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Cloning and Expression of a Gene Encoding *N*-Glycosyltrasferase (*ngt*) from *Saccharothrix aerocolonigenes* ATCC39243

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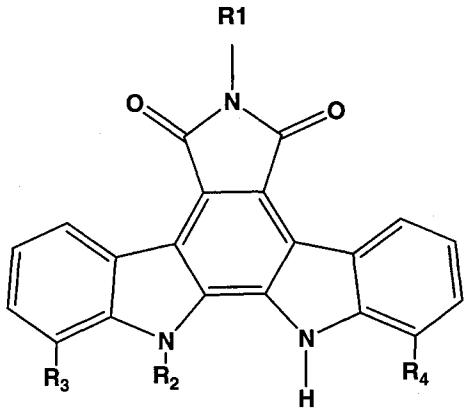
In the course of our bioconversion studies on the derivatives of an indolocarbazole, J-104303, *Saccharothrix aerocolonigenes* ATCC39243 was found to convert J-104303, which was added into the culture medium, to its glycosylated derivative, J-109384. In order to clone the gene having the ability to convert J-104303 to J-109384, a library of *Saccharothrix aerocolonigenes* ATCC39243 DNA fragments was constructed using *Streptomyces lividans* TK21 and pIJ702 as host strain and vector, respectively. By examining more than 5,000 transformants, one was found to convert J-104303 to J-109384. Sequence analysis of the inserted DNA fragment revealed an open reading frame with 1,245 base pairs, named *ngt*. The transformant containing this *ngt* gene was also found to introduce a D-glucose moiety into 6-*N*-methylarcyriaflavin C. Furthermore, when *ngt* was introduced into *Streptomyces mobaraensis* BA13793, a producer of J-104303, the resulting transformant produced J-109384 directly.

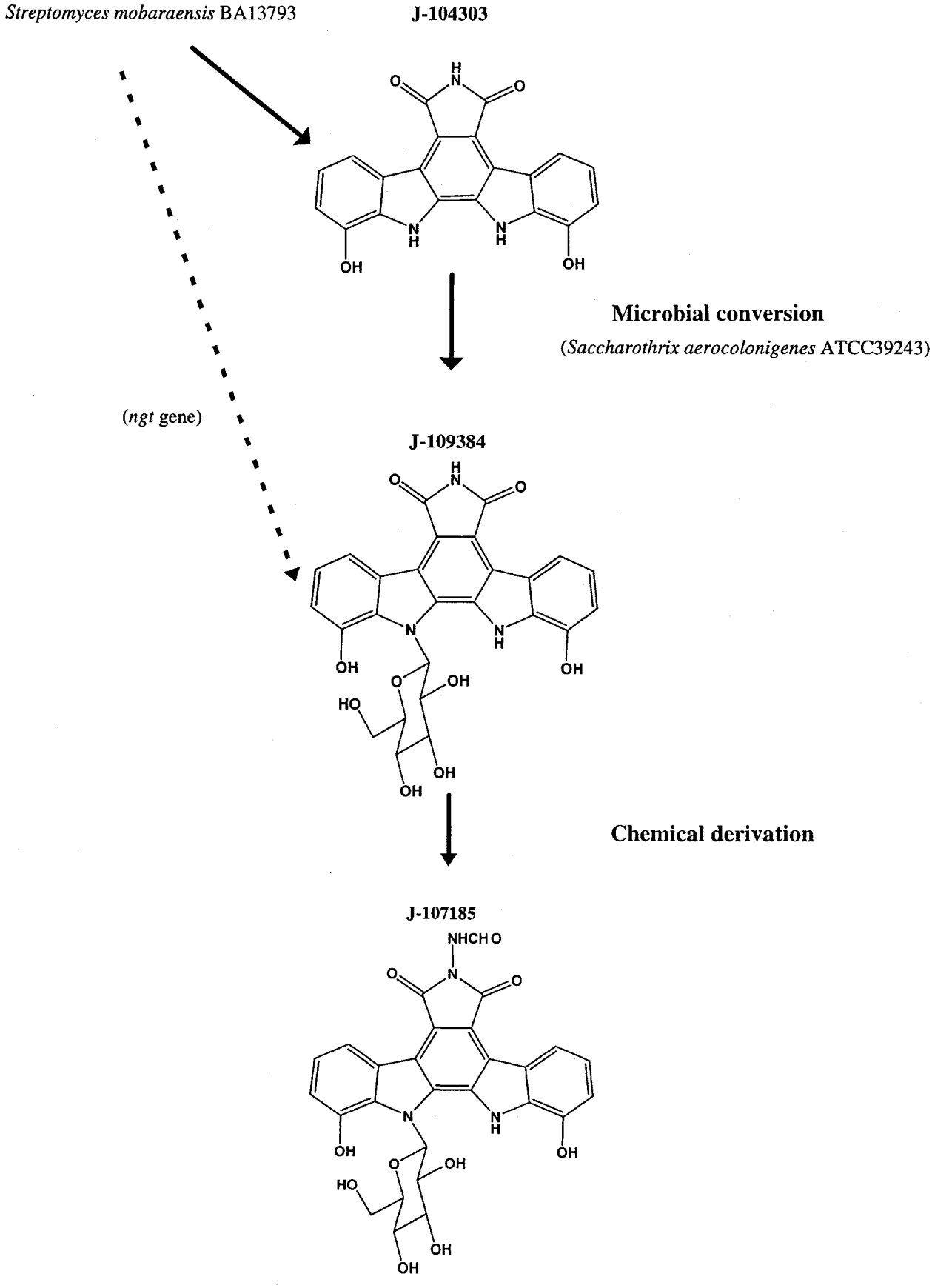
Isolation of J-104303 (BE-13793C; 12,13-dihydro-1,11-dihydroxy-5*H*-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6*H*)-dione), from the culture broth of *Streptomyces mobaraensis* BA13793 was first reported in 1991[1]. J-104303 showed topoisomerase I and II inhibitory activity and inhibited the growth of doxorubicin-resistant (P388/ADR) and vincristine-resistant (P388/VCR) murine leukemia cell lines *in vitro* but not *in vivo*, although, it did exhibit antitumor activity against Ehrlich ascites tumor transplanted in mice[1]. Since J-104303 was seemed to have

Fig. 1. Structures of J-104303, J-109384, rebeccamycin and J-107185.



	R1	R2	R3	R4
J-104303	H	H	OH	OH
J-109384	H	β-D-glucose	OH	OH
Rebeccamycin	H	4-methoxy-β-D-glucose	Cl	Cl
J-107185	NHCHO	β-D-glucose	OH	OH

Fig. 2. Preparation route of J-107185.



The solid arrows indicate the current method. The dotted arrow shows one step microbial production of J-109384.

potential as an antitumor lead compound, derivation studies were carried out. J-109384 (ED-110) a derivative of J-104303 having a D-glucose moiety at N-13, showed effective growth inhibition on various murine and human tumor cells *in vitro* with remarkable *in vivo* antitumor effects not only on murine leukemias but also on murine and human solid tumors transplanted into mice[2]. Biochemical studies of J-109384 showed that it inhibited topoisomerase I but not topoisomerase II[3]. Further modification studies for J-109384 resulted in a still more effective compound, J-107185 (NB-506, 6-*N*-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5*H*-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6*H*)-dione)[4~6], which selectively inhibited topoisomerase I[4]. As shown in Fig. 2, J-107185 was easily derived chemically from the key intermediate, J-109384, but chemical production of the latter from J-104303 proceeded with low over all yield[7]. Since we had earlier shown that *Saccharothrix aerocolonigenes* ATCC39243, producer of rebeccamycin[8,9], could convert J-104303 to J-109384, it was decided to clone the gene responsible for such conversion in order to facilitate efficient production of J-109384 by *Streptomyces mobaraensis* BA13793 (Fig. 2).

In this paper, we report the cloning and sequencing of the gene encoding *N*-glycosyltransferase (*ngt*) from *Saccharothrix aerocolonigenes* ATCC39243 and the direct production of J-109384 by a transformant of strain BA13793 carrying *ngt*. Glycosylation of 6-*N*-methylarcyriaflavin C by *S. lividans* TK21 carrying the *ngt* gene was also observed.

Materials and Methods

Bacterial Strains, Plasmids, Media and Reagents

Streptomyces mobaraensis BA13793 was obtained from our laboratory strain collection as reported previously[1]. *S. mobaraensis* 725M is a high level producer of J-104303 derived from BA13793. *Saccharothrix aerocolonigenes* ATCC39243, *S. lividans* TK21 and *S. lividans* ATCC35287 carrying pIJ702 were obtained from the American Type Culture Collection (ATCC). Plasmid pIJ702[10] was prepared from *S. lividans* TK21. The *E. coli* cloning vectors pUC118 and pUC119 and *E. coli* strain JM109 were purchased from Takara Shuzo.

TSB (Trypticase Soy Broth) medium (Becton Dickinson) was used to grow mycelia of *S. mobaraensis* BA13793, *S. lividans* TK21 and *Saccharothrix aerocolonigenes* ATCC-39243. For protoplasting, *S. mobaraensis* BA13793 and *S. lividans* TK21 were grown in TSB medium containing glycine at 0.8% (w/v) and 0.5% (w/v), respectively. Detection medium (MM-2) for the conversion of J-104303 to J-109384 contained (per litre): 5 g glucose, 80 g dextrin, 3 g yeast extract (Difco), 2.5 g L-threonine, 1 g magnesium sulfate, 0.5 g calcium chloride, 2 g calcium carbonate, 2 mg ferrous sulfate, 0.4 mg cupric chloride, 0.4 mg manganese chloride, 0.4 mg cobalt chloride, 0.8 mg zinc sulfate, 0.8 mg sodium borate, 2.4 mg ammonium molybdate and 5 g 3-(*N*-morphorino)propanesulfonic acid (pH adjusted to 7.2 with NaOH). SOB medium for growth of *E. coli* strain JM109 contained (per litre): 20 g Tryptone (Difco), 5 g yeast extract, 0.58 g sodium chloride, and 0.19 g potassium chloride. Ampicillin (100 μg/ml) was additionally added to SOB medium for the culture of the transformants of *E. coli*.

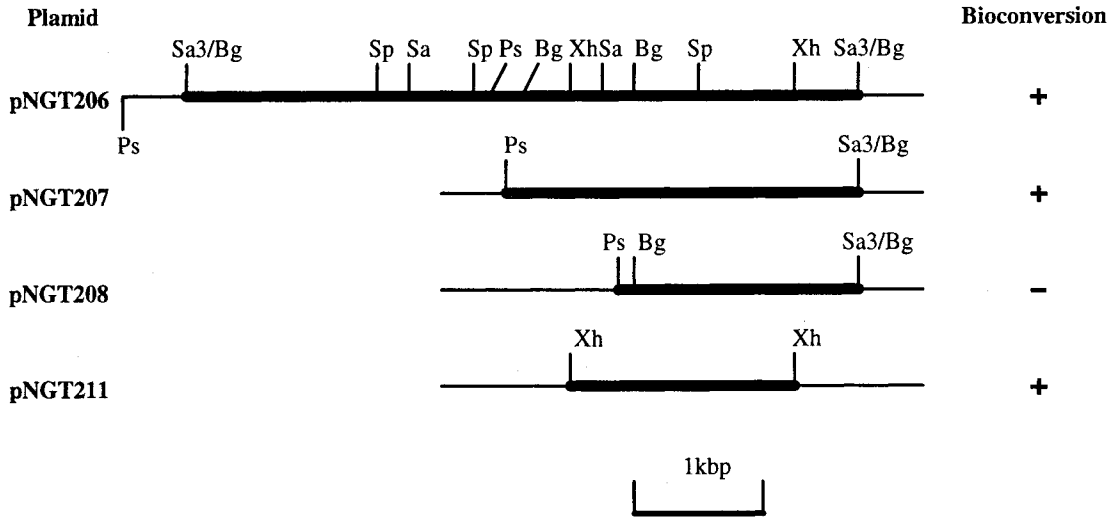
J-104303 was isolated from the culture of *S. mobaraensis* BA13793[1]. 6-*N*-methylarcyriaflavin C was synthesized in our institute[11].

DNA Techniques

Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Syuzo or Toyobo. DNA was isolated and manipulated as described in MANIATIS *et al.* (1982)[12] for *E. coli*, and in HOPWOOD *et al.* (1985)[10] for *S. lividans*. DNA fragments were isolated by GENECLEAN II (Bio 101). DNA was sequenced by BcaBEST™ Dideoxy Sequencing Kit (Takara Syuzo), according to the manufacturer's instructions.

The optimized procedure to obtain transformants of *S. mobaraensis* BA13793 was as follows: the strain was cultured in a 500-ml flask containing 110 ml of TSB medium containing 0.8% (w/v) glycine on a rotary shaker with 180 rpm at 28°C for 2 days. The mycelial pellet was harvested by centrifugation of 40 ml of the culture broth at 3,500 rpm for 10 minutes followed by washing 2 times with 0.5 M sucrose and suspended in 20 ml of modified P3 medium (17.1% (w/v) sucrose, 0.4% (w/v) sodium chloride, 0.2% (w/v) magnesium chloride hexahydrate, 0.294% (w/v) calcium chloride dihydrate and 0.573% (w/v) TES buffer (pH 7.2) adjusted with NaOH)[13]. Then lysozyme solution (1 ml containing 16.5 mg) and acromopeptidase solution (1 ml containing 11 mg) were added to the cell suspension and the mixture was incubated at 37°C for 60 minutes with occasional shaking. The resultant protoplasts were filtered through a 50-ml syringe barrel containing 1 cm height of absorbent cotton. The filtrate was centrifuged at 3,500 rpm for 10 minutes at 4°C and the protoplasts were washed twice followed by resuspension in 20 ml of cooled modified PWP medium (17.1% (w/v) sucrose, 0.4% (w/v) sodium chloride, 0.1% (w/v) magnesium chloride hexahydrate, 0.074% (w/v) calcium chloride dihydrate and 0.573% (w/v)

Fig. 3. Analysis of the essential region for the *ngt* gene.



The single line and the solid line indicated the DNA sequence of pIJ702 and that of the cloned DNA of ATCC39243, respectively. "+" and "-" denote activity of conversion. Plasmid pNGT207 was constructed by *Pst*I digestion and ligation of pNGT206. Plasmid pNGT208 was constructed by *Bgl*II digestion and ligation of pNGT207. Construction of PNGT211 is shown in Fig.4. Abbreviations: Sa3:*Sau*3AI, Sp:*Sph*I; Sa:*Sal*I, Ps:*Pst*I, Bg:*Bgl*II, Xh: *Xho*I.

TES buffer (pH 7.2) adjusted with NaOH). The concentration of the protoplasts was adjusted to $10^9 \sim 10^{10}$ viable protoplasts/ml using the same medium and 100 μl samples of the protoplasts were mixed with 1 μg of plasmids and 0.5 ml of PWP medium containing 20% PEG2000. After 1 minute, transformation was terminated by the addition of 5 ml of PWP medium. Protoplasts were then collected by centrifugation, suspended in 1 ml of PWP medium and 100 μl of the protoplast suspension was dispensed on modified R3 lower layer medium containing (instead of agar) 1.0% (w/v) gellan gum (Wako Pure Chemical) and 0.5 M sucrose and was spread by modified R3 upper layer medium (also agar-free) containing 0.4% (w/v) Low Melting Point Agarose and 0.5 M sucrose. After incubation at 28°C for 2 days, the regeneration plates were overlaid with 2.5 ml of Nutrient broth (Difco) containing 0.4% (w/v) agar and 100 μg/ml of thiostrepton (Sigma). Thiostrepton-resistant (Thio^r) transformants were isolated from these plates after incubation at 28°C for about 2 weeks.

Construction of Plasmids for the Expression of *ngt*

The structure of pNGT206, pNGT207, pNGT208 (experimental construction data are not shown) and pNGT211 are shown in Fig. 3.

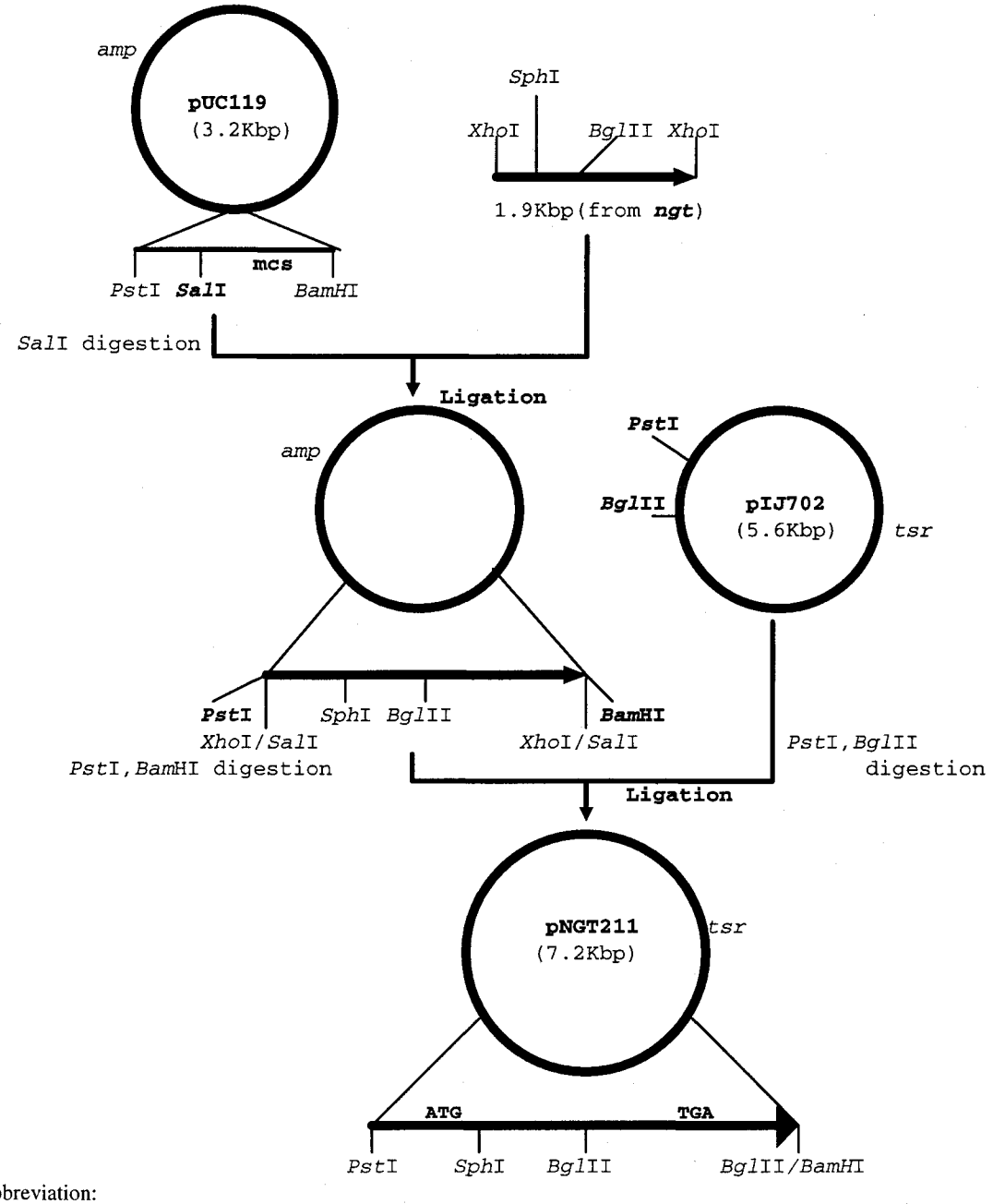
pNGT211 was prepared as follows: First, *Xho*I-*Xho*I fragment involving the *ngt* region was inserted into pUC119 digested with *Sal*I. Then plasmid pNGT211was constructed by the ligation of *Pst*I-*Bam*HI fragment involving *ngt* obtained from recombinant plasmid and pIJ702 digested with *Pst*I plus *Bgl*I, as summarized in Fig. 4.

Cloning of *N*-Glycosyltransferase (*ngt*)

Chromosomal DNA of *S. aerocolonigenes* ATCC39243 was digested partially with *Sau*3AI and resultant DNA fragments of 2~10 Kbp were size-fractionated by using GENECLEAN II Kit. After the insertion of the DNA fragments into the *Bgl*II site of pIJ702, ligated plasmids were transferred into *S. lividans* TK21.

Each Thio^r-Mel$^-$ transformant was inoculated into a 20-ml glass tube containing 5 ml of the detection medium (MM-2) plus thiostrepton (20 μg/ml) and cultivated on a shaker with 180 rpm at 28°C for 3 days. Then 40 ml of J-104303 solution (5 mg/ml) dissolved in a mixture of metanol and tetrahydrofuran (THF) (2 : 1) was added to each tube and cultured under the same condition for 2 days. Each culture broth was extracted with 5 ml of methyl ethyl ketone (MEK) and the extract was concentrated to dryness. The concentrate was dissolved in 50 μl of THF and then

Fig. 4. The summary of construction of pNGT211.



Abbreviation:
amp: Ampicillin-resistant gene, *tsr*: Thiostrepton-resistant gene, *ngt*: *N*-glycosyltransferase gene. mcs: multicloning site. Direction of transcription is shown by arrows. **ATG** and **TGA** indicate start codon and stop codon, respectively.

thin layer chromatography (TLC) was performed on silica gel plates (Merck) with a developing solvent of chloroform - methanol - THF (3 : 1 : 1). The Rf values of J-109384 and J-104303 were 0.36 and 0.73, respectively. This allowed identification of transformants capable of producing J-109384.

Analytical Techniques

Quantitative analyses of the starting material, J-104303, and of J-109384 produced in the culture broth were done as follows: Two ml of the culture was extracted with 28 ml of tetrahydrofuran (THF) by shaking on a reciprocating shaker for 30 minutes, and a part of the extracted THF layer was used for the quantitative analyses. Each sample was

analyzed by HPLC equipped with a YMC-Pack ODS-A column S-5 (4.6 mm×150 mm Yamamura Chemical). A 50 μl portion of the THF extract was injected and eluted with a linear gradient ((9 : 1~9 : 16) of 0.01 M KH_2PO_4 : CH_3CN in 30 minutes, flow rate 1.2 ml/minute) and UV absorbance was monitored at 240 nm.

Quantitative analyses of 6-*N*-methylarcyriaflavin C and 13-(β-D-glucopyranosyl)-6-*N*-methylarcyriaflavin C produced in the culture broth were done as follows: Two ml of the culture was extracted with 28 ml of tetrahydrofuran (THF) by shaking on reciprocating shaker for 30 minutes, and a part of the extracted THF layer was used for the quantitative analyses. Each sample was analyzed by HPLC by using an ODS column similar to that used for the analyses of J-109384. A 50 μl portion of the THF extract was injected and eluted with a linear gradient (50% methanol to 100% methanol in 30 minutes, flow rate 1.0 ml/minute) and UV absorbance was monitored at 305 nm.

Fermentation

Fermentation for producing J-109384 by *S. mobaraensis* BA13793 carrying *ngt* was as follows: Culture medium contained (per litre): 1 g glucose, 20 g dextrin, 10 g corn gluten meal, 5 g fish meal, 1 g yeast extract, 1 g sodium chloride, 0.5 g magnesium sulfate, 0.5 g calcium chloride, 2 mg ferrous sulfate, 0.4 mg cupric chloride, 0.4 mg manganese chloride, 0.4 mg cobalt chloride, 0.8 mg zinc sulfate, 0.8 mg sodium borate, 2.4 mg ammonium molybdate and 5 g 3-(*N*-morphorino)propanesulfonic acid (pH adjusted to 6.7 with NaOH). A 500-ml conical flask containing 110 ml of its medium was inoculated with the transformant strain of *S. mobaraensis* BA13793 grown on an agar slant medium. This flask was then incubated on a rotary shaker (180 rpm) at 28°C for 72 hours. Then one ml aliquot of the culture was inoculated into a 500 ml conical flask with containing 110 ml of a production medium. Production medium contained (per litre): 2 g glucose, 40 g dextrin, 15 g corn gluten meal, 30 g fish meal, 1.5 g yeast extract, 1 g sodium chloride, 0.5 g magnesium sulfate, 0.5 g calcium chloride, 2 mg ferrous sulfate, 0.4 mg cupric chloride, 0.4 mg manganese chloride, 0.4 mg cobalt chloride, 0.8 mg zinc sulfate, 0.8 mg sodium borate, 2.4 mg ammonium molybdate and 5 g 3-(*N*-morphorino)propanesulfonic acid (pH adjusted to 6.7 with NaOH). Then it was incubated on a rotary shaker with 180 rpm at 28°C for 14 days.

Results

Cloning of *N*-Glycosyltransferase Gene (*ngt*) from *Saccharothrix aerocolonigenes* ATCC39243 and Glycosylation of J-104303 by the Transformants Carrying *ngt*

The initial intention was to utilize reverse genetics in order to isolate the gene encoding the enzyme responsible for *N*-glycosylation. However, neither broth filtrates nor cell-free extracts of *S. aerocolonigenes* ATCC39243 converted J-104303 to J-109384 so that the desired enzyme could not be readily purified. Attempts to construct a library of *S. aerocolonigenes* ATCC39243 DNA fragments using *S. mobaraensis* BA13793 as host and pIJ702 as vector were also unsuccessful since the efficiency of transformation (10^3 per μg of plasmid DNA) was too low. It was therefore decided to generate a library of *S. aerocolonigenes* ATCC39243 DNA fragments in *S. lividans* TK21 using pIJ702 as vector. This was successful and, during testing of >5000 thio^r transformants, one was found to convert J-104303 to its glycosylated derivative, J-109384. The plasmid (pNGT206) isolated from this transformant contained a 4.15 Kbp DNA insert from which a 1.9 Kbp *Xho*I fragment that still expressed *N*-glycosylation activity was eventually isolated by sub-cloning. (Fig. 3).

Nucleotide Sequence of the Glycosylating Enzyme Gene, *ngt*

The *Xho*I fragment subcloned in pNGT211 was shown to comprise of 1,898 nucleotide(nt)s. Analysis of this sequence by CODONPREFERENCE analysis[14], revealed one complete open reading frame (ORF) designated *ngt*. The most likely deduced start codon (ATG) and stop codon (TGA) of *ngt* were located at nt 285 (A) and 1529 (T) respectively, suggesting that this ORF encoded a polypeptide chain of 414 amino acid residues (M_r, 45,383). Analysis of the deduced Ngt sequence with the BLAST program[15] revealed similarities to *Streptomyces* glycosyltransferases such as macrolide glycosyltransferase (Mgt)[16], oleandomycin glycosyltransferases (OleD[17] and OleI[18]) and daunosamine glycosyltransferase (DnrS)[19]. These glycosyltransferases are known to catalyze the addition of a selective sugar moiety to specific hydroxyl residues of corresponding aglycones. On the other hand, Ngt catalyzes the addition of a glucose moiety to the nitrogen atom at position 13 in the structure of J-104303. As far as we know, no enzyme with the ability to transfer a glucose moiety to an NH group has ever been reported. Previously, it was reported that UDP (TDP)-sugar transferase[16~19] contained

Fig. 5. DNA sequence of the region containing *ngt*.

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XhoI SacI
CTCGAGCTCGTGCGCGGCCACCAGACCTGCCACACCGGCGCCCAGAACAGTGATCTTCTTGTGTCCGCGTGACATCCCGCCCCCAGTTCGACGTCGTTTC   100

GCCCGTCCATGCTCCTGGCGTGACCGTTGCCCGCACATCACGCGAACAGGCTCGCCCCGAGCGGTTCCCAGCCTCTTCCCCGGTGGTCCAGCGACCTTTC   200

CGACCCGCCTGACCCCGCGTGGTCGAGGGGGTTTTGCCCGCGACTGCCGTGCCGCGGCGGGAATGGTGGCTACCGTCGGACGGCATGGGGGCACGAGTGC   300
                                                                                    ngt>  M  G  A  R  V

TGGTGGCGACCACACCGGGGGACGGGCACGTCAACCCGATGGTGCCGGTCGCCCAGGAGATGGTCAGCCGTGGACACGAGGTGCGGTGGTACACGGGAAA   400
L  V  A  T  T  P  G  D  G  H  V  N  P  M  V  P  V  A  Q  E  M  V  S  R  G  H  E  V  R  W  Y  T  G  K
                                                                                    SphI
GGCGTTCCGCTCCACAGTGGAACGAACAGGAGCCCGCCACGAGCCGATGCGCGACGCCCACGACTTCGGCGGCATGCCGAGGGAGGAGGCGTTTCCCCAG   500
  A  F  R  S  T  V  E  R  T  G  A  R  H  E  P  M  R  D  A  H  D  F  G  G  M  P  R  E  E  A  F  P  Q

CACGCGGGGCTGACCGGGATCACGGGGATGATCGCGGGGTTCAGGGACATCTTCATCGAACCCGCGGCCGACCAGATGACAGACCTGCTGGCACTGCTGG   600
 H  A  G  L  T  G  I  T  G  M  I  A  G  F  R  D  I  F  I  E  P  A  A  D  Q  M  T  D  L  L  A  L  L

AGGACTTCCCGGCCGACGTGCTCGTGACCGACGAGACCTTCTTCGGCGCGGGTTTCGTCAGTGAGCGCACGGGAATCCCCGTCGCCTGGATCGCCACGTC   700
E  D  F  P  A  D  V  L  V  T  D  E  T  F  F  G  A  G  F  V  S  E  R  T  G  I  P  V  A  W  I  A  T  S

GATCTACGTCTTCAGCAGCCGCGACACGGCACCGCTCGGGCTGGGCCTGCCGCCCAGCAGCTCCAGGCTGGGCAGGCTGCGCAACACCGTGCTCAAACAG   800
  I  Y  V  F  S  S  R  D  T  A  P  L  G  L  G  L  P  P  S  S  S  R  L  G  R  L  R  N  T  V  L  K  Q
                BglII
CTGACAGACCGTGTCGTCATGCGAGATCTCCGGCGGCACGCGGACGTGGTGCGCGACCGCGTCGGCCTGCCCCGCATCCGCAAGGGGGCGTTCGAGAACA   900
 L  T  D  R  V  V  M  R  D  L  R  R  H  A  D  V  V  R  D  R  V  G  L  P  R  I  R  K  G  A  F  E  N

TCATGCGCACGCCCGACCTCTACCTGCTGGGCACCGTGCCGTCCTTCGAGTACCCGCGAGGCGACATGCCACCCGAGGTGAGGTTCGTCGGCCCGTTCGT  1000
I  M  R  T  P  D  L  Y  L  L  G  T  V  P  S  F  E  Y  P  R  G  D  M  P  P  E  V  R  F  V  G  P  F  V
                                                SacI
GAGCCCTGCTCCGCCGGACTTCACCCCACCGGCGTGGTGGGGCGAGCTCGACTCCGGCCGGCCCGTCGTCCACGTCACCCAGGGCACCGTCGGCCAACGA  1100
  S  P  A  P  P  D  F  T  P  P  A  W  W  G  E  L  D  S  G  R  P  V  V  H  V  T  Q  G  T  V  G  Q  R

CGCGGAACGCTGCTGCTCCCCGCCATCCGAGCGCTGGCAGCCGAAGACGTGCTCGTGGTCGCGACCACCGGTGCCCCTCTGGAACTGGAGCCGATGCCGG  1200
 R  G  T  L  L  L  P  A  I  R  A  L  A  A  E  D  V  L  V  V  A  T  T  G  A  P  L  E  L  E  P  M  P

CCAACGTGCGGGTGGAACGGTTCATCCCGCATCACGCATTGCTTCCCCACGTGGACGCCATGGTGACCAACGGGGGATACGGCGGCGTCAACACGGCGCT  1300
A  N  V  R  V  E  R  F  I  P  H  H  A  L  L  P  H  V  D  A  M  V  T  N  G  G  Y  G  G  V  N  T  A  L

CGCACACGGCGTGCCGCTGGTCGTCGCGCGCACCGAGGAGAAGCACGAGGTCGCGGCCAGAGTGAGCTGGTCAGGTGCGGGTGTTCACCTGAAGAAGCGC  1400
  A  H  G  V  P  L  V  V  A  R  T  E  E  K  H  E  V  A  A  R  V  S  W  S  G  A  G  V  H  L  K  K  R

AGGCTGTCCGAACGGGACATCAGACGGGCCGTGCGCGCCGTTCTCGACGAGCCGCGCTTCCGGGTCCACGCGGCGCGGCTGCGGGACGAGTACGCGGCGC  1500
 R  L  S  E  R  D  I  R  R  A  V  R  A  V  L  D  E  P  R  F  R  V  H  A  A  R  L  R  D  E  Y  A  A
                SalI
GCGACGCGGTCGTGGACGCGTCGACCTGATCGAGGGCCTCGTCTGAACTCCTCGCTCGTACCACCTTGCGTCTCCTGCCCCCGGTCACCTCGGCTGGATC  1600
R  D  A  V  V  D  A  S  T  *

GGTGACGACGACCTTGGGTCACGAGCGCGTGCGCGGGCCCAGCAGGCTGCGGCGCGAGGTTCTCCATCAGCGCGGTGATGCCGAACGTCCAGTCTTCGGT  1700

GTCCTCGGCGGTGTTCACGACGTTCGTCAACGTGCCCAGTCGGGGCGAGGAGATGCGAACGACGTCCCCGATCTTGTGGGTGAACCCTTCGCCGGGCTGG  1800
                                                                                    XhoI
TCCCGGTCCTCGGTCGGAGCGAACATCGTGCCGGTGAACAGCACGAAGCCGTCGGGATAGCGGTGGTGGGCGCGAACGTGGGACACGAGGTCCTCGAG
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The proposed-translational start site of the *ngt* is shown by bold type. Stop codon is indicated by an asterisk. Amino acid translations are shown for *ngt* gene product. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB023953.

Fig. 6. PILEUP analysis of the Ngt protein.

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Ngt       4RVLVATT  PGDGHVNPMV  PVAQEMVSRG  HEVRWYTGKA  FRSTVERTG
Mgt      24 HIAMFSI  AIHGHVNPSL  EVIRELVARG  HRVTYAIPRL  LADKVAEAG
OleD      9 HIAMFSI  AAHGHVNPSL  EVIRELVARG  HRVTYAIPPV  FADKVAATG
DnrS       2KVLVTA  AMDAHFNGVV  PLAWALRAAG  HDVRVASQPA  LTDSITRAG
Consen     HI-M--I  A--GHVNP--  ---R-L-ARG  HRV--A--P-  --D-V---G
                       *          *   *  * *

Ngt  301 LEPMPA  NVRVERFIPH  HALLPHVDAM  VTNGGYGGVN  TALAHGVPLV  -VARTEEKHE  VAARVSWSGA  GVHL
Mgt  293 LGDVPD  NVEVRTWVPQ  LAILQQADLF  VTHAGAGGSQ  EGLATATPMI  AVPQAADQFG  NADMLQGLGV  ARTL
OleD 278 LGELPD  NVEVHDWVPQ  LAILRQADLF  VTHAGAGGSQ  EGLATATPMI  AVPQAVDQFG  NADMLQGLGV  ARKL
DnrS 296 LPPLPG  NVRVVDSLSL  HVVLPSCAAV  VHHGGAGTWA  TAALHGVPQL  ALAWQWDDVF  RAGQLEKLGA  GIFL
Consen   L--LP-  NV-V--W-P-  -AIL-----F  V-H-GAG---  --LA---P-I  A-P-A-DQF-  NAD-L--LG-  ---L
         *   *   ** *          *        *   * *          *                    *             *
```

Comparison of the Ngt protein with glycosyltransferase from *Streptomyces* spp. (Mgt, OleD and DnrS). Only the most conserved regions are shown. The number represent the position in the sequence. Highly conserved residues are marked by asterisks. Consen, consensus sequence

conserved amino acid sequence in both the *N*-terminal and *C*-terminal regions. Ngt resembles Mgt, OleD and OleI in possessing such conserved sequences (Fig. 6) and appears to belong to the same family of enzymes although it may differ in the mechanism of transfer of the sugar moiety.

One Step Production of J-109384 by the Transformant of *Streptomyces mobaraensis* BA13793 Carrying pNGT207

In order to achieve one step fermentation production of J-109384, pNGT207 contains *ngt* (Fig. 3) was transferred into *S. mobaraensis* BA13793. The resultant produced directly J-109384 at the expected level (data not shown). Next, pNGT207 was transferred into a high level J-104303 producer, *S. mobaraensis* 725M, which produced J-104303 at up to 14.6 mM. The productivity of J-109384 by this transformant reached 2.5 mM after 14 days of culture. Thus, one step fermentation production of J-109384 has become practical (Fig. 8).

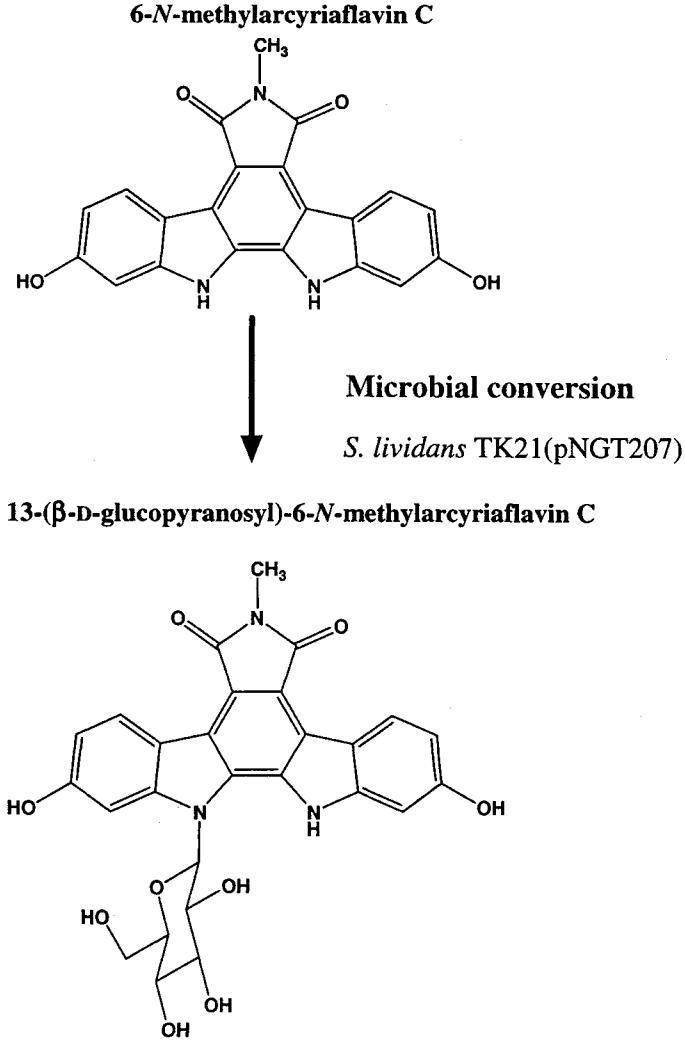
Glycosylation of 6-*N*-Methylarcyriaflavin C by *S. lividans* TK21 (pNGT207)

Arcyriaflavin C (12,13-dihydro-2,10-dihydroxy-5*H*-indolo-[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6*H*)-dione), is an indolocarbazole produced by *Arcyria denudata*[20]. To examine whether *S. lividans* TK21 carrying pNGT207 (*S. lividans* TK21 (pNGT207)) can transfer a D-glucose moiety to the nitrogen atom in the indole ring of an indolocarbazole having hydroxyl groups at different positions from J-104303 assessed possible conversion of 6-*N*-methylarcyriaflavin C by this transformant was as described in Materials and Methods. The microbial conversion of 6-*N*-methylarcyriaflavin C by the transformant was successful in yielding the product indicated in Fig. 7.

Discussion

The J-109384 is the key intermediate to prepare

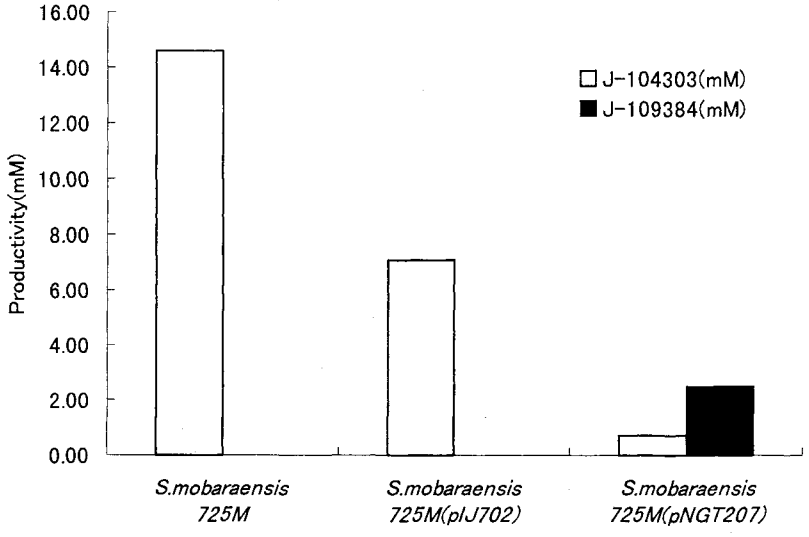
Fig. 7. Microbial conversion of 6-*N*-methylarcyriaflavin C by *S. lividans* TK21(pNGT207).



antitumor substances having indolocarbazole skeleton such as J-107185, which is currently attracting much attention by its efficacy. To create improved antitumor agents, enhanced production of J-109384 was strongly needed. Development studies of the derivatives of J-104303 and arcyriaflavin C were described in the United States Patent No. 5,668,271(Sept. 16,1997).

In this paper, the isolation and characterization of the gene (*ngt*) encoding *N*-glycosyltransferase from *S. aerocolonigenes* ATCC39243 and practical usage of this gene for the production of J-109384 are described. Computer analyses of the deduced Ngt sequence using the BLAST program, revealed conserved amino acid regions in the *N*-terminal and *C*-terminal regions, reminiscent of macrolide glycosyltransferases (Mgt)s. All Mgts described so far are *O*-glycosyltransferases. In contrast, the Ngt reported here is an *N*-glycosyltransferase. Although Mgts and Ngt may differ from each other in their reaction mechanisms, these enzymes appear to belong to the same family from the view point of sequence similarities. SASAKI *et al.*[21] reported that 15 out of 32 streptomycete strains, each of which produced different macrolide or polyether antibiotics, have Mgt activities. Also JENKINS *et al.*[16] reported that even macrolide non-producing *S. lividans* had Mgt activity. These results indicated that glycosyltransferase genes are widely distributed among *Streptomycetes* although none encoding *N*-glycosyltransferase have yet been described. Purification of the product protein of *ngt* has not yet been successful, because of the low solubility of J-104303 in water, which hindered the enzyme activity measurement *in*

Fig. 8. Productivity of J-104303 and J-109384 by *S. mobaraensis* 725M, *S. mobaraensis* 725M harboring pIJ702 and *S. mobaraensis* 725M harboring pNGT207.



Productivity after 14 days of culture in MM-2 medium are shown. Open column indicates productivity of J-104303 and closed column indicates productivity of J-109384.

vitro. A more soluble derivative of J-104303 may make possible the measurement of such enzyme activity. It remains to be elucidated whether the Ngt enzyme is located in the inner membrane, because only mycelia of *S. aerocolonigenes* ATCC39243 and that of *S. lividans* TK21 carrying the *ngt* gene could convert insoluble J-104303. If the enzyme is indeed buried in the membrane, the possible of detergent for its stability may hinder the purification of the enzyme[21]. Even so, once Ngt is obtained in partially purified form, enzymological studies including substrate specificity will become possible.

The productivity level of J-109384 by *S. mobaraensis* 725M carrying *ngt* (2.5 mM at present) was expected lower, since parental strain produced J-104303 at 14.6 mM. Various reasons could be speculated for the lesser productivity of J-109384 by *S. mobaraensis* 725M carrying the *ngt* gene as follows. 1) J-109384 played negative effect on the biosynthesis pathway of J-104303. 2) The enzyme Ngt was inhibited by an intermediate of J-104303, and consequently the intermediate could not enter to the next biosynthesis step of J-104303.

Studies to solve these speculations will open the way to increase the productivity of J-109384 in future. As another speculation, direct insertion of the *ngt* gene into the genome of *S. mobaraensis* 725M may enhance the production of J-109384. Other possible trial is the replacement of the promoter of the *ngt* gene with other inducible promoters.

Because the Ngt enzyme introduced a D-glucose moiety into indolocarbazoles such as J-104303 or 6-*N*-methylarcyriaflavin C, it will be used for the production of these compounds and also for the preparation of other *N*-glycosylated indolocarbazoles. Our research reported in this paper experimentally exemplified a typical example that such technology is able to contribute for the efficient production of microbial products in a practical level.

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