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In Vivo Labeling Method of Cyclosporin A with [^{14}C-Methyl]-S-Adenosyl-L-Methionine Can Evaluate a Potency of Cyclosporin A-Producing Mutant

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ABSTRACT

Cyclosporin A synthetase activity of *Tolypocladium inflatum* can be estimated by measuring its N-methyltransferase activity. In vivo N-methyltransferase activity of cyclosporin A synthetase of cells was measured by in vivo [^{14}C-methyl] labeling assay, which was designed for actively growing cells. After the cells were incubated with 0.025 μCi of [^{14}C-methyl]-S-adenosyl-L-methionine, [^{14}C-methyl] labelled cyclosporin A and its analogs inside the cells were extracted with ethylacetate and ^{14}C radioactivity of the ethylacetate extract of the cells was counted. When various mutant cells grown on agar plate medium after ultraviolet irradiation or N-methyl-N'-nitroso-guanidine treatment were applied to in vivo [^{14}C-methyl] labeling assay, these mutants showed a broad range of in vivo N-methyltransferase activity. Poor correlation was found between in vivo N-methyltransferase activity of cyclosporin A synthetase of the mutant grown on agar plate and the actual amount of cyclosporin A production in shake-flask culture. However, when the cells grown on the shake-flask culture were applied in the in vivo [^{14}C-methyl] labeling assay, a better correlation was resulted. In vivo N-methyltransferase activity reached the maximum value at about 150 h, and then declined quickly, but cyclosporin A was synthesized for 200 h during fer-

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mentation. Specific in vivo N-methyltransferase activity was not greatly influenced by culture age during fermentation. The major product of in vivo [^{14}C-methyl] labeling assay was identified as cyclosporin A, and only trace amounts of other cyclosporin analogues were detected. Therefore, the results suggest that in vivo labeling method with [C^{14}-methyl]-S-adenosyl-L-methionine can easily compare a potency of cyclosporin A-producing mutant during fermentation.

Index Entries: Cyclosporin A; *Tolypocladium inflatum;* cyclosporin A synthetase; in vivo [C^{14}-methyl] labeling method; fermentation; mutagenesis.

Abbreviations: Abu, 2-aminobutyric acid; AdoMet, S-adenosyl-L-methionine; Bmt, (2S,3R,4R,6E)-2-amino-3-hydroxy-4-methyl-6-octenoic acid (=(4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine); Sar, sarcosine.

INTRODUCTION

Cyclosporin A (Fig. 1) is a cyclic undecapeptide that contains three unusual amino acids (Bmt in position 1, Abu in position 2, and D-Ala in position 8) and seven N-methylated peptide bonds. Twenty-five naturally occurring cyclosporins, which have substitutions of amino acids in positions 1, 2, 3, 4, 5, 6, 7, and 11, and/or contain unmethylated peptide bonds in positions 1, 4, 6, 9, 10, or 11, were also reported *(1,2)*. Cyclosporin A and its homologs are produced by the fungus *Tolypocladium inflatum.* Beside these naturally occurring cyclosporins, some cyclosporins, differing in positions 1, 2, and 8 from cyclosporin A, could be produced by feeding their amino acid precursors to culture medium of the fungus. *(3)* Cyclosporin A is synthesized by a multifunctional enzyme, cyclosporin A synthetase *(4)*, which catalyzes at least 40 reactions from their primary precursors to cyclosporin A under ATP and AdoMet consumption. Like the nonribosomal peptide synthesis *(5,6)*, occurring in various other peptides and depsipeptides syntheses *(7,8)*, cyclosporin A synthetase binds to the constitutive amino acids as thioesters, via aminoadenylation. At this stage, seven amino acids are N-methylated, then elongation of the peptide chain and cyclization reactions occur.

Cyclosporin A exhibits anti-inflammatory, immunosuppressive, antifungal, and antiparasitic activities. Because of its immunosuppressive activity, it is widely used in transplantation surgery and treatment of autoimmune disease *(2,3)*. Despite the numerous clinical studies of cyclosporins, there is only a limited amount of published information on the microbial characteristics and strain improvement program of cyclosporin

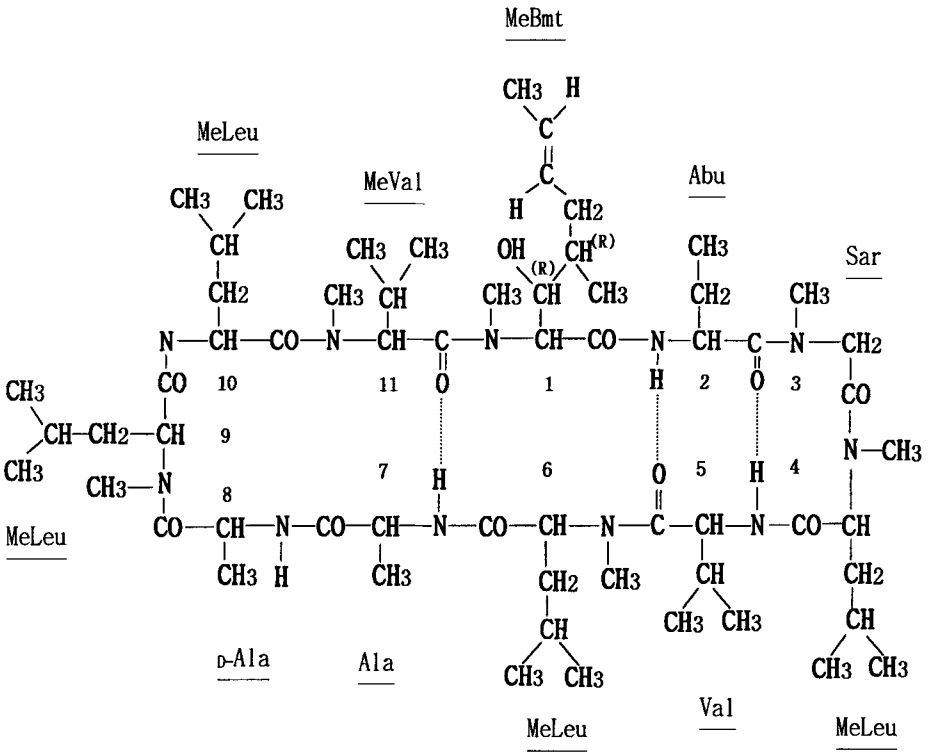

Fig. 1. Structure of cyclosporin A.

A-producing fungus, *T. inflatum.* Pharmaceutical companies producing cyclosporin A by microbial fermentation have placed great emphasis on production cost, which chiefly depends on the potency of the strain used in the manufacturing plant. A simple and direct screening method, such as agar-piece method *(9)*, and agar-diffusion assay has played a major role in the strain-improvement technique for antibiotic-producing strain over the years. However, these primary screening methods cannot be applied for cyclosporin A-producing strain. Therefore, research on strain improvement of cyclosporin A-producing fungus calls for laborious and tedious effort, since numerous mutants must be tested for determining cyclosporin A-producing potency in small-scale fermentation. Moreover, cyclosporin A fermentation requires at least a 10-d cultivation. Thus, a novel rapid screening method for cyclosporin A-producing mutant at an early phase of fermentation is of considerable interest.

The present paper describes in vivo [^{14}C-methyl] labeling method of cyclosporin A with [^{14}C-methyl]-S-adenosyl-L-methionine, for a rapid screening of superior cyclosporin A-producing mutant, and also discusses the relationship between in vivo N-methyltransferase activity of cyclosporin

A synthetase of the mutants and the actual amount of cyclosporin A production by the mutants.

MATERIALS AND METHODS

Radioisotopes and Chemicals

[^{14}C-methyl]S-adenosyl-L-methionine (AdoMet) was purchased from Amersham (Braunschweig, F.R.G.). USP reference standard of cyclosporin A was used throughout this study. All other chemicals used were purchased from Sigma (St. Louis, MO) or Merck (Darmstadt, F.R.G.), and were reagent grade.

Organisms and Culture Conditions

Tolypocladium inflatum M531, derived from *T. inflatum* ATCC 34921, was used as a parent strain in this study. The strain was cultured on slant medium consisting of 1% peptone (Difco, Detroit, MI), 2% yeast extract (Difco), 2% dextrose, and 1.5% agar (pH was adjusted to 6.0) at 27°C for 10 d, and then stocked at –20°C. For shake-flask culture, spore suspension was prepared by suspending the cells grown on slant in a physiological saline solution. The spore suspension was used as a preinoculum to inoculate 500-mL Erlenmeyer flask containing 50 mL inoculation medium. The inoculation medium contained 1% peptone (Difco), 2% yeast extract (Difco), and 2% dextrose (pH 6.0). The inoculation preparation was incubated at 27°C on a rotary shaker (NBS model Inova4330, NJ) at 150 rpm for 2-3 d. 5 mL of the resulting inoculum were transferred to 500-mL Erlenmeyer flask containing 50 mL of fermentation medium. The fermentation medium contained 10% fructose, 1% L-valine, 1% $NH_4H_2PO_4$, 0.3% $CaCO_3$, 0.2% KH_2PO_4, 0.1% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05% KCl (pH 6.0). Fermentation was carried out for 10 d under the same culture conditions as inoculation culture.

Mutagenesis

Spore suspension (10^{6-7}/mL) was prepared from slant culture and filtered through a 1-cm-thick layer of glass wool, in order to remove mycelial fragments. In the first mutagenesis, spores were irradiated with a short-wavelength UV light (15 W) at a distance of 30 cm for 60 s. After proper dilution, the spore suspension was plated out on agar plate, which contained the same composition as the slant medium, and incubated at 27°C for 10–14 d. The colonies grown on the agar plate was tested for a potency of cyclosporin A production in the shake-flask culture described previously. The mutant that showed the highest potency was selected from the first mutagenesis, and further treated with 20 μg/mL N-methyl-N'-

nitroso-guanidine (NTG) in 0.1 *M* acetate buffer solution (pH 5.0) for 30 min for the second mutagenesis. The same procedures used in the first mutagenesis were applied for the rest of the experiment.

In Vivo Labeling of Cyclosporin A with [^{14}C-Methyl]AdoMet

After the first mutagenesis with UV light, a piece of each colony grown on the agar plate was inoculated onto water-saturated filter-paper disks (0.5 cm diameter). Each disk was placed in a hole of a 96-well plate (Corning Glass, Corning NY), which was filled with the slant medium and incubated at 27°C for 6–7 d. Then, each disk was transferred to 1.5-mL Eppendorf tube. The cell grown on each disk was washed twice with distilled water to remove residual nutrients. The washed cells were resuspended in 0.6 mL tap water. 0.025 μCi [^{14}C-methyl]AdoMet was added to each tube, and the mixture was incubated at 27°C for 30 min under gentle shaking. After incubation, 0.6 mL ethylacetate was added to the mixture to stop the reaction, and then [^{14}C-methyl]-labeled cyclosporin A and its analogs were extracted from the mixtures by vigorous vortexing for 1 min. The mixture was centrifuged at 12,800*g* for 5 min, to separate the ethylacetate layer from the mixture. After centrifugaration, 200 μL ethylacetate layer was diluted with cocktail mixer, and ^{14}C-radioactivity (cpm) was measured by liquid scintillation analyzer (Model C1600, Packard Instrument, Downers Grove, IL). After the second mutagenesis with NTG, the selected mutant was tested in the shake-flask culture, as described previously. An aliquot of fermentation broth was taken from the shake-flask culture to be used for in vivo [^{14}C-methyl] labeling assay.

Analytical Procedure

[^{14}C-methyl]-labeled cyclosporins synthesized from in vivo [^{14}C-methyl]-labeling assay were separated on a silica gel high-performance thin-layer chromatography (HPTLC) plate (Merck), by using the solvent system of ethylacetate–methanol–water (10:5:5, by volume). R_f value of authentic cyclosporin A was identified under UV light, and the HPTLC plate containing [^{14}C-methyl] cyclosporin was autoradiographed. Cyclosporin A concentration in the fermentation broth was determined by high-performance liquid chromatography (Model LC-10A, Shimadzu, Japan) equipped with UV detector (SPD-10A, Shimadzu) and supelcosil LC-8 column (15 cm × 4.6 mm 5 μm, Supelco, Bellefonte, PA). Column temperature was 75°C, and acetonitrile–water–phosphoric acid (630:370:0.1), pH 2.8) was used as mobile phase. The flow rate was 1 mL/min, and cyclosporin A was detected at a wavelength of 210 nm. Cell mass was measured as optical density at 610 nm.

RESULTS

First Mutagenesis by UV Irradiation

The parent strain, *T. inflatum* M531, producing about 600–800 mg/L of cyclosporin A, was mutagenized by UV light, and then about 100 mutants grown on the agar plate medium were inoculated onto filter-paper disks. The disks were placed on agar medium in a 96-well plate and incubated as described in Materials and Methods. After a 7-d incubation period, in vivo N-methyltransferase activities of cyclosporin A synthetase of the cells grown on the disks were measured by in vivo [^{14}C-methyl]-labeling assay using [^{14}C-methyl]AdoMet. When [^{14}C-methyl]AdoMet was added to the cell suspension, the cells began to N-methylate amino acid precursors existing in the intracellular amino acid pool, and to synthesize cyclosporin A from these [^{14}C-methyl] amino acid precursors by cyclosporin A synthetase.

As shown in Table 1, various mutants showed a broad range of in vivo N-methyltransferase activities of cyclosporin A synthetase (cpm), from 26 to 982 cpm. The cyclosporin A-producing potencies of the mutants were also determined in the shake-flask culture. The mutant UV-14 showed more than a twofold increase in the cyclosporin A-producing potency, from 670 to 1557 mg/L. However, the results showed a poor correlation between in vivo N-methyltransferase activity of cyclosporin A synthetase of the mutant grown on agar plate and the actual amount of cyclosporin A production in the shake-flask culture.

Second Mutagenesis with NTG Treatment

Spore suspension was prepared from the mutant UV-14, which showed the highest cyclosporin A-producing potency in the shake-flask culture in the first mutagenesis, and was treated with NTG, as described in Materials and Methods. Twenty-six mutants were selected among numerous mutants grown on agar plate. These mutants were cultivated in the shake-flask culture, and then the cells in the culture broth were applied for in vivo [^{14}C-methyl]-labeling assay in the second mutagenesis. As shown in Table 2, the mutants exhibiting high cpm value of in vivo N-methyltransferase activity of cyclosporin A synthetase, at the second or third day, also showed high cyclosporin A-producing potencies at the end of fermentation in shake-flask culture. Therefore, the mutant UV14-17 was selected as the most superior strain, with a significant cyclosporin A-producing potency increase from 1562 to 2108 mg/L.

Table 1
In vivo N-Methyltransferase Activity of Cyclosporin A Synthetase of Various Mutants Selected from the First Mutagenesis Using UV Irradiation

Strains	In vivo N-methyltransferase activity of cyclosporin A synthetase (cpm)[a]	Cyclosporin A concentration in fermentation broth (μg/L)[b]
	7 d	10 d
M531	300	670
UV-5	459	1340
UV-7	417	1443
UV-11	130	62
UV-14	476	1557
UV-15	227	817
UV-20	26	827
UV-22	299	932
UV-28	219	729
UV-31	485	1555
UV-34	450	1169
UV-43	982	1186
UV-45	443	996
UV-47	319	1444
UV-55	630	720
UV-56	172	1314
UV-57	547	1083
UV-59	517	1436
UV-63	550	1455
UV-67	634	890
UV-75	455	716
UV-76	478	653
UV-79	574	694

[a] The cells were grown on the filterpaper disc which were placed on the agar plate medium. Details were described in the Materials and Methods section.
[b] Fermentation was carried out in the shake flask culture for 10 d.

Identification of Reaction Products from In Vivo Labeling with [^{14}C-Methyl]AdoMet

After in vivo [^{14}C-methyl]-labeling assay, cyclosporin A and other reaction products in the reaction mixture were identified by HPTLC. After separating the reaction products on HPTLC plate, the plate was autoradiographed. As shown in Fig. 2, authentic cyclosporin A spot could be observed under UV light at R value of 0.53, and the major spot of every

Table 2
In vivo N-Methyltransferase Activity of Cyclosporin A Synthetase and Cyclosporin A Concentration in Fermentation Broth of Various Mutants Selected from the Second Mutagenesis Using NTG Treatment

	In vivo N-methyltransferase activity of cyclosporin A synthetase according to culture age (cpm)					Cyclosporin A concentration in fermentation broth (μg/L)	
Strains	2 d	3 d	5 d	7 d	10 d	7 d	10 d
U14	1020	1498	2728	4091	7075	890	1562
U14-1	312	156	316	556	334	93	117
U14-2	255	124	385	304	171	33	50
U14-3	1152	1075	2103	5181	6520	1045	2013
U14-4	342	200	220	263	150	44	42
U14-5	1120	947	2229	4963	5965	989	1675
U14-6	1083	940	2169	4476	5904	969	1837
U14-7	935	1080	1654	3768	5568	825	1531
U14-8	964	1070	1920	4162	6207	923	1828
U14-9	1032	896	2156	4627	6670	1023	1948
U14-10	1015	1096	2436	5709	5258	979	1918
U14-11	1080	1063	2570	4581	6658	1024	1886
U14-12	1133	964	2269	5324	6427	1118	2100
U14-13	1063	1083	2348	4355	6680	998	1870
U14-14	993	1055	2132	5062	6460	984	1887
U14-15	94	295	816	3217	4918	293	867
U14-16	912	1014	1992	4891	6587	933	1910
U14-17	1011	1194	2360	5392	7233	1142	2108
U14-18	1098	1166	2617	5417	5886	1041	2046
U14-19	752	1292	2285	5163	7581	917	1752
U14-20	868	1237	2410	4680	7347	902	1809
U14-21	853	1416	2363	5125	7759	915	1753
U14-22	917	1343	2053	4299	7057	854	1677
U14-23	887	1411	2428	3855	4107	629	765
U14-24	447	679	273	471	267	77	82
U14-25	1020	1498	2728	4091	7075	890	1562
U14-26	538	1322	3217	3822	4476	1276	1417
U14-27	548	1056	3308	3787	5824	896	1496

mutant was also detected at the same R value with authentic cyclosporin A. Only trace amounts of other cyclosporins were detected in some mutants. These results indicated that most mutants derived from the first and second mutagenesis produced only cyclosporin A as a major product.

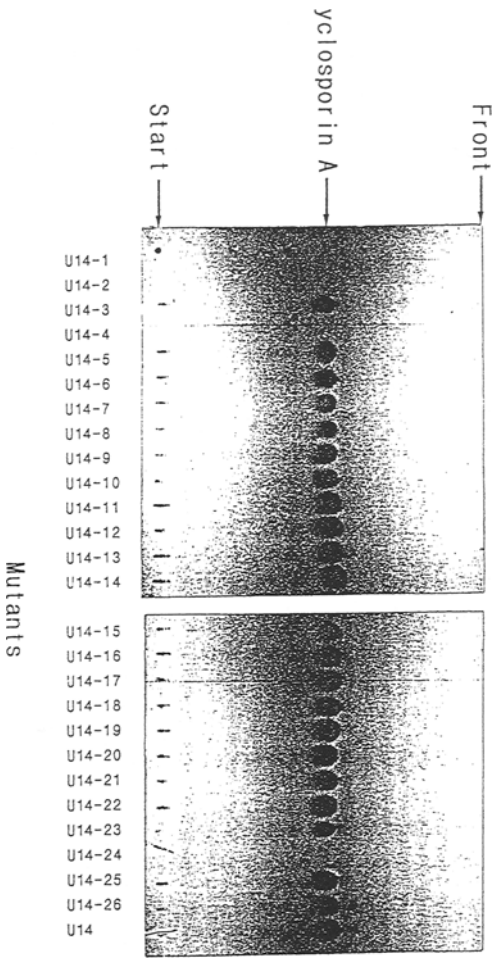

Fig. 2. Identification of reaction products from in vivo labeling with [^{14}C-Methyl]AdoMet. Cyclosporin A and other reaction products in the reaction mixture were identified by HPTLC and autoradiography.

Relationship of In Vivo N-Methyltransferase Activity of Cyclosporin A Synthetase to Cyclosporin A Production During Fermentation

The typical time-course of cyclosporin A production and in vivo N-methyltransferase activity of cyclosporin A synthetase during the shake-flask culture are shown in Fig. 3. In vivo N-methyltransferase activity reached the maximum value at about 150 h, and then decline quickly, but cyclosporin A was synthesized for 200 h during fermentation. Specific in vivo N-methyltransferase activity was not greatly influenced by culture age during fermentation. The results indicated that the actual amount of

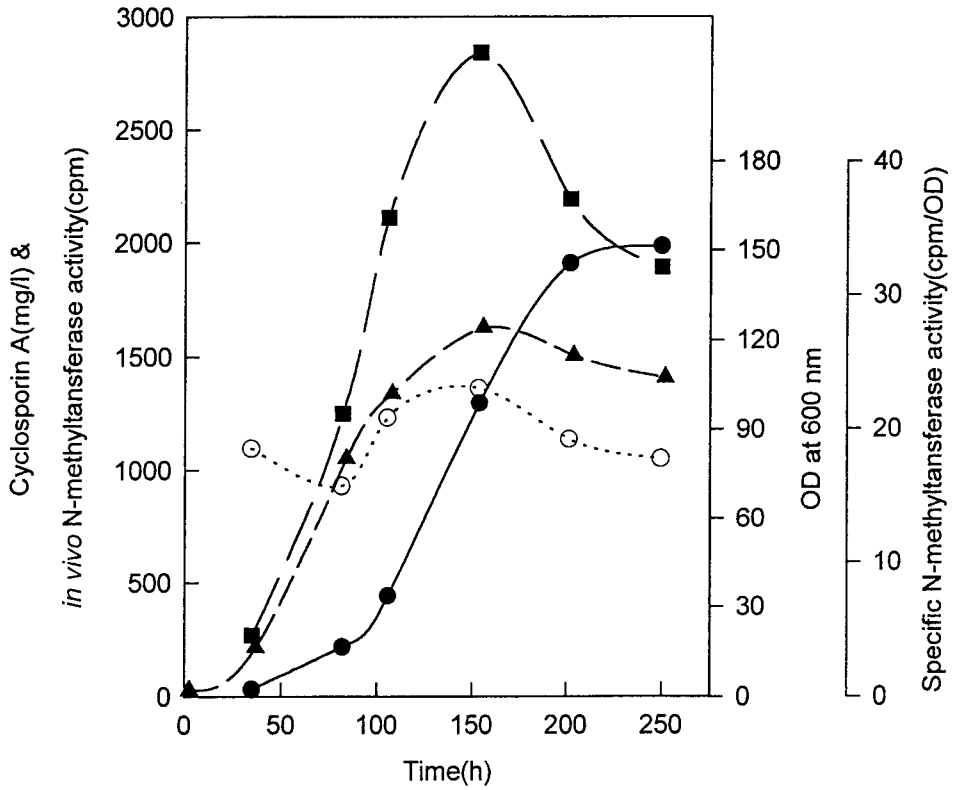

Fig. 3. Typical time-course of cyclosporin A production and in vivo N-methyltransferase activity of cyclosporin A synthetase during fermentation. Detailed culture conditions are described in Materials and Methods. (●) Cyclosporin A concentration, (■) in vivo N-methyltransferase, (▲) optical density, and (○) specific N-methyltransferase activity.

cyclosporin A production was relatively well correlated with in vivo N-methyltransferase activity of cyclosporin A synthetase of the cells during early phase of fermentation, from 50 h up to 150 h, and the correlation is maximum at 7 d. The purpose of this study is to develop a simple method measuring cyclosporin A-producing potency at the early stage of fermentation. From this point of view, it is possible that the cyclosporin-producing potency of the mutant can be compared by in vivo [C^{14}-methyl]-labeling assay after 2–3 d cultivation in the shake-flask culture.

DISCUSSION

The cyclosporin A molecule is synthesized by a total of 40 steps, including 11 aminoadenylation reactions, 11 transesterification reactions to enzyme-bound thioesters, seven N-methylation reactions, 10 peptide-

binding reactions, and one cyclization reaction. These steps do not include transesterification steps from one thioster form to another by 4'-phosphopantetheine on the enzyme *(5)*.

Like enniatin synthetase *(10)*, cyclosporin synthetase accepts only the unmethylated amino acid precursors of cyclosporins while bound to the enzyme as thioesters *(11)*. Methyltransferases responsible for the N-methylation reactions are very similar to enniatin synthetase, which can be considered as a model system of cyclosporin synthetase in many aspects *(12,13)*.

There are no known rate-limiting steps in cyclosporin biosynthesis. However, methyltransferases are known to be an integral part of cyclosporin synthetase. Thus, the simple way to measure activity of cyclosporin synthetase during cyclosporin biosynthesis is to measure the amount of N-methylation, because the cyclosporin A molecule contains 7 N-methylated components. In this study, methyl group of [^{14}C-methyl]AdoMet were transferred to activated amino and hydroxy acids for N-methylation by methyltransferase during in vivo labeling assay.

When the cells grown on the agar plate medium were used for in vivo [^{14}C-methyl] labeling assay in the first mutagenesis, a low level of cyclosporin A production was achieved by some mutants in the shake-flask culture, even though in vivo N-methyltransferase activity (cpm) of those mutants was high in in vivo [^{14}C-methyl] labeling assay. Therefore, there was a poor correlation between in vivo N-methyltransferase activity of cyclosporin A synthetase of the mutant and the actual amount of cyclosporin A produced in the shake-flask culture. The poor correlation seemed to be the result of the different growth rates of the mutants on the filterpaper disks, which might have caused difficulty in obtaining an equal amount of cells from various mutants for in vivo labeling assay. However, when the cells grown on the shake-flask culture were used for in vivo [^{14}C-methyl] labeling assay, it was possible to obtain a better correlation between in vivo N-methyltransferase activity of cyclosporin A synthetase of the mutant and the actual amount of cyclosporin A produced in the shake-flask culture.

As seen from the results of HPTLC and autoradiography, it was evident that the total cpm values of the mutants were principally affected by the amount of [^{14}C-methyl]-labeled cyclosporin A rather than by other [^{14}C-methyl]-labeled cyclosporin A and its analogs. Moreover, the specific in vivo N-methyltransferase activity of cyclosporin A synthetase remained fairly constant during fermentation. Therefore, the results suggest that the in vivo labeling method with [C^{14}-methyl]-S-adenosyl-L-methionine can easily compare a potency of cyclosporin A-producing mutant during fermentation.

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