Isolation of a Constitutive *N*-Acetylneuraminate Lyase-producing Mutant of *Escherichia coli* and Its Use for NPL Production

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An *E. coli* mutant (M8328) was isolated by NTG mutagenesis from *E. coli* KMS3207 which has an inducible *N*-acetylneuraminate lyase (NPL). The mutant was resistant to catabolite repression and produced NPL constitutively. To improve the productivity of NPL, a hybrid plasmid, pMK6, carrying the *npl* gene was introduced into the mutant, M8328. The NPL activity level was increased more than 5-fold in the pMK6-harboring mutant compared with that in the mutant, when the cells were grown in inducer (*N*-acetylneuraminic acid)-free medium. The NPLs produced by the mutant and the pMK6-harboring cells were structurally and immunologically identical with that purified from the parent strain (KMS3207).

N-Acetylneuraminate lyase (NPL) [EC 4.1.3.3] converts *N*-acetylneuraminic acid (NANA) to pyruvate and *N*-acetyl-D-mannosamine. The enzyme is distributed in a wide variety of bacteria belonging to genera such as *Escherichia*, *Pseudomonas*, *Enterobacter*, *Proteus*, *Micrococcus*, *Sarcina*, *Brevibacterium*, *Corynebacterium*, *Arthrobacter*, *Bacillus*, *Hafnia*, *Vibrio* and *Clostridium*.^{1~3)} NPL is the key enzyme for the diagnostic assaying of NANA in serum or urine,^{4,5)} and efficient production methods for NPL have been sought by the present authors,^{6,7)} Kawakami *et al.*⁸⁾ and Aisaka *et al.*⁹⁾ independently.

As reported previously,⁶⁾ NPL is an inducible enzyme in *E. coli* and is formed only in the presence of an inducer (NANA). However, the use of NANA is limited because of its high cost. To overcome this disadvantage, we attempted to obtain an *E. coli* mutant capable of producing NPL in the absence of an inducer.

In this paper, we report the construction of a mutant that produces NPL constitutively and

the production of NPL by the mutant and/or the mutant transformed with pMK6 carrying the *npl* gene.

Materials and Methods

Microorganisms and cultivation. *Escherichia coli* KMS3207, an NPL-producing strain, was used as the parent strain. *E. coli* cells were grown on nutrient broth (yeast extract 1%, meat extract 1%, pH 7.2), L broth (peptone 1%, yeast extract 0.5%, NaCl 0.5%, pH 7.2) or minimal medium (carbon source 0.2%, K₂HPO₄ 0.7%, KH₂PO₄ 0.2%, (KH₄)₂SO₄ 0.1%, MgSO₄·7H₂O 0.01%). YS medium (yeast extract 2%, succinic acid 1%, pH 7.0) was also used for assaying NPL production. Cultivation was carried out at 30°C with reciprocation unless otherwise noted.

Mutagenesis and selection. Mutagenesis was performed with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) according to the method of Adelberg *et al.*¹⁰⁾ Cells in the early log phase in nutrient broth were harvested and then resuspended in a saline solution (10⁸ cells/ml), followed by treatment with NTG (100 µg/ml) at 37°C for 30 min. The mutagenized cells were collected, washed with saline and then suspended in glucose minimal medium (10⁸ cells/ml).

The cell suspension was incubated for about 3 hr and then transferred to NANA-containing minimal medium, followed by incubation for about 3 hr. This process was repeated three times. The treated cells were spread on glucose minimal agar plates to form colonies. About 100 colonies were randomly picked up and the NPL activities of the mutant cells were determined. The mutant showing the highest activity was chosen and designated as M8328.

Assay for NPL. After cultivation for 10 hr at 30°C, the cells were harvested by centrifugation at $15,000 \times g$ for 10 min, washed twice with a 0.85% saline solution and then resuspended in 20 mM potassium phosphate buffer (pH 7.3). The cells were disrupted ultrasonically at 20 kHz and 0°C for 2 min, and then the homogenate was centrifuged at $20,000 \times g$ for 10 min. The cell extract thus obtained was used for assaying NPL. The detailed conditions for the preparation of cell extracts and the NPL activity assay were given in our previous paper.¹¹⁾ One unit of NPL activity is defined as the amount releasing 1 μ mol of *N*-acetyl-D-mannosamine per min at 37°C. Protein was determined by the method of Lowry *et al.*¹²⁾

Transformation and selection. Transformation of *E. coli* M8328 was carried out as described by Norgard *et al.*¹³⁾ Transformants were selected on agar plates of L-broth containing 10 μ g/ml of ampicillin.

Purification of NPL. NPL was purified from mutant M8328 and pMK6-transformed cells, respectively. The purification method was essentially the same as that described by Uchida *et al.*¹¹⁾

Electrophoresis. Sodium dodecyl sulphate (SDS) poly-

acrylamide gel electrophoresis was performed with a separating gel of 12% acrylamide according to the method of Laemmli.¹⁴⁾

Immunological test. Anti-serum against the *E. coli* NPL was obtained from a rabbit immunized against purified NPL with complete Freund's adjuvant (Wako Pure Chem., Osaka, Japan). The immuno-double diffusion test was performed in 1% agarose containing 50 mM sodium phosphate buffer (pH 7.0).

Results and Discussion

Induction and selection of an NPL constitutive mutant

When *E. coli* KMS3207 cells were grown on the minimal medium containing NANA (0.2 %) as a sole source of carbon, NPL formation was induced, the maximum being reached at the early stationary phase, and then the level remained constant throughout the culture (Fig. 1a). However, when glucose was added to the medium together with NANA, the formation of NPL was significantly repressed, although the growth of *E. coli* KMS3207 was not inhibited (Fig. 1b). The NPL formation increased in parallel with the decrease in glucose concentration, but the level of NPL activity was only one-third of that in the case of the NANA-minimal medium (Fig. 1a). This

Fig. 1. Effect of Glucose on NPL Production by *E. coli* KMS3207.

Cells were incubated at 30°C in 100 ml of the minimal medium containing NANA 0.2% (a) or NANA 0.2% and glucose 0.5% (b).

●—●, cell growth; ○—○, NANA; △—△, glucose; ▨, NPL activity.

Table I. NPL ACTIVITY OF MUTANT M8328

The activity was determined using cell extracts as described under **Materials and Methods**.

Media	Strains	
	NPL activity (units/mg protein)	
	Wild (KM3207)	Mutant (M8328)
NANA minimal	0.51	1.27
Nutrient broth	0.00	1.32

Table II. NPL ACTIVITY OF THE TRANSFORMANT

All cells were grown on YS medium and the activity was determined using cell extracts as described under **Materials and Methods**.

Strains	NPL activity (U/mg protein)
Wild (KMS3207)	0.0
Mutant (M8328)	1.9
Transformant (M8328/pMK6)	10.3

repressive effect of glucose on NPL formation was reversed by the addition of cyclic adenosine 3',5'-monophosphate (data not shown), suggesting that the NPL formation is controlled by catabolite repression. If a mutant freed from catabolite repression can be obtained, it must be an NPL-constitutive strain and must produce the enzyme without an inducer. According to the procedure described under **Materials and Methods**, we obtained one NPL-constitutive strain, which was designated as M8328. As shown in Table I, M8328 was capable of producing NPL without NANA, demonstrating that it was an NPL-constitutive mutant. When M8328 was grown in the minimal medium containing an inducer (NANA), the NPL activity increased approximately 2-fold in comparison with that of the wild strain. Furthermore, the mutant cells produced a larger amount of NPL in nutrient broth without the inducer (NANA), whereas the wild strain produced no detectable amount of NPL under the same conditions. These results indicated that the formation of NPL by mutant M8328 was insensitive to catabolite repression and that NPL could be produced irrespective of the presence or absence of an inducer (NANA).

Production of NPL by pMK6-transformed M8328

In order to further improve the productivity of NPL, hybrid plasmid pMK6^{6,7)} containing the *npl* gene for NPL was introduced into *E. coli* M8328. The frequency of transformation of M8328 by the plasmid was approximately

Fig. 2. Analysis of Extracts of Wild, Mutant and Transformant Cells by SDS-Polyacrylamide Gel Electrophoresis.

The conditions are given under **Materials and Methods**. 1, marker proteins (from top): phosphorylase *b* 94 K, bovine serum albumin 67 K, ovalbumin 45 K, chymotrypsinogen *A* 25 K, cytochrome C 12.5 K; 2, purified NPL; 3, transformant (M8328/pMK6); 4, mutant (M8328); 5, wild (KMS3207).

10^6 per μg of DNA. When one of the transformants (pMK6/M8328) was grown in the YS medium, a marked increase in NPL activity (10.3 U/mg protein) was observed, as shown in Table II. Cell extracts of M8328/pMK6, M8328 and the wild strain were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). Each

Table III. PURIFICATION OF NPLs FROM *E. coli* M8328 AND M8328/pMK6

Strains	Steps	Total units (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Activity yield (%)
M8328	Crude extract	4,400	2,316	1.9	1.0	100
	DEAE-Cellulose	3,564	125	28.5	15.0	81
	Gel filtration	3,080	69	44.6	23.5	70
	Lyophilization	2,904	63	46.1	24.3	66
M8328/pMK6	Crude extract	22,660	2,200	10.3	1.0	100
	DEAE-Cellulose	20,847	601	34.7	3.4	92
	Gel filtration	19,941	415	48.1	4.7	88
	Lyophilization	19,261	396	48.6	4.7	85

sample gave a band corresponding to that of the purified NPL, but the intensity differed with the strain. Among these strains, M-8328/pMK6 gave the densest protein band, corresponding to a M_w of 33,000, a value similar to the M_w of the NPL subunit, and the amount of NPL produced by M8328/pMK6 was calculated to comprise about 20% of the total cellular soluble protein. Thus the productivity of NPL was greatly improved by the introduction of a hybrid plasmid containing the *npl* gene into a mutant insensitive to catabolite repression. Since the inducer (NANA) is very expensive, the efficient and enhanced accumulation of NPL in a simple medium (YS medium) without an inducer is advantageous for the production of NPL on an industrial scale.

Purification and properties of NPLs

Typical purification procedures for NPLs from M8328/pMK6 and M8328 cells on 1 l culture of broth scale are summarized in Table III. The NPLs of M8328/pMK6 and M8328 were purified approximately 4.7- and 24.3-fold, and the activity yields were 85% and 66%, respectively. The purified NPLs from these strains were found to be homogeneous on polyacrylamide gel electrophoresis and showed the same mobility as that purified from the wild strain (KMS3207) (data not shown). Although the detailed physicochemical properties are not shown, the NPLs purified from the two strains were the same as that in the wild strain

Fig. 3. Double Diffusion Test of the Purified NPLs.

Center well, anti-serum against NPL of *E. coli* KMS3207; Well 1, NPL from *E. coli* KMS3207; Well 2, NPL from the mutant (M8328); Wells 3 and 5, 20 mM phosphate buffer (pH 7.3); Well 4, NPL from *Arthrobacter ureafaciens*; Well 6, NPL from the transformant (M8328/pMK6).

with respect to the optimum pH (7.5~8.0), pH stability (6.0~9.0), temperature stability (up to 65°C), K_m value (3.6 mM for NANA) and behaviour toward inhibitors (Ag^+ and Hg^{++}).

Double diffusion tests were also carried out to determine the immunochemical characteristics of NPLs produced by M8328/pMK6 and M8328 (Fig. 3). The precipitin lines formed by the M8328 and M8328/pMK6 NPLs completely fused with that of the parent, indicating that the NPLs have the same immunochemical properties.

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