NMP in wastewater treatment reactor by physiological adaptation [15].

SMIC-3 catabolized NMP as a substituted carbon source for glucose in the condition without glucose but did not catabolize NMP in the condition with glucose [16]. This physiological selectivity of SMIC-3 for a specific substrate corresponds to biochemical reactions that the crude enzyme isolated from SMIC-3 grown on glucose did not catalyze oxidative degradation of NMP. Differences of 1-methyl succinimide and succinate produced by growing cells of SMIC-3 (Table 2) and crude enzyme catalysis (Table 3) may be a clue that methylamine may be a metabolite, but 1-methyl succinimide and succinate may be intermediates for successive metabolism. On the other hand, both crude enzymes isolated from SMIC-3 grown on glucose and NMP commonly catalyzed dehydrogenation of pyruvate, isocitrate, and malate even though the specific activities were significantly different depending on sources of the crude enzyme (Table 4) [17]. It may be assumed that metabolism of SMIC-3 for initial oxidation of NMP is induced by NMP itself, and the metabolic intermediate (succinate) generated from NMP is catabolized through same metabolic pathway with that from glucose because isocitrate dehydrogenase and malate dehydrogenase are essential enzymes for TCA cycle.

The physiological variation of SMIC-3 grown on glucose and NMP may be caused by the differently expressed enzymes. NMP is a substrate to induce physiological function of SMIC-3 to catabolize itself [18]. Change of substrate from glucose to NMP induced the protein expressions to be greatly changed based on 2D-SDS-PAGE patterns (Fig. 3). Protein spots n1, n4, n6, and n7 were selectively expressed in SMIC-3 grown on NMP, but protein spots n2, n3, n5, n8, and n9 were weakly expressed in SMIC-3 grown on glucose. Meanwhile, protein spots from g2 to g7 were selectively expressed in SMIC-3 grown on NMP, but protein spot g1 was commonly expressed in SMIC-3 grown on glucose and NMP. Four proteins selectively expressed in SMIC-3 grown on NMP are possible to be catalysts for initial metabolism of NMP; however, the proteins could not be identified because the proteins were not released in database. Six proteins selectively expressed in SMIC-3 grown on glucose were very possible to catalyze initial oxidation of glucose; however, the proteins also were not released (Table 5). The difference of protein spots may be a clue that physiological function or metabolic activity of SMIC-3 may be switched to NMP-specific mode during growing on NMP. However, it was limited to inform enzymes catalyzing each step of metabolic pathway from NMP to succinate and from succinate to carbon dioxide, because not all of the protein spots could be identified. Accordingly, the metabolic pathway for catabolism of NMP has to be estimated on the basis of metabolite and metabolic intermediates generated from NMP (Table 2 and 3).

The metabolic pathway for initial oxidative degradation of NMP was designed using methyl amine, 1-methyl succinimide, and succinate (Table 2 and 3). From the results in Tables 2 and 3, it can be deduced that SMIC-3 may oxidize NMP to 1-methyl succinimide by oxygenation and may split 1-methy succinimide into methylamine and succinate by hydration. The biochemical homogeneity of dehydrogenase activities for pyruvate, isocitrate, and succinate suggests the possibility that the metabolic intermediate generated from glucose and NMP may be completely oxidized through a common pathway (TCA cycle). Most metabolic intermediates required for synthesis of building blocks are generated through glycolysis and TCA cycle in *Pseudomonas* genus, from which succinate generated from NMP may be a common intermediate for SMIC-3 growing on glucose and NMP. In the proposed pathway of NMP (Fig. 4), methylamine is a metabolite that is not catabolized by SMIC-3 and succinate is a metabolic intermediate that is catabolized by SMIC-3. The metabolite production is a clue that reducing power yield from NMP is lower than glucose by metabolite production. This may cause growth yield of SMIC-3 on NMP to be lower than that on glucose.

Conclusively, *Pseudomonas* sp. SMIC-3 incompletely oxidized NMP and produced methylamine (acetonitrile) as a final product. Methylamine itself is a modestly toxic compound but can be converted to a strongly toxic compound, hydrogen cyanide, by animal metabolism. Methylamine is a one carbon compound that can be completely oxidized or converted to methane by methylotrophic bacteria and methanogens [19,20]. Accordingly, the methylamine can be biochemically oxidized by co-culture of SMIC-3 and methylotrophic bacteria, since methylotrophic bacteria are aerobes. Chemical absorption or oxidative degradation of methyl amine may be a useful technique for removing the methyl amine by systematic combination with bioreactor operated by SMIC-3 [21,22].

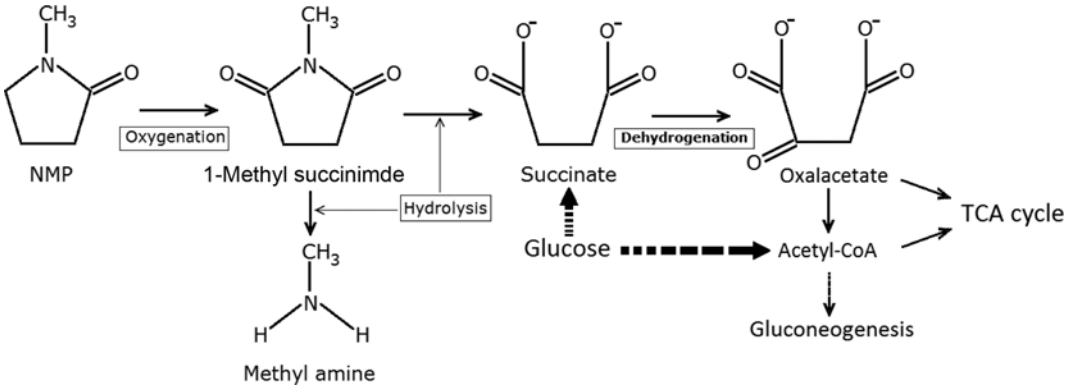

Fig. 4. Proposed pathway of NMP metabolism estimated based on metabolites produced by growing cells, catalysis of crude enzyme, and dehydrogenation activity of crude enzyme.

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Fraction-Spot-Run ID: **g1**
MS-Fit search selects **187** entries (results displayed for top **10** matches).

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 123 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	629	4/4/3	37.3	3.3	-9.44	21.7	No		9535/5.6	M	RHODOBACTER SPHAEROIDES ATCC 17025	hypothetical protein Rsph17025_0429
2	204	4/4/3	23.2	3.3	0.617	20.0	No		17023/5.2		ACINETOBACTER BAUMANNII ATCC 17978	putative O-methyltransferase protein
3	373	4/4/3	23.4	3.3	14.3	9.56	No		19669/6.3	M	ERWINIA TASMANIENSIS ET1/99	Putative capsid completion protein
4	278	4/4/3	17.9	3.3	-3.49	6.67	No		30820/9.8	M	PASTEURELLA DAGMATIS ATCC 43325	cell division protein
5	201	4/4/3	9.1	3.3	6.48	19.5	No		33898/7.7	M	RHODOBACTER SPHAEROIDES ATCC 17029	hypothetical protein Rsph17029_3454
6	273	4/4/3	6.3	3.3	-4.22	21.8	No		81238/5.1	M	DESULFITOBACTERIUM HAFNIENSE Y51	hypothetical protein DSY4901
7	215	5/5/4	5.1	4.1	-3.39	25.9	No		86202/7.6	M	NATRANAEROBIUS THERMOPHILUS JW/NM-WN-LF	diguanylate cyclase and metal dependent phosphohydrolase
8	269	4/4/3	5.3	3.3	2.64	32.6	No		102853/5.4	M	SHEWANELLA BALTICA OS223	TraG domain protein

Fraction-Spot-Run ID: **g2**
MS-Fit search selects **187** entries (results displayed for top **10** matches).

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 123 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	629	4/4/3	37.3	3.3	-9.44	21.7	No		9535/5.6	M	RHODOBACTER SPHAEROIDES ATCC 17025	hypothetical protein Rsph17025_0429
2	204	4/4/3	23.2	3.3	0.617	20.0	No		17023/5.2		ACINETOBACTER BAUMANNII ATCC 17978	putative O-methyltransferase protein
3	373	4/4/3	23.4	3.3	14.3	9.56	No		19669/6.3	M	ERWINIA TASMANIENSIS ET1/99	Putative capsid completion protein
4	278	4/4/3	17.9	3.3	-3.49	6.67	No		30820/9.8	M	PASTEURELLA DAGMATIS ATCC 43325	cell division protein
5	201	4/4/3	9.1	3.3	6.48	19.5	No		33898/7.7	M	RHODOBACTER SPHAEROIDES ATCC 17029	hypothetical protein Rsph17029_3454
6	273	4/4/3	6.3	3.3	-4.22	21.8	No		81238/5.1	M	DESULFITOBACTERIUM HAFNIENSE Y51	hypothetical protein DSY4901
7	215	5/5/4	5.1	4.1	-3.39	25.9	No		86202/7.6	M	NATRANAEROBIUS THERMOPHILUS JW/NM-WN-LF	diguanylate cyclase and metal dependent phosphohydrolase
8	269	4/4/3	5.3	3.3	2.64	32.6	No		102853/5.4	M	SHEWANELLA BALTICA OS223	TraG domain protein

Fraction-Spot-Run ID: **g3**
MS-Fit search selects **651** entries (results displayed for top **10** matches).

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 153 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	323	5/4/3	20.0	2.6	3.73	29.5	No		15547/5.5	M	STREPTOMYCES GRISEOFLAVUS TU4000	hypothetical protein SgriT_35369
2	236	4/4/3	16.9	2.6	-4.95	16.8	[1]		24786/6.2	M	OXALOBACTER FORMIGENES OXCC13	nitroreductase
3	269	4/4/3	18.6	2.6	-0.454	26.3	No		32491/7.7	M	PSEUDOMONAS FLUORESCENS PF0-1	LysR family transcriptional regulator
4	209	4/4/3	12.9	2.6	-3.57	23.2	No		35811/7.1	M	LACTOBACILLUS ANTRI DSM 16041	conserved hypothetical protein
5	232	5/5/3	7.0	3.3	6.64	30.3	No		41079/7.8	M	MEIOTHERMUS SILVANUS DSM 9946	glutamate 5-kinase
6	317	4/4/3	10.1	2.6	2.10	32.7	No		54919/5.5	M	ALCANIVORAX BORKUMENSIS SK2	AAA family ATPase
7	273	4/4/3	6.3	2.6	-8.10	18.1	No		81238/5.1	M	DESULFITOBACTERIUM HAFNIENSE Y51	hypothetical protein DSY4901
8	450	4/4/3	8.2	2.6	2.85	27.9	No		92205/5.8	M	LACTOBACILLUS JENSENII JV-V16	DNA gyrase, subunit A

Fraction-Spot-Run ID: **g4**
MS-Fit search selects **4** entries.

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 39 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	11.2	5/4/10	24.9	12.8	1.57	31.8	No		32703/9.7	M	YARROWIA LIPOLYTICA	YALI0A20988p

2	11.1	4/4/10	10.4	10.3	3.49	1.78	No		84042/5.5	M	PHOTORHABDUS LUMINESCENS SUBSP. LAUMONDII TTO1	fused phosphoenolpyruvate-protein phosphotransferase PtsP/GAF domain-containing protein
3	3.58	4/4/10	8.0	10.3	10.3	21.1	No		101243/5.7	M	ZYMOMONAS MOBILIS SUBSP. MOBILIS ATCC 10988	ATP-binding region, ATPase domain protein
4	19.9	4/4/10	5.2	10.3	-7.29	27.0	No		118898/8.5	M	CLOSTRIDIUM BOLTEAE ATCC BAA-613	hypothetical protein CLOBOL_04835

Fraction-Spot-Run ID: **g5**
MS-Fit search selects **6** entries.

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 59 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	4050	4/4/7	5.8	6.8	0.00869	26.9	No		35921/8.3	M	BORRELIA DUTTONII LY	vlp protein, delta subfamily
2	9.84	4/4/7	8.1	6.8	6.15	22.3	No		57514/7.1	M	THERMOANAEROBACTER TENGCONGENSIS MB4	ABC-type sugar (aldose) transport system, ATPase component
3	32.9	4/4/7	8.8	6.8	-0.574	36.2	No		90975/7.6	M	ENTEROBACTER CANCEROGENUS ATCC 35316	outer membrane usher protein psac
4	2.24	4/4/7	4.7	6.8	-1.49	31.3	No		106649/5.7		STAPHYLOCOCCUS CARNOSUS	beta-D-galactosidase
5	93.1	4/4/7	5.3	6.8	13.8	9.93	No		107987/8.6	M	CORYNEBACTERIUM MATRUCHOTII ATCC 14266	NADH:ubiquinone oxidoreductase subunit 5
6	47.6	4/4/7	5.7	6.8	10.4	17.0	No		120682/5.7	M	STAPHYLOCOCCUS AUREUS SUBSP. AUREUS COL	hypothetical protein SACOL1859

Fraction-Spot-Run ID: **g6**
MS-Fit search selects **14** entries (results displayed for top **10** matches).

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 53 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	161	4/4/8	21.2	7.5	-2.35	19.2	No		28127/5.2	M	CLOSTRIDIUM DIFFICILE 630	cobalt-precorrin-6a reductase
2	169	4/4/8	17.1	7.5	-0.0787	21.3	No		33344/8.9	M	BREVIBACILLUS BREVIS NBRC 100599	transcriptional regulator
3	33.4	4/4/8	15.7	7.5	1.63	8.57	No		36980/9.7	M	ENTEROCOCCUS FAECIUM 1,231,408	integrase
4	117	4/4/8	7.8	7.5	16.1	5.65	No		75185/8.9	M	STAPHYLOCOCCUS AUREUS A9299	transposase B
5	38.3	4/4/8	9.4	7.5	-3.41	32.3	No		77865/6.3	M	VARIOVORAX PARADOXUS S110	malate synthase G
6	11.1	4/4/8	10.4	7.5	3.79	8.97	No		84042/5.5	M	PHOTORHABDUS LUMINESCENS SUBSP. LAUMONDII TTO1	fused phosphoenolpyruvate-protein phosphotransferase PtsP/GAF domain-containing protein
7	8.86	4/4/8	9.0	7.5	-0.988	1.68	No		85300/5.1		CLOSTRIDIUM DIFFICILE QCD-23M63	putative ATP-binding protein
8	6.73	4/4/8	7.1	7.5	-5.13	33.2	No		87790/9.1	M	PREVOTELLA MELANINOGENICA D18	conserved hypothetical protein
9	10.6	4/4/8	6.5	7.5	-1.68	20.0	No		89004/5.5	M	ALICYCLOBACILLUS ACIDOCALDARIUS LAA1	Alpha-glucosidase
10	22.9	4/4/8	8.2	7.5	-3.69	30.8	No		91074/4.9	M	CLOSTRIDIUM PHYTOFERMENTANS ISDG	FG-GAP repeat-containing protein

Fraction-Spot-Run ID: **g7**
MS-Fit search selects **1** entry.

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 45 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	72.1	4/4/9	11.2	8.9	-4.06	22.4	No		46946/10.2	M	FRANKIA SP. CCI3	acyltransferase 3

Fraction-Spot-Run ID: **n1**
MS-Fit search selects **58** entries (results displayed for top **10** matches).

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 84 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	65.2	4/4/5	17.8	4.8	2.93	24.1	No		46402/7.1	M	BEIJERINCKIA INDICA SUBSP. INDICA ATCC 9039	hypothetical protein Bind_3675
2	117	4/4/5	8.5	4.8	2.76	16.7	No		46892/5.7	M	ACIDAMINOCOCCUS FERMENTANS DSM 20731	thiamine biosynthesis protein ThiC
3	110	4/4/5	10.2	4.8	10.4	26.8	No		47066/6.3	M	MICROSCILLA MARINA ATCC 23134	bacterial regulatory protein, DeoR family

4	769	5/5/6	18.8	6.0	-3.56	30.6	No		50487/9.1	M	DICHELOBACTER NODOSUS VCS1703A	signal recognition particle protein
5	56.7	4/4/5	10.4	4.8	7.59	37.0	No		60116/6.3		NEISSERIA MENINGITIDIS ALPHA153	Glucose-6-phosphate isomerase
6	1119	4/4/5	10.4	4.8	-7.64	18.8	No		62724/8.3	M	YARROWIA LIPOLYTICA	YALI0E10395p
7	103	4/4/5	6.8	4.8	0.424	32.2	No		65260/9.0	M	ENTEROCOCCUS FAECALIS T3	predicted protein
8	77.5	6/4/5	10.0	4.8	-1.63	31.3	No		94895/5.1	M	BACTEROIDES SP. 1_1_6	glutaminase
9	148	4/4/5	8.8	4.8	-7.02	15.5	No		98264/6.6	M	LEISHMANIA MAJOR STRAIN FRIEDLIN	hypothetical protein

Fraction-Spot-Run ID: **n3**
MS-Fit search selects **118** entries (results displayed for top **10** matches).

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 64 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	321	4/4/6	4.2	6.2	-2.85	23.2	1		31100/8.7	M	PARABACTEROIDES JOHNSONII DSM 18315	hypothetical protein PRABACTJOHN_02797
2	177	4/4/6	2.3	6.2	1.30	23.2	No		56968/8.3		STREPTOCOCCUS MUTANS	Ffh
3	150	4/4/6	2.1	6.2	1.30	23.2	No		64327/5.1	M	AZORHIZOBIUM CAULINODANS ORS 571	chemotaxis sensory transducer
4	180	4/4/6	5.9	6.2	4.98	20.0	No		64758/4.9		BACTEROIDES SP. D2	hypothetical protein BacD2_07398
5	73.0	4/4/6	6.0	6.2	1.39	19.7	No		68263/4.9	M	BACTEROIDES SP. D20	conserved hypothetical protein
6	135	4/4/6	7.1	6.2	-0.869	42.6	No		72933/7.0	M	LISTERIA INNOCUA CLIP11262	hypothetical protein lin2209

Fraction-Spot-Run ID: **n4**
MS-Fit search selects **31** entries (results displayed for top **10** matches).

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 55 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	58.5	4/4/7	31.6	7.3	-3.50	21.4	No		8854/11.2		SALMONELLA ENTERICA SUBSP. ENTERICA SEROVAR TYPHI STR. 404TY	sensor protein ZraS
2	22.2	4/4/7	12.8	7.3	-0.494	25.3	No		33471/9.6	M	BRADYRHIZOBIUM SP. ORS278	putative Acyl-CoA N-acyltransferase
3	11.7	4/4/7	10.9	7.3	-6.21	15.7	No		39139/10.0		RHIZOBIUM ETLI BRASIL 5	hypothetical protein RetlB5_19868
4	25.4	4/4/7	9.0	7.3	0.393	18.3	No		47007/4.7	M	RUBROBACTER XYLANOPHILUS DSM 9941	NAD(P)(+) transhydrogenase (AB-specific)
5	42.1	4/4/7	6.3	7.3	-5.60	10.8	No		66789/6.0	M	COXIELLA BURNETII RSA 331	aspartyl-tRNA synthetase
6	10.9	5/5/9	6.7	9.1	-6.37	22.7	No		92239/6.3	M	YERSINIA KRISTENSENII ATCC 33638	Penicillin-sensitive transpeptidase
7	17.5	4/4/7	6.0	7.3	-4.62	24.3	No		93017/8.7	M	HAEMOPHILUS SOMNUS 129PT	nitrate reductase
8	33.8	4/4/7	4.8	7.3	-3.09	24.0	No		113317/5.2	M	COLLINSELLA STERCORIS DSM 13279	hypothetical protein COLSTE_01723

Fraction-Spot-Run ID: **n5**
MS-Fit search selects **155** entries (results displayed for top **10** matches).

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 100 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	452	4/4/4	11.4	4.0	11.5	15.1	1		25438/11.0		MYCLE	RecName: Full=Uncharacterized protein ML0869
2	276	4/4/4	10.1	4.0	-0.846	12.5	No		40697/5.3	M	STREPTOCOCCUS INFANTARIUS SUBSP. INFANTARIUS ATCC BAA-102	hypothetical protein STRINF_00857
3	735	5/5/5	15.0	5.0	2.52	29.5	No		44264/5.4	M	PSEUDOALTEROMONAS TUNICATA D2	Tyrosyl-tRNA synthetase II
4	713	4/4/4	11.8	4.0	10.3	25.7	No		45615/4.7	M	VIBRIO ANGUSTUM S14	isocitrate dehydrogenase
5	507	7/5/5	15.3	5.0	6.15	19.1	No		45949/9.2		STREPTOMYCES VIRIDOCHROMOGENES DSM 40736	regulatory protein
6	90.3	4/4/4	12.8	4.0	5.38	24.9	No		51843/4.9	M	THERMOSIPHO AFRICANUS TCF52B	F0F1 ATP synthase subunit beta
7	169	4/4/4	8.6	4.0	2.86	16.5	No		93572/5.9	M	DESULFOVIBRIO AESPOEENSIS ASPO-2	peptidase S16 lon domain protein
8	108	4/4/4	6.2	4.0	1.50	28.8	No		117973/5.8	M	SINORHIZOBIUM MEDICAE WSM419	adenylyl cyclase class-3/4/guanylyl cyclase
9	122	4/4/4	6.8	4.0	-0.145	13.4	No		123887/8.6		VERRUCOMICROBIUM SPINOSUM DSM 4136	hypothetical protein VspiD_23355

Fraction-Spot-Run ID: **n6**
MS-Fit search selects **406** entries (results displayed for top **10** matches).

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 110 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index # Protein MW (Da)/pI	Accession #	Species	Protein Name
1	630	4/4/4	59.1	3.6	4.33	19.3	No	9456/5.0		MAGNETOSPIRILLUM MAGNETOTACTICUM MS-1	COG0539: Ribosomal protein S1
2	335	4/4/4	8.7	3.6	0.459	10.1	No	32160/5.8	M	THERMUS THERMOPHILUS HB27	arginase
3	349	4/4/4	17.3	3.6	16.0	6.95	No	34321/9.1	M	PSEUDOMONAS AERUGINOSA 2192	hypothetical protein PA2G_03003
4	283	4/4/4	9.9	3.6	-2.52	8.85	No	43087/6.9	M	SULFOLOBUS ISLANDICUS M.14.25	acetyl-CoA acetyltransferase
5	279	4/4/4	9.4	3.6	3.48	30.3	No	43632/10.3	M	NEISSERIA MUCOSA ATCC 25996	putative membrane protein

Fraction-Spot-Run ID: **n7**
MS-Fit search selects **145** entries (results displayed for top **10** matches).

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 79 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index # Protein MW (Da)/pI	Accession #	Species	Protein Name
1	77.9	4/4/5	15.9	5.1	6.49	19.2	No	27276/5.2		STREPTOMYCES SP. SPB78	GntR family transcriptional regulator
2	95.1	4/4/5	17.6	5.1	4.06	18.9	No	32527/9.4	M	VIBRIO CORALLIILYTICUS ATCC BAA-450	ribosomal protein S6 glutaminyl transferase
3	67.3	4/4/5	15.8	5.1	1.93	19.1	No	37004/5.5	M	STAPHYLOCOCCUS EPIDERMIDIS ATCC 12228	putative homoserine-o-acetyltransferase
4	62.5	4/4/5	15.1	5.1	12.0	17.7	No	37502/8.4		LISTERIA MONOCYTOGENES FSL J2-064	hypothetical protein LmonocytoFSL_14784
5	160	4/4/5	11.4	5.1	0.908	23.2	No	54823/5.1	M	CHLOROBACULUM PARVUM NCIB 8327	prolyl-tRNA synthetase
6	69.6	4/4/5	7.3	5.1	9.29	18.1	No	75067/5.5	M	GARDNERELLA VAGINALIS 409-05	AMP-binding enzyme
7	91.6	4/4/5	6.6	5.1	2.65	14.2	No	102942/5.1	M	SILICIBACTER LACUSCAERULENSIS ITI-1157	negative regulator of genetic competence ClpC/mecB
8	71.6	4/4/5	6.1	5.1	-11.0	13.8	No	104234/5.9	M	STREPTOMYCES GRISEUS SUBSP. GRISEUS NBRC 13350	LuxR family transcriptional regulator

Fraction-Spot-Run ID: **n8**
MS-Fit search selects **6** entries.

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 60 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index # Protein MW (Da)/pI	Accession #	Species	Protein Name
1	140	4/4/7	22.4	6.7	0.395	24.4	No	31293/5.5	M	RHIZOBIUM ETLI CFN 42	aminoglycoside N3`-acetyltransferase protein
2	26.8	4/4/7	7.9	6.7	-5.51	11.6	No	66283/4.9	M	DESULFOTALEA PSYCHROPHILA LSV54	sigma factor SigB regulation protein (RsbU)
3	27.8	4/4/7	6.3	6.7	-1.84	33.8	No	91345/9.5	M	TRYPANOSOMA CRUZI STRAIN CL BRENER	DNA-repair protein
4	55.9	4/4/7	6.2	6.7	11.4	10.6	No	104554/7.4	M	MYCGA	RecName: Full=Alanyl-tRNA synthetase; AltName: Full=Alanine--tRNA ligase; Short=AlaRS

Fraction-Spot-Run ID: **n9**
MS-Fit search selects **11** entries (results displayed for top **10** matches).

[+] **Results Summary**

Protein Hit Number	MOWSE Score	# pep # mat % mat 68 pks	% Cov	% TIC	Mean Err ppm	Data Tol ppm	# Hom Prot	MS-Digest Index # Protein MW (Da)/pI	Accession #	Species	Protein Name
1	81.0	4/4/6	19.1	5.9	-5.46	13.9	No	31371/6.1	M	BACILLUS THURINGIENSIS SEROVAR HUAZHONGENSIS BGSC 4BD1	hypothetical protein bthur0011_33920
2	50.1	4/4/6	10.9	5.9	2.66	23.2	No	44738/5.4	M	PARVIBACULUM LAVAMENTIVORANS DS-1	Rieske (2Fe-2S) domain-containing protein
3	80.1	4/4/6	10.0	5.9	1.33	13.0	No	46123/5.3	M	METHYLOBACTERIUM NODULANS ORS 2060	hypothetical protein Mnod_3408
4	4.00	4/4/6	7.5	5.9	2.62	22.9	No	85638/8.9	M	VIBRIO SP. EX25	glycerol-3-phosphate acyltransferase
5	3.99	4/4/6	8.5	5.9	10.9	19.0	No	85994/8.2	M	BRACHYSPIRA HYODYSENTERIAE WA1	translation initiation factor IF-2
6	9.04	4/4/6	4.7	5.9	9.33	21.3	No	87389/9.4	M	BACILLUS CEREUS AH187	permease, putative
7	11.0	5/4/6	5.6	5.9	-0.648	29.9	No	117985/8.4	M	FRANKIA SP. EUI1C	ATP-dependent transcriptional regulator protein-like protein
8	6.24	4/4/6	3.6	5.9	1.37	23.1	No	118415/6.6	M	HALOTHIOBACILLUS NEAPOLITANUS C2	Protein of unknown function DUF2309