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Genetic and Molecular Analyses of *Escherichia coli* *N*-Acetylneuraminate Lyase Gene

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Two plasmids containing the *N*-acetylneuraminate lyase (NALase) gene (*nanA*) of *Escherichia coli*, pNL1 and pNL4, were constructed. Immunoprecipitation analysis indicated that the 35,000-dalton protein encoded in pNL4 was NALase. The synthesis of NALase in *E. coli* carrying these plasmids was constitutive.

N-Acetylneuraminic acid (NANA) is cleaved to pyruvate and *N*-acetyl-D-mannosamine in a reversible reaction by *N*-acetylneuraminate lyase (NALase) (*N*-acetylneuraminic acid aldolase [EC 4.1.3.3]). NALase is an inducible enzyme in *Escherichia coli* that is produced only in the presence of NANA. The purification of NALase from *E. coli* and its enzymatic properties were reported previously (12). The K1 capsular polysaccharide produced by *E. coli* K1 is composed of polysialic acid (poly-*N*-acetylneuramic acid). Silver et al. cloned the K1 capsular polysaccharide genes of *E. coli* and proposed that the A region included a NANA operon concerned with NANA synthesis (10, 11). Recently, Vimr and Troy reported that mutations affecting either the transport or degradation of sialic acid were designated *nanT* and *nanA*, respectively, and that NALase regulated the intracellular concentration of sialic acid (13, 14). We cloned the *nanA* gene to attempt to analyze genetically NANA catabolism. This paper deals with the genetic and molecular analyses of the NALase gene (*nanA*) of *E. coli*.

The strains and plasmids used are listed in Table 1. Nutrient broth containing thymine (4), LB medium (5), and M9 minimal medium (5) were used. NALase induction medium contained 1 g of KH_2PO_4, 0.5 g of $MgSO_4$, 2 g of yeast extract (Difco Laboratories, Detroit, Mich.), 5 g of polypeptone, and 5 g of NANA (Nakarai Chemical Co., Kyoto, Japan) in 1 liter of distilled water adjusted to pH 7.0. NALase activity was assayed as described previously (9). One unit of NALase activity was defined as the amount of enzyme that liberated 1 μmole of pyruvate per min under the reaction conditions used.

E. coli K-12 JE1011 cells were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and the mutagenized colonies were replica plated onto an M9 minimal medium plate containing NANA as a carbon source and onto an M9 minimal medium plate containing pyruvate instead of NANA. Colonies which formed on the latter plates but not on the former plates were selected. Of 4,000 mutagenized cells, 9 mutants were isolated and classified into two groups according to the incorporation of ^{14}C-NANA (Table 2). The mutants of the first group (group 1, 22-1 and 22-2) were deficient in NALase, whereas the group 2 mutants seemed to be deficient in the NANA transport system.

For cloning of the *nanA* gene of *E. coli*, the chromosomal DNA of *E. coli* JE1011 (4) and plasmid pBR322 DNA (5) were digested with *Hin*dIII and ligated with T4 DNA ligase. The ligation mixtures were used to transform *E. coli* 22-1 (a *nanA* mutant). The recombinant plasmid pNL1 was obtained from an Ap^r *nanA*$^+$ transformant which contained a 7.7-kilobase-pair (kb) *Hin*dIII fragment. *Eco*RI and *Hin*cII sites were mapped within the 7.7-kb *Hin*dIII fragment. A subcloning experiment indicated that a 1.3-kb *Eco*RI-*Hin*dIII fragment in pBR322 was indispensable for NALase activity (pNL4). Plasmid pNL1 could transform group 1 and 2

TABLE 1. Strains and plasmids used

Strain and plasmid	Properties	Reference
HB101	*pro leuB thi lacY hsdR hsdM ara-14 galK2 xyl-5 mtl-1 supE44* F$^-$ *Endo*A *recA* Str^r	8
JE1011	F$^-$ *thr leu trp his thy thi ara lac gal xyl mtl* Str^r	4
CSR603	*thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44* F$^-$	2a
22-1 and 22-2	Group 1 mutants derived from JE1011 (*nanA* mutants)	This study
22-4, 22-5, 24-4, 25-2, 25-3, 25-4, and 25-5	Group 2 mutants derived from JE1011	This study
CGSC 4245	Hfr KL-16[a] *thi-1 relA1 spoT1*	2
KL-14	Hfr KL-14[b] thi-1 *relA1*	2
CGSC 4311	Hfr KL226[c] *relA1 tonA22* Tc^r *pit-10 spoT1*	2
CGSC 4316	Hfr pK191[d] Δ(*gpt-lac*) *5 supE44 relA1 thi-1*	2
pBR322	Ap^r Tc^r	8
pNL1	pBR322 plus 7.7-kb *Hin*dIII fragment of *E. coli*	This study
pNL4	pBR322 plus 1.3-kb *Eco*RI-*Hin*dIII fragment of *E. coli*	This study

[a] Hfr KL-16 transfers *lysA* first and *serA* last.
[b] Hfr KL-14 transfers *argG* first and *metC* last.
[c] Hfr KL226 transfers *purE* first and *lip* last.
[d] Hfr pK191 transfers *supD* first and *cheC* last.

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TABLE 2. Time course of ^{14}C-NANA uptake[a]

Strain	Incorporation of ^{14}C-NANA (nmol/mg of protein) at:		
	0 min	15 min	30 min
JE1011	0.09	2.86	5.01
22-1	0.25	18.3	20.9
22-2	0.94	10.6	9.03
22-4	0.27	0.09	0.20
22-5	0.08	0.08	0.10
24-4	0.06	0.25	0.35
25-2	0.09	0.08	0.17
25-3	0.19	0.16	0.09
25-4	0.06	0.09	0.14
25-5	0.11	0.08	0.11

[a] The cells (15- to 16-h culture in NALase induction medium without NANA) were collected and suspended in 50 mM phosphate buffer (pH 7.0). After preincubation for 10 min at 37°C, 3 μl of ^{14}C-NANA (244 Ci/mol; Amersham Corp., Arlington Heights, Ill.) and 30 μl of 2 mM NANA were added to 0.6 ml of each suspension, and the suspensions were incubated for 0, 15, and 30 min at 37°C. The samples were filtered through membrane filters (0.45-μm HA), and the radioactivity on the dried filters was determined (3).

mutants to Ap^r Nan$^+$ at a high frequency. On the other hand, plasmid pNL4 could transform group 1 mutants to Ap^r Nan$^+$ but could not transform group 2 mutants to Ap^r Nan$^+$.

For the identification of plasmid-encoded polypeptides, a modification of the maxicell method described by Sancar et al. was used (7). No incorporation of label into protein occurred in the parent strain, CSR603, after irradiation. Strains carrying pBR322 produced proteins with molecular weights of 35,000 and 28,000, corresponding to the products of the Tc^r and Ap^r genes, respectively (Fig. 1, lane 4). Cells carrying pNL4 also produced proteins with molecular weights of 35,000 and 28,000, but the protein with a molecular weight of 35,000 did not correspond to the Tc^r gene product, since pNL4 was tetracycline sensitive, owing to the insertion of the 1.3-kb fragment. The supernatant of such cells after lysozyme treatment was immunoprecipitated with antiserum against *E. coli* NALase (6, 8). The immunoprecipitate was solubilized and analyzed by gel electrophoresis. The molecular weight of the band detected on the gel was 35,000 (Fig. 1, lane 3). The size of the band on the gel was consistent with that of the purified protein. These results indicated that the gene product from pNL4 was NALase.

The expression and enzymatic properties of NALase from *E. coli* (pNL4) were examined. *E. coli* HB101(pBR322), *E. coli* HB101(pNL1), and *E. coli* HB101(pNL4) were aerobically grown in nutrient broth containing thymine for 24 h at 37°C, and the NALase activities in the cells were assayed. No NALase activity was observed in *E. coli* HB101 and *E. coli* HB101(pBR322) (<0.01 U/ml). The NALase activities in *E. coli* HB101(pNL1) and *E. coli* HB101(pNL4) were 0.20 and 0.22 U/ml, respectively. These results indicated that the synthesis of NALase in *E. coli* (pNL1) or *E. coli* (pNL4) was constitutive. NALase was purified from *E. coli* (pNL4) grown in LB medium (constitutive NALase) or *E. coli* grown in NALase induction medium (inducible NALase) by protamine treatment, fractionation with ammonium sulfate, heat treatment, column chromatography on DEAE-Sepharose, and gel filtration on Sephacryl S-200. The results of an Ouchterlony double-diffusion test with both enzymes indicated that the constitutive NALase immunologically cross-reacted with the inducible NALase. The constitutive NALase had the same enzymatic properties as the inducible NALase in terms of molecular weight (35,000), optimal pH (pH 7.5), and K_m (2.5 mM). Preliminary mapping of the *nanA* gene of *E. coli* was carried out (4). The results of crosses between various Hfr strains (KL-14, KL-16, KL226, and pK191) and *nan* mutants indicated that *nan* genes were located at approximately 70 and 80 min on the genetic map (1).

Two groups of mutants were obtained, as judged by the incorporation of ^{14}C-NANA into cells. Group 1 seems to be *nanA* mutants, and group 2 seems to be *nanT* mutants, as reported by Vimr and Troy (13). Immunoprecipitation analysis confirmed that the gene product from pNL4 was the NALase enzyme and that plasmid pNL4 could transform group 1 mutants (22-1 and 22-2) to Nan$^+$ but could not transform group 2 mutants to Nan$^+$. These results strongly indicated that the 1.3-kb *Eco*RI-*Hin*dIII fragment contained the *nanA* gene and that group 1 mutants were deficient in NALase (*nanA* mutants). On the other hand, plasmid pNL1 could transform both group 1 and group 2 mutants to Nan$^+$, suggesting that the cloned 7.7-kb fragment contained both *nanA* and *nanT* genes, as reported by Vimr and Troy (13). The preliminary mapping data for these genes were also consistent with the location reported by Vimr and Troy (13).

The synthesis of NALase in *E. coli* (pNL1) or *E. coli* (pNL4) was constitutive. There are two possibilities: either the cloned fragment lacks the regulatory region or the *nanA* gene is derepressed by high-copy-number plasmids.

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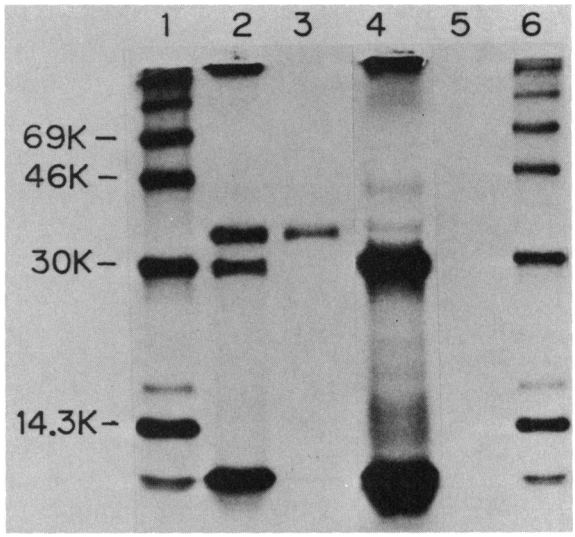

FIG. 1. Autoradiogram of immunoprecipitated [^{35}S]methionine-labeled proteins made in *E. coli* CSR603 carrying various plasmids. The 12% sodium dodecyl sulfate-polyacrylamide gels for protein separation were prepared by the method of Laemmli (see reference 5). Lanes: 1 and 6, ^{14}C-labeled proteins used as molecular size markers (lysozyme [14.3 kilodaltons], carbonic anhydrase [30 kilodaltons], ovalbumin [46 kilodaltons], and bovine serum albumin [69 kilodaltons]); 2, autoradiogram of plasmid-encoded proteins obtained from *E. coli* CSR603(pNL4); 3, autoradiogram of immunoprecipitate of plasmid-encoded proteins obtained from *E. coli* CSR603(pNL4); 4, autoradiogram of plasmid-encoded proteins obtained from *E. coli* CSR603(pBR322); 5, autoradiogram of immunoprecipitate of plasmid-encoded proteins obtained from *E. coli* CSR603(pBR322). K, Kilodaltons.

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