Microbial Glycosylation of Macrolide Antibiotics by *Streptomyces hygroscopicus* ATCC 31080 and Distribution of a Macrolide Glycosyl Transferase in Several *Streptomyces* Strains

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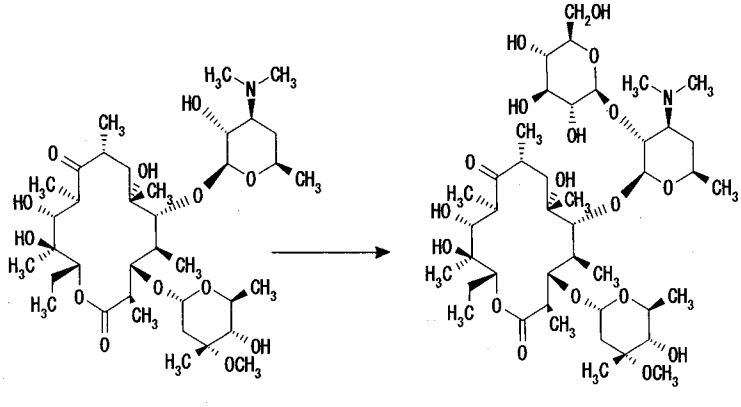
(Received for publication February 29, 1996)

In the course of our microbial transformation study on erythromycin derivatives, *Streptomyces hygroscopicus* ATCC 31080, which produces a polyether antibiotic carriomycin, was found to transform erythromycin derivatives to their inactivated derivatives. The structures of inactivated derivatives prepared by enzyme reaction using the cell extract, UDP-glucose (or UDP-galactose) and Mg^{2+} (or Mn^{2+}) were elucidated on the basis of analysis of their spectral data to be the compounds glycosylated at C-2′ of a desosamine moiety, indicating that the enzyme is a macrolide glycosyl transferase (MGT). The MGT activity of cell extract from *S. antibioticus* ATCC 11891, a producing organism of oleandomycin, could be distinguished from that of ATCC 31080, based on the ability to glycosylate tylosin. We examined 32 actinomycete strains producing such polyketides as macrolide and polyether antibiotics, and found that 15 strains of *Streptomyces* have macrolide glycosyl transferase activity. It suggests that the MGTs have been distributed among at least polyketide producing *Streptomyces* strains.

CELMER *et al.* first found that 2′-*O*-glucosyl oleandomycin is an inactivated form of oleandomycin, and that *Streptomyces antibioticus* ATCC 11891 produces intracellularly 2′-*O*-glucosyl oleandomycin and converts extracellurarly 2′-*O*-glucosyl oleandomycin to oleandomycin, and pointed out that the glucosylation system could protect the organism from high production of the antibiotic and its antibacterial activity[1]. Later, this mechanism of resistance was supported by SALAS *et al.* They proposed that the oleandomycin-glycosylating enzyme gene of *Streptomyces antibioticus* ATCC 11891 located upstream of the oleandomycin biosynthesis gene cluster and expression of the enzyme activity was coupled with production of oleandomycin[2]. It is well known that the self-produced oleandomycin of ATCC 11891 is intracellularly converted and inactivated to 2′-*O*-glucosyl oleandomycin by the glycosylating enzyme, and the product is extracellurarly converted to oleandomycin by another hydrolysis enzyme[3].

It was also reported that two *Streptomyces* strains have the ability of glycosylating and inactivating some macrolide antibiotics although they are not known to produce macrolide antibiotics. One is *S. vendargensis* ATCC 25507 which is able to convert erythromycin A to an inactive derivative 2′-(*O*-[β-D-glucopyranosyl])-erythromycin A[4] as shown in Fig. 1. Another is

Fig. 1. Microbial transformation of erythromycin A (**1**) to 2′-*O*-glucosyl-erythromycin A (**5**).



1 **5**

S. lividans TK21 which shows inducible resistance to macrolide antibiotics. It was suggested that the resistance is caused by the involvement of a macrolide glycosyl transferase (MGT) because of its utilization of UDP-glucose as a cofactor and its specificity for 2'-OH groups of the saccharide moieties of macrolide antibiotics in their inactivated derivatives[5]. This MGT gene has been cloned and sequenced[6].

In this paper, we report the glycosylation products of erythromycin derivatives by the MGT of *Streptomyces hygroscopicus* ATCC 31080[7], a producing strain of a polyether antibiotic carriomycin, and the characterization of the MGT activities from *S. hygroscopicus* ATCC 31080 and *S. antibioticus* ATCC 11891.

From the viewpoint of the role of the MGT, some discussion on the distribution of the MGT activity to several *Streptomyces* strains will be given.

Materials and Methods

Microorganisms

Actinomycete strains used for MGT assay were obtained from ATCC, IFO, JCM, and the culture collection in our laboratory (denoted by TA-).

Chemicals

Erythromycin A and oleandomycin phosphate were purchased from Sigma Chemical Co. Erythromycin B was generously gifted from Abbott Laboratory. UDP-glucose, UDP-galactose and UDP-mannose were purchased from Nakarai Tesque Co. UDP-[^{14}C]-glucose (9.25 MBq) was obtained from New England Nuclear Co. Desosamine was prepared by degradation of erythromycin A[8]. Kujimycin B was isolated from the fermentation broth of *Streptomyces spinichromogenes* var. *kujimyceticus* TPR-885 as reported previously[9]. According to the methods as previously reported, picromycin, chalcomycin and angolamycin were obtained from the fermentation broth of *Streptomyces* sp. TA0001, *Streptomyces* sp. TA0005, and *Streptomyces* sp. TA0006, respectively[10]. Midecamycin A1 was purified from "Miokamycin" of Meijiseika Kaisha. Leucomycin A1 was prepared from "Leucomycin Intravenous" of Toyo Jozo Co. (Asahikasei Co.). Josamycin was isolated from "Josamycin" of Yamanouchi Pharmaceutical Co. Tylosin tartrate was purchased from Mercian Co. Clarithromycin[11], azithromycin[12], *N*-demethyl erythromycin A[13], decladinosyl erythromycin A[13], erythromycin A enolether[13], erythromycin A oxime[13] and erythromycin B oxime[13] were prepared synthetically from erythromycins A and B in our laboratory.

General

Mass spectra were obtained with a Jeol JMS-SX102 Mass Spectrometer. NMR spectra were taken in C_5D_5N on a Jeol GX-400 spectrometer at ambient temperature at 400 MHz (^{1}H) and 100.4 MHz (^{13}C) using the solvent peaks as internal references downfield of TMS at 0 ppm. The multiplicities of signals in the ^{13}C NMR spectra were determined by the DEPT experiments.

Thin-layer chromatography (TLC) was used to examine glycosylation products. The TLC plate (Merck Art. 5715, Germany) was developed with chloroform-methanol-25% aqueous ammonia (100:10:1). Detection of conversion products was carried out by spraying anisaldehyde-H_2SO_4-acetate (1:2:100) or 2% $CeSO_4$ in 2 N H_2SO_4 onto the plate and heating it at 105°C for 3 minutes. The detection limit of the conversion products by the spraying reagents was 0.1 μg/ml of reaction mixture. The percentage yield of products on the TLC plate was determined using a Shimadzu High Speed TLC Scanner. Conversion products labeled with ^{14}C were analyzed by a radio scanner system consisting of a Noland Radio Isotope Analyzer LB 283 and an Ortec MCA IT-5400. The detection limit of conversion products labeled with ^{14}C was 1 ng/ml of reaction mixture.

Preparation of Cell Extract from *S. hygroscopicus* and *S. antibioticus*

S. hygroscopicus ATCC 31080 and *S. antibioticus* ATCC 11891 were grown with continuous rotary agitation in 500-ml Erlenmeyer flasks containing 100 ml of medium composed of 0.3% glycerol, 0.7% lactose, 0.3% beef extract, 0.1% peptone and 0.1% Brain Heart Infusion Broth (Eiken) at pH 7.0, for 96 hours at 30°C. The mycelium was harvested by centrifugation, washed several times with 10 mM Tris-HCl (pH 7.5) and stored at −20°C. The mycelium yield was calculated to be approximately 10 g (wet weight) per 100 ml of the cultured medium.

The procedures of the cell extract preparation were carried out at 4°C as follows. Intact mycelium (10 g, wet weight) of each of *S. hygroscopicus* ATCC 31080 and *S. antibioticus* ATCC 11891 was suspended in 100 ml of 25 mM Tris-HCl (pH 7.5) containing 7 mM 2-mercaptoethanol, 1% Triton X-100 and 10% glycerol, and disrupted by sonication for 5 minutes with Branson Sonifier 450, followed by centrifugation at 28,000 g for 15 minutes. The resulting supernatant was fractionated by ammonium sulfate (20 to 40%). The cell extract was concentrated to 2.0 ml solution by dialysis against polyethyleneglycol #6,000 and dialyzed again for an additional 12 hours against 25 mM Tris-HCl (pH 7.5) containing 7 mM 2-mercaptoethanol, 0.1% Triton X-100 and 50% glycerol.

Assay of Enzyme Activities

The cell extract mentioned above (100 μl) was incubated at 30°C for 24 hours with 1.0 mg of UDP-glucose and 0.1 mg of a macrolide antibiotic in reaction mixture (1.0 ml) containing 50 mM bicine (pH 7.0), 7 mM $MgCl_2$ and 100 mM NaCl. After incubation, the reaction mixture was extracted with chloroform. The chloroform

layer was concentrated *in vacuo* for examination by TLC. To distinguish between UDP-glucose (or UDP-galactose) dependent and non-dependent reactions, incubation with no donor substrate (*i.e.* without UDP-glucose and UDP-galactose) was also carried out as a control experiment. To determine the effect of divalent cations ($MgCl_2$, $MnCl_2$, $CaCl_2$, $CoCl_2$, $NiCl_2$, $CdCl_2$, $ZnCl_2$, $CuCl_2$ or $FeCl_2$: 2 mM) on the transglycosylation of erythromycin B, the activities were measured in the presence of 1 mM EDTA. The transglycosylation activity was measured at various pHs from 5.0 to 10.0. The reaction buffer, 50 mM bicine (pH 7.0) was replaced with 50 mM sodium phosphate (pH 5.0~6.5), 50 mM HEPES (pH 6.5~7.5), and 50 mM Tris-HCl (pH 7.5~10.0), respectively.

Preparative-scale Enzyme Reaction and Isolation of the Conversion Product

To isolate the conversion products, the enzyme reaction was performed in a 500 ml volume.

The reaction mixture was adjusted to pH 9.0 and extracted with an equal volume of chloroform. The chloroform layer was washed with saturated sodium chloride solution, dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was subjected to silica gel column chromatography (column: Lobar Column size B, Merck Art. 10401, 25 mm i.d. × 310 mm). The chromatography was developed with 400 ml of the solvent of chloroform - methanol - 25% aqueous ammonia (100 : 10 : 1). The fractions containing the glycosylation product were collected and concentrated to dryness. The products thus obtain were recrystallyzed from organic solvent (*i.e.* methylene chloride).

Antibacterial Activity

The test organisms were selected from culture collections in our laboratory. The MICs were determined by the 2-fold serial agar plate dilution method using Sensitivity Test Agar (Eiken). The final inoculum size in the test medium was approximately 10^6 cfu/ml. The test agar plates containing antibiotics were inoculated with the microbes and incubated at 37°C for 18 hours.

Results

Microbial Glycosylation of Macrolide Antibiotics by Using Cell Extracts of *S. hygroscopicus* ATCC 31080 and *S. antibioticus* ATCC 11891

Microbial glycosylations of macrolide antibiotics were carried out using cell extracts of *S. hygroscopicus* ATCC 31080 and *S. antibioticus* ATCC 11891. The reaction mixtures were incubated with UDP-glucose, and without UDP-glucose as a control experiment. The reaction mixtures of azithromycin and 14-membered macrolide antibiotics except for erythromycin A enolether and clarithromycin gave new spots on the TLC plate, respectively. Furthermore, these spots could be detected only in reactions with UDP-glucose but not without UDP-glucose, suggesting that these spots were due to glycosylation products of individual macrolide antibiotics. Thus, it was revealed that the biocatalyst responsible for the reaction products was an UDP-glucose dependent macrolide glycosyltransferase. These two strains were found to have the same MGT activity in the transglycosylation of 14 and 15 membered macrolide antibiotics possessing a desosamine or a chalcose as shown in Table 1.

Table 1. Results of assay using enzyme preparation from *S. hygroscopicus* ATCC 31080 and *S. antibioticus* ATCC 11891 with various substrate.

Compounds	Glycosylation product by using:	
	ATCC 31080	ATCC 11891
Erythromycin A	+	++
Erythromycin B	++	+++
Erythromycin A oxime	++	+++
Erythromycin B oxime	++	+++
N-Demethyl-erythromycin A	+	++
Decladinosyl-erythromycin A	+	++
Erythromycin A enolether	N.D.	N.D.
Clarithromycin	N.D.	N.D.
Azithromycin	++	+++
Oleandomycin	+	++
Picromycin	T.A.	T.A.
Narbomycin	T.A.	T.A.
Kujimycin B	T.A.	T.A.
Chalcomycin	N.D.	N.D.
Midecamycin A1	N.D.	N.D.
Leucomycin A1	N.D.	N.D.
Angolamycin	N.D.	N.D.
Spiramycin	N.D.	N.D.
Tylosin	N.D.	++
Desosamine	N.D.	N.D.

+++: >50% yield, ++: 25~50% yield, +: ~25% yield (0.1~25 μg product/ml reaction mixture), T.A.: Trace amount only detected by radio isotope experiments, N.D.: not detected, detection limit: 1 ng/ml reaction mixture.

On the other hand, the cell extract of *S. hygroscopicus* ATCC 31080 showed no activity of transglycosylation for 16-membered macrolide antibiotic such as tylosin. The specific spot on the TLC plate corresponding to the glycosylation product could be detected in the reaction mixture of tylosin with the cell extract of *S. antibioticus* ATCC 11891. The MGT activity of cell extract from *S. antibioticus* ATCC 11891, a producing organism of oleandomycin, was distinguished from that of ATCC 31080, based on the ability to glycosylate tylosin.

Characterization of Macrolide Glycosyl Transferase Activity from *S. hygroscopicus* ATCC 31080

The MGT activity of cell extract of *S. hygroscopicus* ATCC 31080 as well as that of *S. antibioticus* ATCC 11891 could be observed to express intensively at the stationary phase from 48 to 96 hours' cultivation. Activity could be detected slightly at the logarithmic phase until 48 hours' cultivation. On the other hand, carriomycin was observed to be produced by *S. hygroscopicus* ATCC 31080 at the stationary phase and hardly at the logarithmic phase, Oleandomycin production by *S. antibioticus* ATCC 11891 was also detected at the stationary phase. This phenomenon suggests that the expression of the MGT activity and secondary metabolism occurred simultaneously in *S. hygroscopicus* ATCC 31080 as well as in *S. antibioticus* ATCC 11891.

As the MGT activity was stable under extraction with Triton X-100 from the mycelium but unstable without detergent, the enzyme was presumed to be located and buried in the inner membrane.

The MGT activity of cell extracts of *S. hygroscopicus* ATCC 31080 was assayed for the utilization of various donor substrates (*e.g.* UDP-glucose) by incubating with erythromycin B and tylosin as acceptor substrate. As shown in Table 2, the transglycosylation enzyme reaction utilized UDP-glucose and UDP-galactose as donor substrates. The ability to utilize UDP-glucose and UDP-galactose was similar in the transglycosylation (data not shown).

The effect of divalent cations on the transglycosylation of erythromycin B was examined. As shown in Table 2, $MgCl_2$ was required and essential, but could be completely replaced by $MnCl_2$. In the absence of such divalent cations, no activity was observed.

Table 2. Characteristics of MGT activity of cell extract from ATCC 31080.

Substrate (acceptor)[a]:	Fourteen membered macrolide antibiotics (excluding clarithromycin and erythromycin A enolether)
Substrate (donor)[b]:	
Utilize:	UDP-glucose, UDP-galactose
Not utilize:	UDP-glucuronic acid, UDP-galacturonic acid, UDP-*N*-acetylglucosamine, UDP-mannose
Cofactor of divalent ion:	Mg^{2+} or Mn^{2+} dependence (2 mM)
Optimum pH:	7.0~7.5
Reaction pH:	6.0~9.5

[a] Assay was incubated with UDP-glucose as donor substrate.
[b] Assay was incubated with erythromycin B as acceptor substrate.

The transglycosylation activity of cell extracts was also examined at various pHs from 5.0 to 10.0. The strongest activity was detected at pH 7.0~7.5 but no activity was observed at below pH 6.0 and above pH 9.5 as summarized in Table 2.

To compare the characteristics of the MGT activity from ATCC 31080 and ATCC 11891, the MGT activity in cell extracts of *S. antibioticus* ATCC 11891 was examined, and was observed to have almost the same properties as that of ATCC 31080 shown in Table 2 except for its ability to glycosylate tylosin. UDP-glucose could be completely replaced by UDP-galactose for transglycosylation of tylosin using cell extract from ATCC 11891.

Production and Isolation of Glycosylation Products by Using Cell Extract of *S. hygroscopicus* ATCC 31080

500 ml preparative-scale enzyme reactions with erythromycin A (**1**), erythromycin B (**2**), erythromycin A oxime (**3**) and azithromycin (**4**) were carried out by using cell extract of *S. hygroscopicus* ATCC 31080 with UDP-glucose as a donor substrate. The conversion products **5**~**8** from individual substrates were examined on TLC. From 100 mg of each of substrate, 20 mg of **5**, 95 mg of **6**, 50 mg of **7**, 95 mg of **8** were isolated, respectively.

We also carried out the preparative-scale reaction of **2** with UDP-galactose as a donor by using cell extract of *S. hygroscopicus* ATCC 31080 (*vide supra*) and obtained 80 mg of **9**.

Characterization and Structure Elucidation of Conversion Products by Using Enzyme from *S. hygroscopicus* ATCC 31080

Comparison of the spectral data of **5** with those of 2'-(*O*-[β-D-glucopyranosyl])erythromycin A[4] demonstrated that they were identical to each other. Thus, **5** was identified as 2'-(*O*-[β-D-glucopyranosyl])erythromycin A.

As depicted in Table 3, **6**~**9** were isolated as colorless amorphous powder or crystalline needles. They are soluble in water, lower alcohols, DMSO and acetone and slightly soluble in chloroform and ethyl acetate, but insoluble in *n*-hexane. Their molecular weights, determined by high resolution FAB-MS spectra, indicated an increment of molecular formula for **6**~**9** corresponding to $C_6H_{11}O_5$ from the substrate, respectively. The

Table 3. Physicochemical properties of products glycosylated by MGT_{shy}.

Compound	6	7	8	9
Appearance	Colorless amorphous	Colorless amorphous	Colorless needles	Colorless amorphous
Formula	$C_{43}H_{77}NO_{17}$	$C_{43}H_{78}N_2O_{18}$	$C_{44}H_{77}N_2O_{17}$	$C_{43}H_{77}NO_{17}$
MP (°C)	130.5~131.5	191.5~192.5	143.0~144.0	110.0~111.0
HR-FAB-MS:	$(M+K)^+$	$(M+Na)^+$	$(M+K)^+$	$(M+K)^+$
Calcd. for	918.483	933.515	949.525	918.483
Found	918.486	933.516	949.526	918.484
Solubility				
Soluble:	DMSO, acetone, H_2O, MeOH, EtOH	DMSO, acetone, H_2O, MeOH, EtOH	DMSO, acetone, H_2O, MeOH, EtOH	DMSO, acetone, H_2O, MeOH, EtOH
Slightly Soluble:	EtOAc, $CHCl_3$	EtOAc, $CHCl_3$	EtOAc, $CHCl_3$	EtOAc, $CHCl_3$
Insoluble:	*n*-Hexane	*n*-Hexane	*n*-Hexane	*n*-Hexane

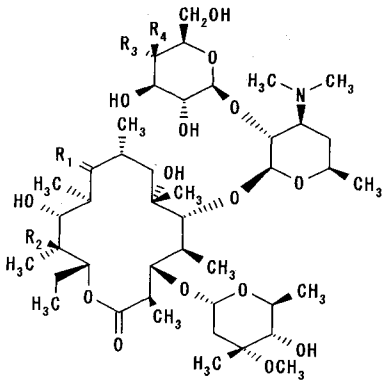
Table 4. ^{13}C NMR chemical shifts (δ) assignments of **6**, **7**, **8** and **9**.

Position	6	7	8	9	Position	6	7	8	9
1	176.7 s	176.1 s	178.7 s	176.8 s	1′	101.1 d	101.5 d	100.8 d	100.9 d
2	44.8 d	45.5 d	45.3 d	44.8 d	2′	79.4 d	81.9 d	81.7 d	79.4 d
3	81.8 d	79.9 d	78.1 d	81.9 d	3′	65.5 d	65.9 d	65.4 d	65.5 d
4	39.5 d	40.4 d	42.5 d	39.5 d	4′	29.4 t	30.2 t	29.4 t	29.4 t
5	82.2 d	82.9 d	82.3 d	82.2 d	5′	69.5 d	71.7 d	67.9 d	69.4 d
6	75.4 s	75.0 s	73.7 s	75.3 s	6′ (5′-CH_3)	21.4 q	21.6 q	22.0 q	21.3 q
7	38.1 t	39.3 t	42.2 t	39.0 t	3′-N(CH_3)$_2$	40.5 q	40.4 q	40.5 q	40.4 q
8	44.7 d	35.0 d	26.5 d	44.7 d	1″	96.3 d	96.7 d	94.1 d	96.2 d
9	219.9 s	170.9 s	70.2 t	220.0 s	2″	35.0 t	35.6 t	34.6 t	34.4 t
10	39.3 d	26.2 d	62.4 d	39.3 d	3″	72.7 s	73.5 s	72.9 s	72.6 s
11	68.3 d	67.9 d	73.6 d	68.3 d	4″	77.9 d	78.7 d	77.4 d	77.9 d
12	40.2 d	75.5 s	74.2 s	40.1 d	5″	65.8 d	66.0 d	65.6 d	65.7 d
13	74.9 d	77.3 d	77.3 d	75.0 d	6″ (5″-CH_3)	18.8 q	19.2 q	18.3 q	18.8 q
14	25.4 t	25.7 t	21.3 t	25.4 t	7″ (3″-CH_3)	21.5 q	22.0 q	21.6 q	21.5 q
15 (14-CH_3)	10.4 q	11.2 q	8.3 q	10.4 q	3″-OCH_3	49.6 q	49.7 q	49.6 q	49.6 q
16 (2-CH_3)	15.3 q	15.4 q	21.3 q	15.3 q	1‴	106.7 d	107.6 d	106.8 d	106.6 d
17 (4-CH_3)	8.8 q	9.7 q	7.3 q	8.7 q	2‴	75.2 d	76.6 d	74.8 d	75.2 d
18 (6-CH_3)	27.3 q	27.5 q	27.5 q	27.1 q	3‴	77.6 d	78.9 d	77.0 d	75.8 d
19 (8-CH_3)	18.5 q	16.6 q	16.4 q	18.5 q	4‴	70.3 d	72.2 d	70.5 d	70.2 d
20 (10-CH_3)	9.1 q	11.6 q	11.2 q	9.0 q	5‴	75.8 d	77.9 d	75.5 d	77.4 d
21 (12-CH_3)	9.4 q	17.5 q	14.5 q	9.4 q	6‴	62.8 t	63.9 t	62.7 t	62.7 t

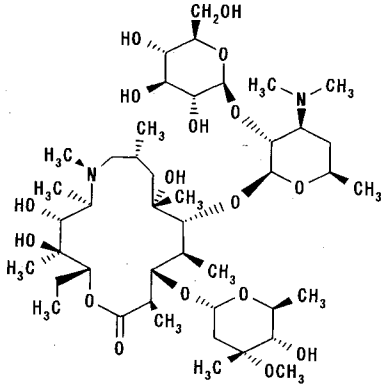
difference suggests that **6**~**9** are tentatively assigned to be monosaccharides of their respective substrates. This is supported by the fact that these compounds are the products of the UDP-glucose and UDP-galactose dependent reaction. In order to get further structural information, the ^{13}C NMR spectra were compared. ^{13}C Chemical shift assignments of **6**~**9** are shown in Table 4. The increase of six signals was observed commonly in the ^{13}C NMR spectra of **6**~**8**, compared with those of the respective substrates. These signals were resonated approximately at the same regions. These chemical shift values of **6**~**8** were identical with those of β-D-glucopyranoside[14]. The chemical shift values of the signals corresponding to C-1‴~6‴ of **9** were also compatible with those of β-D-galactopyranoside[14]. These results indicated that one of the hydroxyl groups was glycosylated in **6**~**9**.

Furthermore, their ^1H-^{13}C COSY spectra demonstrated a downfield shift by *ca.* 10 ppm of the C-2′ signals, which was consistent with the typical β-glycosylation shift[15]. In the HMBC spectra, H-2′ of a desosamine moiety in **6**~**9** showed a long-range coupling to C-1‴ of a glucose unit in **6**~**8** and a galactose unit in **9**. It was revealed that a sugar moiety was located at C-2′ of a desosamine moiety through a β 1,2 linkage. No other differences were observed in the ^{13}C NMR data between **6**~**9** and their respective substrates, indicating that the rest of the molecules of **6**~**9** was not altered. Therefore, the structures of **6**~**9** were elucidated as depicted in Fig. 2.

Fig. 2. Structures of transglycosylation products **6**~**9**.



	6	7	9
R_1	O	NOH	O
R_2	H	OH	H
R_3	OH	OH	H
R_4	H	H	OH



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6; 2′-(*O*-[*β*-D-glucopyranosyl])erythromycin B, **7**; 2′-(*O*-[*β*-D-glucopyranosyl])erythromycin A oxime, **8**; 2′-(*O*-[*β*-D-glucopyranosyl])azithromycin, **9**; 2′-(*O*-[*β*-D-galactopyranosyl])erythromycin B.

Table 5. *In vitro* antibacterial activity of erythromycin B, erythromycin A oxime, azithromycin and their transglycosylation products.

Organisms	MIC (μg/ml)					
	2	**3**	**4**	**6**	**7**	**8**
Staphylococcus aureus 209P-JC	0.20	0.20	0.39	12.5	100	>100
Staphylococcus aureus Smith 4	0.39	0.20	0.39	12.5	>100	>100
Staphylococcus aureus BB	0.39	0.20	0.20	12.5	100	>100
Staphylococcus aureus J-109	>100	>100	>100	>100	>100	>100
Staphylococcus epidermides sp-al-1	0.39	0.39	0.39	25	>100	>100
Streptococcus faecalis ATCC 8043	0.05	0.05	0.05	1.56	25	50
Bacillus subtilis ATCC 6633	0.20	0.05	0.39	6.25	100	>100
Micrococcus luteus ATCC 9341	N.D.	0.05	<0.05	N.D.	6.25	12.5
Micrococcus luteus NIHJ	0.05	0.05	N.D.	1.56	12.5	N.D.
Escherichia coli NIHJ JC-2	>100	>100	6.25	>100	>100	>100
Escherichia coli K-12	12.5	25	1.56	>100	>100	>100
Klebsiella pneumoniae IFO 3317	N.D.	100	1.56	N.D.	>100	>100
Streptomyces hygroscopicus ATCC 31080	3.13	1.56	1.56	>100	>100	>100
Streptomyces antibioticus ATCC 11891	3.13	3.13	3.13	>100	>100	>100

Inoculum size: 10^{-6} cfu/ml, medium: sensutivity test agar (Eiken), N.D.: not done.

In Vitro Antibacterial Activity of **2**~**4** and Their Glycosylation Products **6**~**8**

MICs of **2**~**4** and their glycosylation products **6**~**8** are shown in Table 5. These results suggested that the glycosylation at C-2′ in macrolide antibiotics caused loss of antibacterial activity.

MICs of **2**~**4** against ATCC 11891 and ATCC 31080 were 1.56~3.13 mg/ml, while MICs of **6**~**8** were >100 mg/ml. This suggests that the MGTs of the organisms were absent or had little activity during the early growth phase (see Discussion).

Distribution of Macrolide Glycosyl Transferase among Several Macrolide and Polyether Producing *Streptomyces* Strains

As shown in Table 6, a total of 32 actinomycete strains producing various polyketide antibiotics was examined for MGT activity. It was found that 15 strains among them could convert erythromycin B to 2′-*O*-glucosyl erythromycin B. The MGT activities of these strains were

Table 6. Distribution of a macrolide glycosyl transferase in Actinomycete strains producing macrolide and polyether antibiotics.

Microorganism	Secondary product	MGT activity[a]
Streptomyces antibioticus ATCC 11891	Oleandomycin	+
Saccharopolyspora erythraea IFO 13426	Erythromycin A	−
Streptomyces fradiae JCM 4439	Tylosin	−
Streptomyces ambofaciens JCM 4204	Leucomycin A3	−
Streptomyces spinchromogenes TPR 885	Kujimycin	−
Streptomyces sp. TA 0029	Lonomycin A	+
Streptomyces sp. TA 0036	Lasalocid	−
Streptomyces sp. TA 0041	Nigericin	−
Streptomyces sp. TA 0050	Moyukamycin	−
Streptomyces sp. TA 0052	Laidlomycin	−
Streptomyces sp. TA 0065	Carriomycin	+
Streptomyces sp. TA 0071	Etheromycin	−
Streptomyces sp. TA 0078	Dianemycin	+
Streptomyces sp. TA 0114	Carriomycin	+
Streptomyces sp. TA 0185	Concanamycin	+
Streptomyces sp. TA 0001	Picromycin	+
Streptomyces felleus JCM4031	Picromycin	−
Streptomyces sp. TA 0003	Narbomycin	+
Micromonospora megalomicea IFO 14114	Megalomicins	−
Streptomyces sp. TA 0005	Chalcomycin	+
Streptomyces sp. TA 0006	Angolamycin	+
Streptomyces avermitilis JCM 5070	Avermectin	−
Streptomyces chartreusis JCM 4570	Chartreusin	−
Streptomyces sp. TA 0173	Virustmycin	−
Streptomyces sp. TA 0225	Oligomycin	+
Streptomyces sp. TA 0212	Antimycins	−
Streptomyces sp. TA 0229	Antimycins	+
Streptomyces sp. TA 0082	Monactin	−
Streptomyces sp. TA 0241	Nonactin	−
Streptomyces sp. TA 0190	Anthracyclines	+
Streptomyces sp. TA 0191	Nogalamycin	+
Streptomyces coelicolor IFO 15146	Actinorhodin	+

[a] MGT activity: macrolide glycosyl transferase activity converting erythromycin B to 2′-*O*-glucosyl erythromycin B, +: detected, −: not detected.

observed at the stationary phase of the growth. Strains in which no macrolides have been found to date, also possessed MGT activity. It is likely that the MGT distributes at least among several *Streptomyces* strains producing polyketides and is not necessarily dependent on production of macrolide antibiotics.

Discussion

We found that cell extract of *Streptomyces hygroscopicus* ATCC 31080 producing carriomycin and cell extract of *S. antibioticus* ATCC 11891 producing oleandomycin had similar MGT activity in the transglycosylation of 14 and 15 membered macrolide antibiotics possessing a desosamine or a chalcose (*e.g.* erythromycin A, oleandomycin, picromycin, azithromycin, kujimycin B). They showed no MGT activity for clarithromycin or erythromycin A enolether. This was attributable to steric hindrance between the MGT and some substituent at C-6 in the molecule of erythromycin A. The cell extract of ATCC 31080 had no activity for 16-membered macrolides whereas that of ATCC 11891 could catalyze glycosylation of tylosin, indicating that the MGT activity of ATCC 31080 could be distinguished from that of ATCC 31080.

The MGT activities of cell extract of ATCC 31080 and ATCC 11891 were characterized by the utilization of UDP-glucose and UDP-galactose, the dependence on Mg^{2+} or Mn^{2+} and the optimum pH range from 7.0 to 7.5. The MGT activities were stable under extraction with 1% Triton X-100 from the mycelia but unstable with no detergent, indicating that the MGT enzymes may be located and buried in the inner membrane.

The structures of inactivated forms of macrolide antibiotics were determined by their spectral data to be 2′-(*O*-[β-D-glucopyranosyl])erythromycin A (**5**), 2′-(*O*-[β-D-glucopyranosyl])erythromycin B (**6**), 2′-(*O*-[β-D-glucopyranosyl])erythromycin A oxime (**7**), 2′-(*O*-[β-D-glucopyranosyl])azithromycin (**8**), and 2′-(*O*-[β-D-galactopyranosyl])erythromycin B (**9**), respectively. Thus, it was found that the location of the MGTs' glycosylation was at C-2′ of a desosamine and that the donor substrate of UDP-glucose could be substituted by UDP-galactose. It was presumed that the glycosylation site of glycosylkujimycin B was at C-2′ of a chalcose although it could

not be isolated because of its low conversion yield.

Antibacterial activity was lost in **6**~**8** by glycosylation at C-2′ in the parent compounds and the MGTs exerted their role as inactivation enzymes of macrolide antibiotics.

HERNANDEZ *et al.*[6] described the existence of the MGT gene in the upstream region of the oleandomycin biosynthetic gene cluster of *S. antibioticus* ATCC 11891 and the relationship of the secondary metabolism with the expression of the MGT gene. The expression of the MGT activity and the production of antibiotics, well known secondary metabolites, occurred simultaneously in *S. hygroscopicus* ATCC 31080 as well as in *S. antibioticus* ATCC 11891, which demonstrated that the MGTs of ATCC 31080 and ATCC 11891 emerged concurrently with the secondary metabolism. It is likely that the emergence of the MGT activity of *S. hygroscopicus* ATCC 31080 is related to the production of a polyketide antibiotic carriomycin. Growth inhibition of *S. hygroscopicus* ATCC 31080 and *S. antibioticus* ATCC 11891 was observed by addition of exogenous macrolide antibiotics at the start of their cultivation whereas addition at the growing phase had no effect on their growth, indicating the emergence of the MGT at the stationary phase. This phenomenon was reflected in the MIC values of erythromycin derivatives against ATCC 31080 and ATCC 11891. As the MGT emerged in the secondary metabolism, the role of MGT in the strains producing macrolide antibiotics could be restricted to the inactivation of the endogenously-produced macrolide antibiotics.

In order to investigate the distribution of the MGT, 32 strains of *Actinomycetes* producing polyketide antibiotics were examined for the MGT activity against erythromycin B. Among them, 15 strains of *Streptomyces* were found to have the similar activity at the stationary phase. The high frequency of the MGT activity distribution among *Streptomyces* strains might be attributable to gene transfer rather than being one of the characters of the genus *Streptomyces*. VINING reported that the sequences of the genes related to the secondary metabolism show much weaker homologies with those related to the primary metabolism in the same organism, and gene transfer is an important factor in the evolution of secondary metabolism[16]. These results indicated that the MGT gene of one *Streptomyces* strain might be transferred into or distributed to the genomes of some other *Streptomyces* strains and that the expression of the MGT gene might be orchestrated in the secondary metabolism, although the cloning of their genes and the alignment of their sequences were not performed.

We think that the archetypal MGT gene might be originated and derived from the organisms producing macrolide antibiotics, which could be endogenously inactivated by the MGT. *S. antibioticus* ATCC 11891 producing oleandomycin could be one of the strains possessing the archetypal MGT.

Acknowledgments

We thank Dr. TAKASHI ADACHI and Mr. TOSHIFUMI ASAKA for preparing macrolides as substrate of MGT, and Mr. MICHIO YAMAGISHI for isolation and identification of microorganisms.

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