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Research Paper

Elucidation of the function of two glycosyltransferase genes (*lanGT1* and *lanGT4*) involved in landomycin biosynthesis and generation of new oligosaccharide antibiotics

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Abstract

Background: The genetic engineering of antibiotic-producing *Streptomyces* strains is an approach that became a successful methodology in developing new natural polyketide derivatives. Glycosyltransferases are important biosynthetic enzymes that link sugar moieties to aglycones, which often derive from polyketides. Biological activity is frequently generated along with this process. Here we report the use of glycosyltransferase genes isolated from the landomycin biosynthetic gene cluster to create hybrid landomycin/urdamycin oligosaccharide antibiotics.

Results: Production of several novel urdamycin derivatives by a mutant of *Streptomyces fradiae* Tü2717 has been achieved in a combinatorial biosynthetic approach using glycosyltransferase genes from the landomycin producer *Streptomyces cyanogenus* S136. For the generation of gene cassettes useful for combinatorial biosynthesis experiments new vectors named pMUNI, pMUNII and pMUNIII were constructed. These vectors facilitate the construction of gene combinations taking advantage of the compatible *Mun*I and *Eco*RI restriction sites.

Conclusions: The high-yielding production of novel oligosaccharide antibiotics using glycosyltransferase gene cassettes generated in a very convenient way proves that glycosyltransferases can be flexible towards the alcohol substrate. In addition, our results indicate that LanGT1 from *S. cyanogenus* S136 is a D-olivosyltransferase, whereas LanGT4 is a L-rhodinosyltransferase. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Urdamycin; Landomycin; Glycosyltransferase; Combinatorial biosynthesis; Vector system

1. Introduction

Many biologically active natural compounds are produced by actinomycetes, most of them by *Streptomyces* species. Since the first report on using genetic engineering techniques to create hybrid antibiotics the potential of manipulating biosynthetic pathways in *Streptomyces* has received much attention [1,2]. Many naturally occurring bioactive metabolites possess unusual carbohydrate moieties, which serve as molecular recognition elements important for biological activities. Without these sugar elements, the biological activities are often either completely abolished or dramatically decreased [3,4].

There is an increasing interest in antibiotics containing oligosaccharide structures. As oligosaccharide antibiotics are difficult to produce by chemical synthesis, the genetic engineering of oligosaccharide structures could be an important and very promising technique. Recently it has been shown that glycosyltransferases, responsible for the formation of oligosaccharide structures, may have a certain degree of flexibility towards nucleotide sugar donor but also towards the acceptor molecule [5–7].

Landomycin A, produced by *Streptomyces cyanogenus* S136, and urdamycin A, produced by *Streptomyces fradiae*

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Tü2717, belong to the angucycline group of antibiotics [8]. In various biological tests landomycin A showed interesting antitumor activities, in particular against prostata cancer cell lines [9], and it was shown that landomycin A inhibits DNA synthesis and G_1/S cell cycle progression [10]. In contrast, urdamycin A possesses only weak antitumor activity. One important difference between both compounds is the oligosaccharide side chain attached to the polyketide moiety. The hexasaccharide side chain of landomycin A is comprising two repeating trisaccharides each consisting of the sequence β-D-olivose-(4→1)-β-D-olivose-(3→1)-α-L-rhodinose (Fig. 1) [11]. The same deoxysugar building blocks are assembled to a trisaccharide chain in urdamycin A forming the sequence β-D-olivose-(3→1)-α-L-rhodinose-(4→1)-β-D-olivose [12]. This trisaccharide is connected to the aglycone by an unusual C-glycosidic bond and urdamycin A contains an additional L-rhodinose attached O-glycosidically at the 12b-position (Fig. 1).

The biosynthetic gene clusters of both compounds have been cloned and sequenced [6,13–16]. Four glycosyltransferase genes have been detected in each cluster. The function of glycosyltransferases involved in urdamycin biosynthesis has been elucidated [15,16] and the generation of a strain of *S. fradiae* Tü2717 with mutations in three of four glycosyltransferase genes producing monoglycosylated intermediates as aquayamycin has been described [16]. Here we report the successful expression of glycosyltransferase genes of the landomycin producer in a mutant of the urdamycin producer *S. fradiae* Tü2717, which led to the formation of various, new urdamycin derivatives with new saccharide side chains. We also introduce a novel cloning vector allowing the simple and convenient set up of gene cassettes.

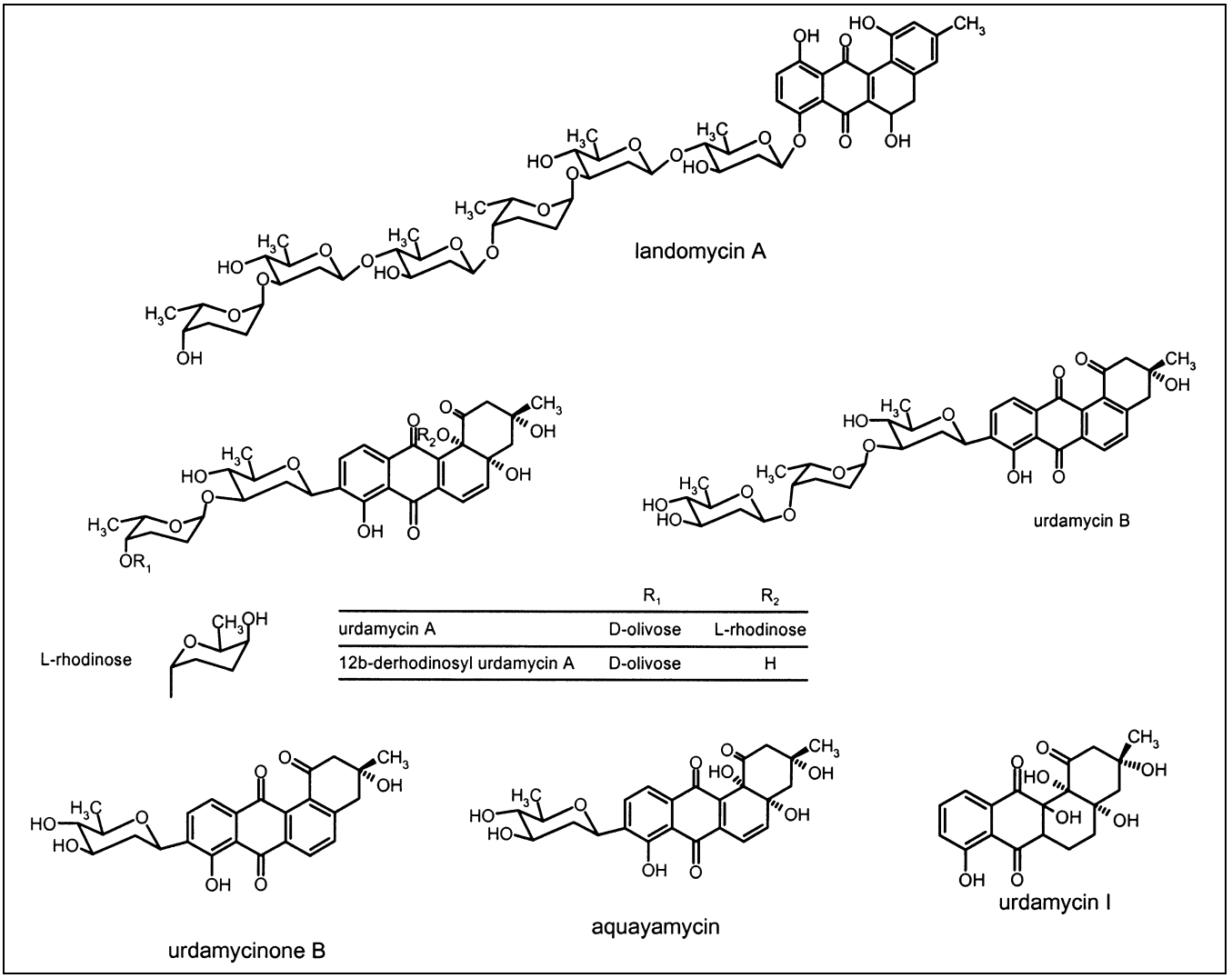


Fig. 1. Structures of landomycin A produced by *S. cyanogenus* S136 and urdamycin derivatives produced by *S. fradiae* and mutants *S. fradiae* A-x and *S. fradiae* A-0. For the urdamycins only A- and B-type aglycones are shown. These are produced as a complex with C- and D-type urdamycins without changes in the sugar moieties [21].

2. Results

2.1. Sequence comparison of glycosyltransferases from the landomycin and urdamycin biosynthetic gene cluster

It has been described that the comparison of the deduced products of all eight putative glycosyltransferase genes from the landomycin and urdamycin biosynthetic gene cluster with proteins in databases clearly implemented that they are involved in glycosylation steps [13,15,16]. Since the functions of the glycosyltransferases UrdGT1a (12b-L-rhodinosyltransferase), UrdGT1b (4A-D-olivosyltransferase), UrdGT1c (3′-L-rhodinosyltransferase) and UrdGT2 (9-D-olivosyltransferase) were determined unambiguously [15,16], we expected to predict the function of the glycosyltransferases LanGT1, LanGT2, LanGT3 and LanGT4 by sequence comparison. As shown in Fig. 2 the results were: (i) UrdGT2 resembles LanGT2, (ii) UrdGT1b and UrdGT1c are closely related to LanGT1 and LanGT3, and (iii) UrdGT1a shows the closest resemblance to LanGT4.

2.2. Generation of a S. fradiae mutant (mutant S. fradiae A-0) lacking all four glycosyltransferase genes of the urdamycin biosynthetic gene cluster

S. fradiae BF-1-1 [15] carrying an in-frame deletion in *urdGT2* was used to generate a mutant lacking all four glycosyltransferase genes from the urdamycin biosynthetic gene cluster. Mutant BF-1-1 was transformed with pKC-12-B2 [16], which has been constructed for the deletion of *urdGT1a*, *urdGT1b* and *urdGT1c*. Apramycin resistant colonies were obtained and single cross-over events were confirmed by hybridization using a ColE1 fragment from pKC1132 as probe. Integrants were grown without apramycin selection to allow for second cross-over. Apramycin sensitive clones were screened by PCR for the deletion of a 4 kb *Bam*HI–*Pst*I fragment which removes *urdGT1a*, *urdGT1b*, *urdInt* and *urdGT1c* from the chromosome. The deletion was confirmed by Southern hybridization. Chromosomal DNA from this mutant named *S. fradiae* A-0 and DNA from mutant BF-1-1 were isolated and digested with *Pst*I. Hybridization was carried out using a 5.2 kb *Sac*I fragment as a probe. Strain BF-1-1 showed the expected bands at 5.2, 2.6 and 2.4 kb whereas mutant A-0 showed one single band at 2.4 kb confirming the deletion (Fig. 3C). Secondary metabolites accumulated by mutant A-0 were urdamycin I (Fig. 1), urdamycin J (major components) and minor amounts of rabelomycin. The genotypes of all strains used in this study are shown in Fig. 3.

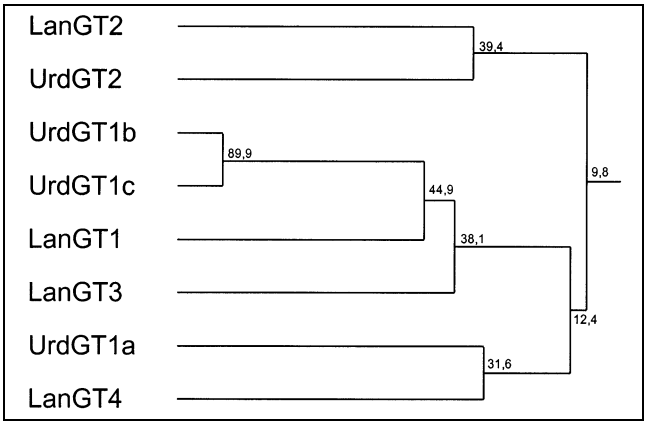


Fig. 2. Dendrogram derived from the comparison of urdamycin and landomycin glycosyltransferase sequences (accession numbers: UrdGT1a AAF00214, UrdGT1b AAF00215, UrdGT1c AAF00217, UrdGT2 AAF00209, LanGT1 AAD13555, LanGT2 AAD13553, LanGT3 AAD13559, LanGT4 AAD13562). The dendrogram was drawn based on an alignment built with DNASIS using the resettings of the program. Genetic similarity is given at points of branching.

Table 1
Important restriction sites located in the polylinker of pMUN vectors

Plasmid	Restriction sites
pMUN1	*Hin*dIII, *Mun*I, *Eco*RV, *Eco*RI, *Nsi*I, *Bam*HI, *Spe*I, *Xba*I
pMUN2	*Hin*dIII, *Mun*I, *Nco*I, *Nsi*I, *Nde*I, *Bgl*II, *Eco*RI, *Spe*I, *Xba*I
pMUN3	*Hin*dIII, *Mun*I, *Nco*I, *Nsi*I, *Nde*I, *Bgl*II, *Eco*RI, *Bam*HI, *Spe*I, *Xba*I

2.3. Construction of a novel vector system allowing a simple set up of gene cassettes

To facilitate the set up of gene cassettes for combinatorial biosynthesis several plasmids named pMUNI, pMUNII and pMUNIII were constructed. These vectors contain a single *Mun*I and a single *Eco*RI restriction site within the polylinker. Genes can be cloned into these vectors between both restriction sites. As *Mun*I and *Eco*RI are compatible, gene cassettes can be generated as depicted in Fig. 4. Important restriction sites of vectors pMUNI, pMUNII and pMUNIII are given in Table 1. pMUNI still retains the intact *lacZ′* from pBluescript allowing blue/white screening. Gene cassettes generated in one of these vectors can be restricted by *Hin*dIII and *Xba*I and can be ligated into the *Hin*dIII and *Xba*I sites of plasmid pUWL201 [17]. This plasmid contains the minimal replicon from pIJ101 [18] and the *tsr* gene from *Streptomyces azureus* conferring resistance to thiostrepton for propagation and selection in *Streptomyces*. The ColE1 replicon and the β-lactamase from pBluescript SK− are used for maintenance in *Escherichia coli*. The strong constitutive *ermE* promoter [19] allows the expression of genes in *Streptomyces* under control of the constitutive erythromycin resistance promoter.

2.4. Expression of single glycosyltransferase genes in S. fradiae A-x and S. fradiae A-0

As listed in Table 2 all genes were expressed singly in *S. fradiae* A-x and *S. fradiae* A-0. *urdGT2*, *lanGT1*, *lanGT2*, *lanGT3* and *lanGT4* were amplified by PCR, first cloned

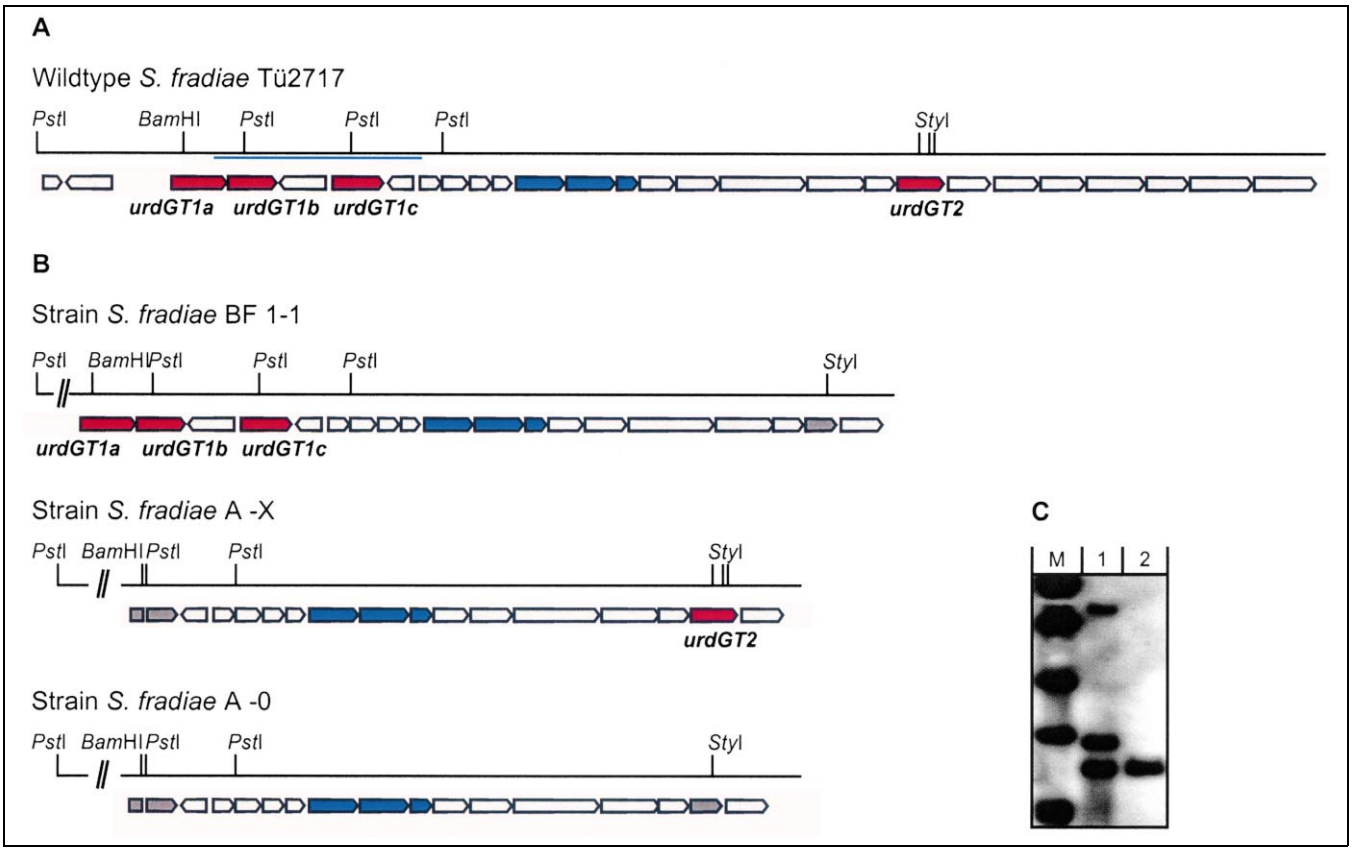


Fig. 3. A: Organization of the urdamycin biosynthetic gene cluster from *S. fradiae* Tü2717. Genes and their direction of transcription are indicated by arrows; active glycosyltransferase genes are shown in red, polyketide synthase genes in blue; the blue bar shows the localization of the fragment used as probe for hybridization; only relevant restriction sites are shown. B: Genotypes of glycosyltransferase gene mutant strains used in this study; inactive glycosyltransferase genes are shown in dark gray. C: Southern hybridization confirming the deletion in *S. fradiae* A-0; lane M: DIG-labelled molecular size marker with the following fragments shown: 1953 bp, 2799 bp, 3639 bp, 4899 bp, 6106 bp; lane 1: chromosomal DNA isolated from *S. fradiae* BF-1-1, digested with *Pst*I; lane 2: chromosomal DNA isolated from *S. fradiae* A-0, digested with *Pst*I (for details see text).

into the vector pMUNII and then transferred to the expression vector pUWL201. These constructs as well as constructs containing *urdGT1a*, *urdGT1b* and *urdGT1c* [16] were used to transform the A-x and A-0 mutants. The expected production of compounds 100-1 and 12b-derhodinosyl-urdamycin G (Fig. 5) was observed when *urdGT1c* was expressed in mutant A-x and compound 100-2 (Fig. 5) was found when *urdGT1a* was expressed. Also as expected, urdamycinone B and aquayamycin (Fig. 1) were produced when *urdGT2* was expressed in mutant A-0.

Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analysis of culture supernatants of mutant A-0 containing either *lanGT1*, or *lanGT2*, or *lanGT3*, or *lanGT4* did not show the formation of any new compound. However, new compounds were produced by mutant A-x containing either *lanGT1* or *lanGT4*. 12b-Derhodinosyl-urdamycin G (and various follow-up products including the previously not described photochemical rearrangement product, named lourdamycin), compound 100-1, and its new 5-hydroxy analog (Fig. 5) were the principal products of mutant A-x after expression of *lanGT4*. By experimental incidents we also isolated one plasmid containing two copies of *lanGT4*. Expression of this plasmid, which is a strong overexpression of the *lanGT4* gene, in mutant A-x resulted in 12b-derhodinosyl-urdamycin G plus large amounts (30% of total production) of urdamycin N and traces of the new urdamycin Q (Fig. 5). Small amounts of novel compounds, presumably ladamycins A_2 and B_2, were detected when extracts of *S. fradiae* A-x containing *lanGT1* were carefully analyzed by HPLC-mass spectrometry (MS).

2.5. *Coexpression of urdGT2, urdGT1a, urdGT1b and urdGT1c in S. fradiae A-0*

The successful implementation of the pMUN vectors requires all genes to be efficiently transcribed from the erythromycin resistance promoter. As a control, plasmids pUWL-u1a-u2 containing *urdGT1a* and *urdGT2* and pUWL-u2-u1a-u1b-u1c containing all four urdamycin glycosyltransferase genes were constructed and expressed in *S. fradiae* A-0. Compound 100-2 was identified when *urdGT1a* and *urdGT2* were expressed and urdamycin A production was restored to similar levels as in the wild-type after coexpression of all four genes (Table 2).

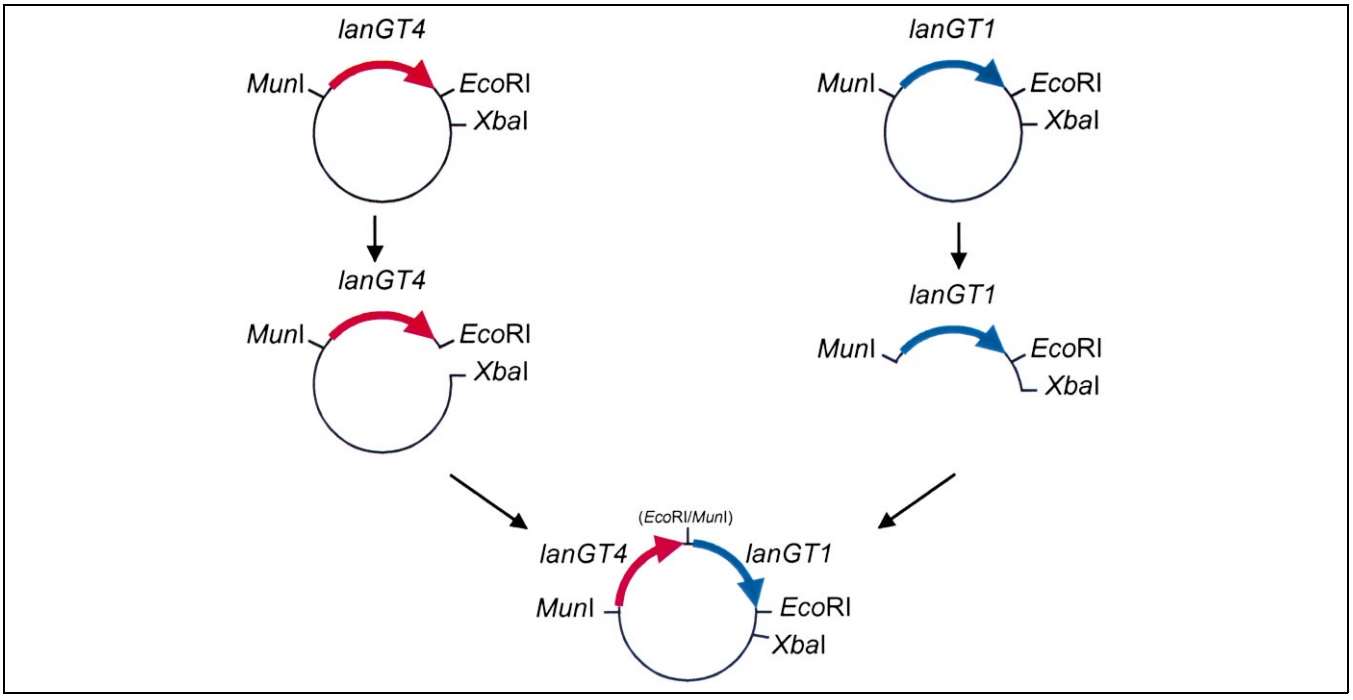


Fig. 4. Generation of a gene cassette containing *lanGT1* and *lanGT4* using the pMUN vector system.

2.6. *Coexpression of landomycin glycosyltransferase genes in S. fradiae A-x*

The expression of *lanGT1* and/or *lanGT4* in *S. fradiae* A-x resulted in the accumulation of hybrid antibiotics (see above). Gene cassettes containing *lanGT1*+either *lanGT2*, or *lanGT3* or *lanGT4* and *lanGT4*+either *lanGT2* or *lanGT3*, respectively, were constructed and expressed in mutant A-x. Analysis of supernatant of the strain containing *lanGT1+lanGT4* showed production of several novel compounds, namely ladamycin A_3, its follow-up product ladamycin D_3 and ladamycin B_3 (Fig. 5) at high levels (90% of total production). These compounds were also produced by mutants containing *lanGT1+lanGT4+lanGT2*, *lanGT1+lanGT4+lanGT3*, and the one which contained all four landomycin glycosyltransferase genes.

Table 2
Mutants, glycosyltransferase gene constructs and resulting metabolites

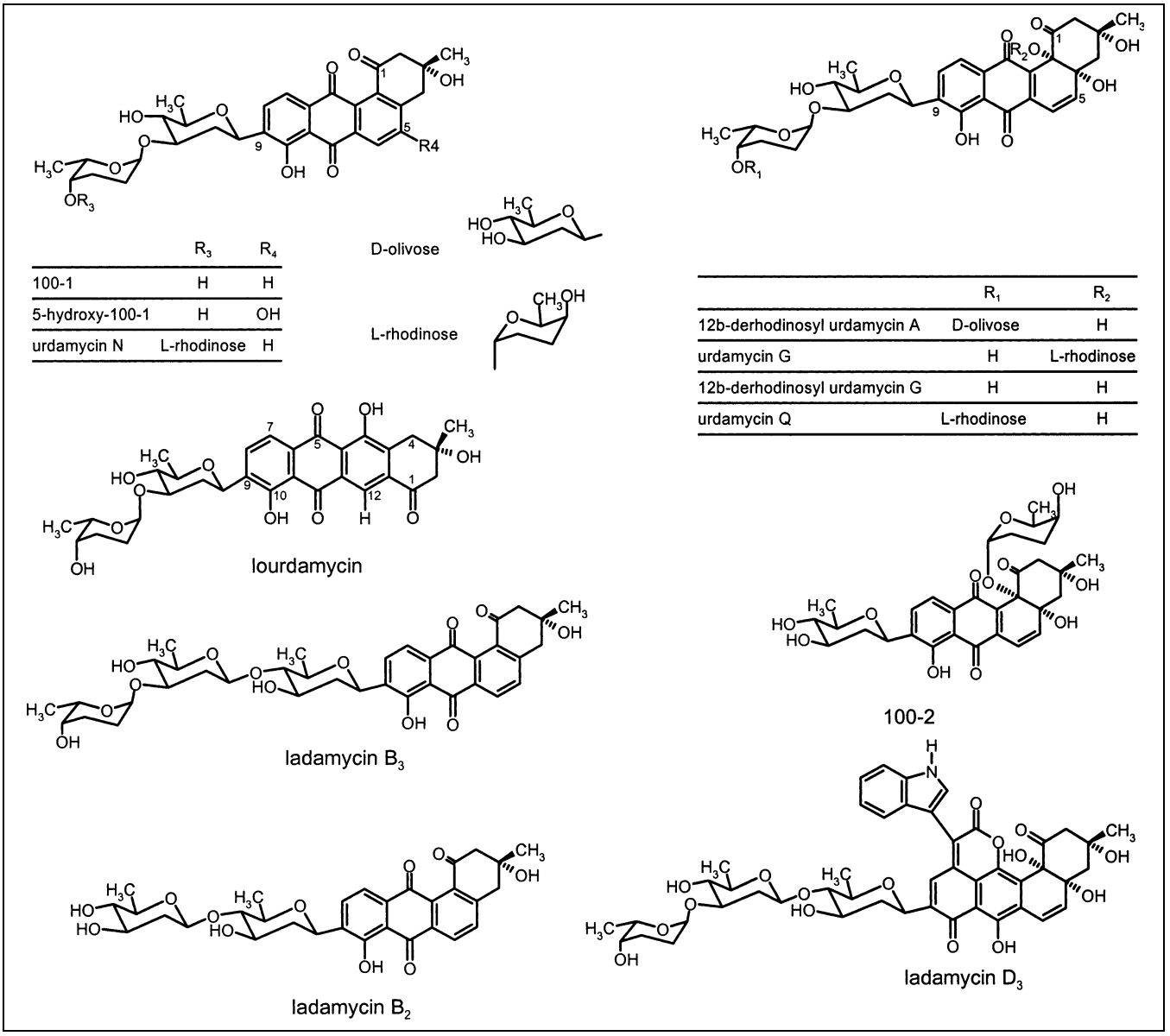
Strain	Plasmid	Expressed glycosyltransferase	Products of the recombinant strain
Wild-type	–		urdamycin A, urdamycin B, 12b-derhodinosyl-urdamycin A
A-0	pUWL201	–	urdamycin I, urdamycin J, rabelomycin
A-0	pUWL-urdGT2	UrdGT2	aquayamycin, urdamycinone B
A-0	pUWL-u1a-u2	UrdGT1a+UrdGT2	100-2, aquayamycin, urdamycinone B
A-0	pUWL-u2-u1b-u1c-u1a	UrdGT2+UrdGT1a+UrdGT1b+UrdGT1c	urdamycin A, 12b-derhodinosyl-urdamycin A, urdamycin G, 100-2, urdamycin B, 100-1
A-x	pUWL201	–	aquayamycin, urdamycinone B
A-x	pUWL-I1	LanGt1	aquayamycin, urdamycinone B, ladamycin A_2, ladamycin B_2
	pUWL-I1-I2	LanGT1+LanGT2	
	pUWL-I1-I3	LanGt1+LanGT3	
	pUWL-I1-I2-I3	LanGT1+LanGT2+LanGT3	
A-x	pUWL-I4	LanGT4	12b-derhodinosyl-urdamycin G, aquayamycin, 100-1, urdamycinone B
	pUWL-I2-I4	LanGT2+LanGT4	
	pUWL-I3-I4	LanGT3+LanGT4	
	pUWL-I2-I3-I4	LanGT2+LanGT3+LanGT4	
A-x	pUWL-I4-I4	LanGT4+LanGT4	12b-derhodinosyl-urdamycin G, 100-1, urdamycin N
A-x	pUWL-I4-I1	LanGT4+LanGT1	ladamycin A_3, ladamycin B_3
	pUWL-I1-I2-I4	LanGT1+LanGT2+LanGT4	
	pUWL-I1-I3-I4	LanGT1+LanGT3+LanGT4	
	pUWL-I1-I2-I3-I4	LanGT1+LanGT2+LanGT3+LanGT4	

Expression of *urdGT1a*, *urdGT1c* and gene cassettes containing various combinations of these genes in mutant A-x and the resulting metabolites have been described [17]. Expression of single glycosyltransferase genes and gene cassettes of the landomycin cluster in mutant A-0 and expression of *lanGT2*, *lanGT3* and *lanGT2+lanGT3* in mutant A-0 did not result in the production of new metabolites.

2.7. Structures

All structures, except those for ladamycins A_2 and A_3 (Fig. 5), were determined using nuclear magnetic resonance (NMR) and MS. Ladamycins A_2 and A_3 were identified through HPLC-MS. In both cases, the HPLC-UV revealed the same 5-hydroxy-1,4-naphthoquinone chromophore known from aquayamycin and urdamycin A, but a different retention time than all known urdamycins. Compared to aquayamycin, the mass of 616 g/mol for ladamycin A_2 (in agreement with $C_{31}H_{36}O_{13}$) indicates an additional olivose moiety, and the mass of 730 g/mol found for ladamycin A_3 indicates an additional olivose plus an additional rhodinose moiety. Ladamycin A_2 was observed in *S. fradiae* A-x containing *lanGT1* and ladamycin A_3 was observed in *S. fradiae* A-x after coexpressing *lanGT1* and *lanGT4*.

The existence of ladamycin A_3 is also confirmed indirectly through the structure of ladamycin D_3, its urdamycin D-type analog, found in *S. fradiae* A-x (*lanGT1*, *lanGT2*, *lanGT3*, *lanGT4*). The non-enzymatic conversion of urdamycin A-type into urdamycin D-type molecules through addition of transaminated tryptophan is well known [20]. The negative fast atom bombardment (FAB) mass spectrum confirms the molecular formula $C_{47}H_{51}NO_{16}$ (885; *m/z* found 885, 100%, M^-) of ladamycin D_3. The 1,4-linkage of the two olivose moieties in ladamycin D_3 follows from the $^3J_{C-H}$ couplings between 1A-H and C-4′ and between 4′-H and C-1A, both observed in the HMBC spectrum (Table 7) as well as from



	R_3	R_4
100-1	H	H
5-hydroxy-100-1	H	OH
urdamycin N	L-rhodinose	H

	R_1	R_2
12b-derhodinosyl urdamycin A	D-olivose	H
urdamycin G	H	L-rhodinose
12b-derhodinosyl urdamycin G	H	H
urdamycin Q	L-rhodinose	H

Fig. 5. Compounds produced by expression of glycosyltransferase genes in mutants *S. fradiae* A-x and *S. fradiae* A-0.

Table 3
Primers used for amplification of glycosyltransferase genes

Amplified gene	Name	Sequence
urdGT2	urdGT2F	TGGGCCGAGGCAATTGCATCCGGCACTC
	urdGT2R	GGTGCCGGATCCGCCCGCCAGCACAAG
lanGT1	lanGT1F	TCGGCACAATTGGCAGGAGACGCATATG
	lanGT1R	CGACTCGTGAGATCTCGCGGTCGGTCA
lanGT2	lanGT2F	CACCTCAAGCTTCAATTGCAGCAGGGAGTATCCGTG
	lanGT2R	CGATGCGAGATATCAGACCAG
lanGT3	lanGT3F	GCGTGCCAATTGCCAAGGCATATGATG
	lanGT3R	GGACCGGCCGAGATCTGACGAGGGG
lanGT4	lanGT4F	GAATCCCAATTGGAGAACCATATGCGTG
	lanGT4R	TCCTGACGGGCGGAGATCTTCGGCGGC

Restriction sites introduced for cloning are underlined.

the ^{13}C downfield shift observed for C-4′ (δ 88.2, see Table 6).

Ladamycin B_2 was discovered in *S. fradiae* A-x (*lanGT1*). Its structure is suggested from significant signals, observed in the ^{1}H NMR spectrum, which reveals two aromatic A–B systems as well as two olivose moieties.

Ladamycin B_3, found in *S. fradiae* A-x following coexpression of *lanGT1* and *lanGT4*, is a structural isomer of urdamycin B [21], as the high-resolved negative FAB-MS reveals ($C_{37}H_{44}O_{13}$, HR calculated 696.2782, found 696.2802). It differs from urdamycin B in the sequence of its saccharide chain, which is α-L-rhodinose-(1→3)-β-D-olivose-(1→4)-β-D-olivose. This saccharide chain sequence follows from the observed long-range couplings (HMBC, Table 7) and the chemical shifts of the carbon signals (δ 88.6 for C-4′, δ 77.3 for C-3A). The $^3J_{C-H}$ couplings between 4′-H and C-1A and between 1A-H and C-4′ prove the 1,4-linkage of the two olivose units, whereas the coupling between 1B-H and C-3A proves the linkage of the terminal rhodinose to 3-OH of olivose A.

The major product of *S. fradiae* A-x containing *lanGT4* is 12b-derhodinosyl-urdamycin G. Its MS data (negative atmospheric pressure chemical ionization (APCI)-MS, *m/z* 600.2, 8%, M^-, *m/z* 582.3, 100%, $M-H_2O$) confirm its molecular formula of $C_{31}H_{36}O_{12}$ (calculated 600.2207). This indicates an additional rhodinose compared to aquayamycin. The NMR data (Table 5 and 6) confirm the presence of a rhodinose moiety, and also its linkage to the 3′-OH group of the C-glycosidically bound D-olivose moiety (^{13}C NMR and HMBC data, Tables 6 and 7). Distinct signals of the rhodinose (sugar A) are for instance the anomeric proton and carbon (δ_H 4.96; δ_C 95.2), which appear in the ^{1}H NMR spectrum as a broad singlet due to its α-glycosidic linkage and in the anomeric region below δ 100 in the ^{13}C NMR spectrum. Another distinct signal of the rhodinose is the broad quartet (δ 4.18, Table 5) of 5A-H. The linkage of this rhodinose to the 3′-position (of the C-glycosidically bound D-olivose) is indicated by the typical downfield shift of this signal in the ^{13}C NMR (δ 78.7, Table 6) and is proven unambiguously by the $^3J_{C-H}$ couplings between 1A-H and C-3′ as well as between 3′-H and C-1A (both were observed in the HMBC spectrum, Table 7).

The structure of the new compound 5-hydroxy-100-1, which was also discovered in *S. fradiae* A-x containing *lanGT4*, was deduced from its high-resolved negative FAB mass spectrum (the $M-H^-$ was observed, calculated for $C_{31}H_{33}O_{11}$ 581.2023, found 581.2027) indicating one oxygen atom more than for the known compound 100-1 [22], and the NMR data (Tables 5–7). The position of the additional OH group in 5-hydroxy-100-1 follows from the 6-H signal, which appears as singlet (δ 7.78) in contrast to 100-1, where this is a doublet of an AB system [22]. That the OH group is located at 5- and not at 6-position could be unambiguously clarified through the HMBC spectrum, in which long-range C–H couplings were observed between this singlet and C-4a, C-5, C-6a, C-7 and C-12.

Another new compound from the same strain, which has never been observed previously, is lourdamycin, which

Table 4
Oligonucleotides used for construction of pMUN vectors

Vector	Oligosequences	Ligated to	Cut with
pMUNI	AGCTTCAATTGGATATCGAATTCATGCATG CTAGCATGCATGAATTCGATATCCAATTGA	pBluescript	*Hin*dIII/*Bam*HI
pMUNII	AATTGCCATGGATGCATATGAGATCTGAATTC GATCGAATTCAGATCTCATATGCATCCATGGC	pMUNI	*Mun*I/*Bam*HI
pMUNIII	AATTGCCATGGATGCATATGAGATCTGAATTCG GATCCGAATTCAGATCTCATATGCATCCATGGC	pMUNI	*Mun*I/*Bam*HI

Overhanging compatible ends used for insertion of the oligonucleotide are underlined.

Table 5
^{1}H NMR data of ladamycin B_3, ladamycin D_3, 5-hydroxy-100-1, 12b-derhodinosyl-urdamycin G and lourdamycin in d_6-acetone at 400 MHz

Proton	Ladamycin B_3	Ladamycin D_3	5-Hydroxy-100-1	12b-Derhodinosyl-urdamycin G	Lourdamycin
2-H_{eq}	2.87 dd (14, 1.5)[a]	2.81 dd (13, 3)[a]	2.82 dd (13.5, 1.5)	2.67 dd (12.5, 3)	2.82 dd (16, 2)[a]
2-H_{ax}	3.08 d (14)[b]	3.28 d (13)	3.05 d (13.5)	2.97 d (12.5)	2.94 d (16)
3-CH_3	1.47 s	1.21 s	1.48 s	1.22 s	1.52 s
4-H_{eq}	3.20 dd (17, 1.5)	1.94–2.12[b]	3.25 dd (18, 1.5)	ca. 2.06[b]	3.36 dd (18, 2)
4-H_{ax}	3.31 d (17)	1.94–2.12[b]	2.99 d (18)	2.23 d (15)	3.08 d (18)
5-H	7.73 d (8)	6.09 d (10)	–	6.44 d (10)	–
6-H	8.30 d (8)	7.00 d (10)	7.78 s	6.81 d (10)	–
7-H	–	–	–	–	7.91 d (8)
8-H	–	–	–	–	8.00 d (8)
10-H	7.92 d (8)	8.15 s	7.88 d (8)	7.88 d (8)	–
11-H	7.60 d (8)	–	7.56 d (8)	7.54 d (8)	–
12-H	–	–	–	–	8.36 s
5″-H	–	8.01 s	–	–	–
7″-H	–	7.58 d (7.5)[d]	–	–	–
8″-H	–	7.23 dd (7.5, 7)	–	–	–
9″-H	–	7.15 dd (7.5, 7)	–	–	–
10″-H	–	7.57 d (7.5)[d]	–	–	–
1′-H	4.90 dd (11, 2)	4.74 d (10)	4.87 dd (11, 1.5)	4.85 dd (11, 1.5)	4.92 dd (11.5, 2)
2′-H_{eq}	2.46 ddd (13, 5, 2)	2.44 ddd (13, 5, 1.5)	2.51 ddd (13, 5, 2)	2.49 ddd (13, 5, 2)	2.57 ddd (13, 5, 2)
2′-H_{ax}	1.30–1.38[b,c]	1.16–1.26[b,c]	1.26–1.43[b,c]	1.26–1.44[b,c]	1.26–1.43[b,c]
3′-H	3.74 ddd (11, 9, 5)	3.68 ddd (11, 9, 5)[b]	3.78 ddd (11, 9, 5)	3.77 ddd (11, 8.5,5)	3.81 ddd (11, 9, 5)
4′-H	3.14 dd (9, 9)	2.90 dd (9, 9)[a]	3.15 dd (9, 9)	3.14 dd (9, 9)	3.17 ddd (9, 9, 3)
5′-H	3.55 dq (9, 6)[b]	3.44 dq (9, 6)[b]	3.48 dq (9, 6)	3.48 dq (9, 6)	3.51 dq (9, 6)[b]
6′-CH_3	1.35 d (6)	1.05 d (6)	1.35 d (6)	1.35 d (6)	1.37 d (6)
1A-H	4.74 dd (10, 2)	4.61 dd (10, 1.5)	4.98 br s	4.96 br s	4.99 br s
2A-H_{eq}	2.41 ddd (13, 5, 2)	2.33 ddd (12.5, 5, 1.5)	1.26–1.43[b,c]	1.26–1.44[b,c]	1.26–1.43[b,c]
2A-H_{ax}	1.40–1.54[b,c]	1.39–1.45[b,c]	1.98–2.09[b,c]	2.02–2.09[b,c]	2.02–2.09[b,c]
3A-H_{eq}	–	–	1.98–2.09[b,c]	2.02–2.09[b,c]	2.02–2.09[b,c]
3A-H_{ax}	3.67 ddd (12, 9, 5)	3.61 ddd (11.5, 9, 5)[b]	1.58–1.64[c]	1.59–1.64[c]	1.59–1.64[c]
4A-H	3.09 ddd (9, 9, 2.5)[b]	3.05 ddd (9, 9, 3)	3.53 br s	3.54 br s	3.55 br s[b]
5A-H	3.43 dq (9, 6)	3.37 dq (9, 6)[b]	4.19 dq (6.5, 1)	4.18 dq (6.5, 1)	4.20 dq (6.5, 1)
6A-CH_3	1.30 d (6)	1.26 d (6)	1.14 d (6.5)	1.14 d (6.5)	1.15 d (6.5)
1B-H	4.95 br s	4.91 br s	–	–	–
2B-H_{eq}	1.40–1.54[b,c]	1.39–1.45[b,c]	–	–	–
2B-H_{ax}	2.00–2.10[b,c]	1.94–2.12[b,c]	–	–	–
3B-H_{eq}	2.00–2.10[b,c]	1.94–2.12[b,c]	–	–	–
3B-H_{ax}	1.59–1.66[c]	1.58–1.65[c]	–	–	–
4B-H	3.54 br s	3.53 br s	–	–	–
5B-H	4.17 dq (6.5, 1)	4.15 dq (6.5, 1)	–	–	–
6B-CH_3	1.12 d (6.5)	1.10 d (6.5)	–	–	–
OH signals[e]	4.66 d (2.5), 4.52, 4.17	5.25, 4.90, 4.72, 4.64 d (3), 4.45, 3.12	4.64	12.33, 5.24, 4.74, 4.63, 4.61	13.10, 13.12, 4.64 d (3), 4.14

δ in ppm, (J in Hz). Coupling partners confirmed by gCOSY experiments.
[a]Obscured by water, visible after addition of D_2O.
[b]Obscured by solvent or other signal(s).
[c]Complex.
[d]Assignments are interchangeable; br = broad signal.
[e]Not all OH signals were observed due to water in the samples.

has a linearly assembled tetracyclic aglycone. This could be deduced from the NMR data. A photochemical rearrangement of aquayamycin into the analogous linearly assembled tetracene-1,6,12-trione derivative has been described by Umezawa et al. [23]. The ^{1}H NMR data of the aglycone moiety of lourdamycin (that includes the C-glycosidically bound D-olivose) are in agreement with those described for the rearranged aquayamycin. Thus, it is likely that lourdamycin derives from 12b-derhodinosyl-urdamycin G, presumably because of the influence of light during the fermentation and/or purification process. The rearranged aglycone structure follows from the long-range C–H couplings observed in the HMBC spectrum, in particular the couplings between 12-H and C-1, C-4a, C-5a and C-11 (Table 7).

After expression of pUWL-l4-l4 in *S. fradiae* A-x the new urdamycin Q was found. Since only about 0.3 mg of the purified compound was available for characterization, only a ^{1}H NMR spectrum in d_6-acetone was obtained. This clearly shows the aromatic (δ 7.93 and 8.01) as well

Table 6
^{13}C NMR data (from HMBC and HSQC spectra) of ladamycin B_3, ladamycin D_3, 5-hydroxy-100-1, 12b-derhodinosyl-urdamycin G and lourdamycin in d_6-acetone (100.6 MHz)

Carbon	Ladamycin B_3	Ladamycin D_3	5-Hydroxy-100-1	12b-Derhodinosyl-urdamycin G	Lourdamycin
1	196.2 s	206.6 s	196.5 s	205.2 s	195.8 s
2	53.3 t	52.0 t	53.1 t	51.9 t	51.2 t
3	71.8 s	75.3 s	71.6 s	76.6 s	70.3 s
3-CH_3	29.5 q	29.4 q	29.8 q	29.5 q	29.4 q
4	43.9 t	43.9 t	37.8 t	43.6 t	36.6 t
4a	149.4 s	80.5 s	137.1 s	81.0 s	138.8 s
5	134.2 d	137.2 d	160.6 s	145.7 d	161.5 s
5a	–	–	–	–	117.3 s
6	128.8 d	118.3 d	112.4 d	116.8 d	188.4 s
6a	133.5 s	123.4 s	134.7 s	138.5 s	132.1 s
7	188.4 s	155.7 s	188.9 s	189.2 s	119.4 d
7a	115.1 s	n.o.	115.2 s	114.3 s	–
8	158.0 s	186.8 s	157.9 s	157.6 s	133.6 d
9	136.9 s	142.8 s	136.4 s	138.2 s	139.2 s
10	133.6 d	135.2 d	133.5 d	133.4 d	159.0 s
10a	–	–	–	–	115.8 s
11	118.7 d	n.o.	118.4 d	118.9 d	188.2 s
11a	134.5 s	116.5 s	134.6 s	131.0 s	n.o.
12	182.7 s	n.o.	181.2 s	182.3 s	115.7 d
12a	136.3 s	128.4 s	127.1 s	139.5 s	138.5 s
12b	136.4 s	78.1 s	139.0 s	76.9 s	–
2″	–	n.o.	–	–	–
3″	–	127.2 s	–	–	–
4″	–	108.2 s	–	–	–
5″	–	131.9 d	–	–	–
6a″	–	136.6 s	–	–	–
7″	–	112.3 d	–	–	–
8″	–	122.8 d	–	–	–
9″	–	121.1 d	–	–	–
10″	–	120.4 d	–	–	–
10a″	–	127.2 s	–	–	–
1′	71.0 d	70.7 d	71.0 d	71.0 d	71.1 d
2′	39.1 t	39.2 t	37.2 t	37.0 t	36.9 t
3′	70.6 d	70.4 d	78.9 d	78.7 d	78.7 d
4′	88.6 d	88.2 d	75.9 d	75.8 d	75.8 d
5′	74.6 d	74.3 d	76.3 d	76.4 d	76.3 d
6′	17.5 q	17.3 q	17.9 q	17.9 q	17.9 q
1A	101.0 d	100.8 d	95.3 d	95.2 d	95.3 d
2A	36.5 t	36.4 t	23.9 t	23.8 t	23.8 t
3A	77.3 d	76.6 d	25.6 t	25.5 t	25.5 t
4A	75.2 d	74.9 d	66.4 d	66.3 d	66.4 d
5A	72.3 d	72.2 d	67.1 d	67.2 d	67.1 d
6A	17.4 q	17.4 q	16.6 q	16.7 q	16.6 q
1B	95.4 d	95.1 d	–	–	–
2B	23.9 t	23.9 t	–	–	–
3B	25.4 t	25.5 t	–	–	–
4B	66.4 d	66.3 d	–	–	–
5B	67.2 d	67.1 d	–	–	–
6B	16.7 q	16.5 q	–	–	–

δ in ppm, ^{13}C multiplicities from gHSQC and gHMBC experiments. n.o. = not observed.

as the olefinic AB system (δ 6.42 and 6.81), characteristic for the aquayamycin aglycone, and signals for three sugars. The C-glycosidically bound D-olivose (e.g. δ 4.94, d, $J = 10$ Hz, 1′-H; δ 4.01, m, 3′-H), and the two rhodinose moieties are identified by typical signals (e.g. δ 5.0 s and 5.19 s, 1A-H and 1B-H; δ 4.23 q and 4.38 q, 5A-H and 5B-H) in comparison with the known urdamycin O [6]. The data also reveal that urdamycin Q lacks the L-rhodinose at C-12-O, which occurs in urdamycin O, and which can easily be identified through its typical ^1H signals [6].

The spectroscopic data (NMR, MS), R_f values and HPLC retention times of all known urdamycin derivatives, such as 100-1 (found in *S. fradiae* A-x after expression of *lanGT4*), and of urdamycin N (found in *S. fradiae* A-x with twice *lanGT4*) were identical with those previously described.

3. Discussion

Many antibiotics have their aglycone moieties assembled first followed by decoration with sugar moieties to produce the final bioactive compound. In recent years several glycosyltransferase genes have been isolated from antibiotic-producing organisms. Among these glycosyltransferase genes are *lanGT1*, *lanGT2*, *lanGT3* and *lanGT4* of the landomycin cluster [13], *urdGT1a*, *urdGT1b*, *urdGT1c* and *urdGT2* of the urdamycin cluster [15,16], those for erythromycin [24,25], megalomicin [26], mithramycin [27,28], elloramycin [29] and oleandomycin biosynthesis [30], *graORF14* of the granaticin cluster [31,32] and *dnrS*/*dnmS* of the daunorubicin biosynthetic pathway [33,34]. Functions of some of these genes have been determined mostly by gene inactivation experiments [15,16, 24,25,33] and in a few cases by heterologous expression [26,29,35]. Based on sequence comparison of the deduced amino acid sequences of the landomycin glycosyltransferase genes with the closely related urdamycin glycosyltransferase genes, whose functions were known, some initial hypothesis could be raised. We expected LanGT1 and LanGT3 to be involved in the elongation of the hexasaccharide chain, LanGT2 to be responsible for the attachment of a D-olivose to the polyketide moiety, and LanGT4 to be a rhodinosyltransferase. Recently, the knowledge on glycosyltransferase genes and other sugar biosynthetic genes has been exploited for the production of novel glycosylated hybrid antibiotics. Systems constructed by different groups rely on feeding of aglycones to a modified host, which harbors suitable glycosyltransferase and sugar biosynthetic genes [36,37]. Liu and coworkers produced several new derivatives of methymycin/pikromycin by deleting endogenous sugar biosynthetic genes [7,38] and coexpression of the *calH* gene from the calicheamine pathway [39]. Two recent examples describe the coexpression of sugar and aglycone biosynthetic genes to generate novel glycosylated anthracyclines or macrolides, respectively [40,41].

Some flexibility of glycosyltransferases especially towards their sugar co-substrates has been described making these glycosyltransferases attractive tools for combinatorial biosynthesis. Only a low number of pharmaceutically important drugs are containing two, three or even more deoxysugars. The chemical synthesis of such compounds is very difficult to proceed and therefore impractical, and nearly impossible to be established in large scale. With glycosyltransferase genes of the landomycin and urdamycin clusters in hand we wanted to explore whether landomycin/urdamycin hybrids carrying modified sugar side chains could be generated by expressing glycosyltransferase gene cassettes.

In addition to mutant *S. fradiae* A-x, in which three of four urdamycin glycosyltransferases had been deleted, we generated a second host (mutant *S. fradiae* A-0) carrying no functional glycosyltransferase gene. Since both mutants are still capable to produce dTDP-D-olivose and dTDP-L-rhodinose, which are likely to serve as co-substrates of the landomycin glycosyltransferases, both hosts were therefore suitable for the heterologous expression of these genes.

New cloning vectors (pMUNI, pMUNII and pMUNIII) were generated based on the compatibility of the restriction sites *Eco*RI and *Mun*I. A major advantage of these vectors is that once a number of biosynthetic genes cloned in either pMUNI, pMUNII or pMUNIII are available, they can be rapidly combined to generate large gene cassettes. Glycosyltransferase genes of the urdamycin cluster were used to test this gene cassette approach. *S. fradiae* A-0 containing a plasmid with *urdGT1a*, *urdGT1b*, *urdGT1c* and *urdGT2* was capable to produce urdamycin A in the same amount as the wild-type supporting the use of the pMUN vector strategy for combinatorial biosynthesis.

Based on this system we tested the possibility of producing novel oligosaccharide antibiotics by expressing landomycin glycosyltransferase genes in mutants A-x and A-0. None of the glycosyltransferases was accepting products of mutant A-0 as substrate, which indicates a high specificity of the glycosyltransferase responsible for the attachment of the first D-olivose to the aglycone. In contrast, aquayamycin and urdamycinone B, the main products of mutant A-x, were accepted as substrates by both LanGT1 and LanGT4. Expression of these genes, alone or in combination, resulted in the accumulation of 12b-derhodinosyl-urdamycin G and other novel compounds, such as urdamycin Q, ladamycin B_2, ladamycins A_3 and D_3. Expression of *lanGT3* and *lanGT2* or coexpression with *lanGT1* and/or *lanGT4* in our hosts did not lead to the production of new compounds with longer saccharide chains. Either these enzymes are more specific towards their alcohol substrates than LanGT1 or LanGT4 and we were not able to provide a suitable substrate yet, or the export system of *S. fradiae* limits the secretion of compounds containing more than three sugars in the 9-side chain.

The expression of *lanGT1* alone or together with *lanGT4* resulted in the formation of novel hybrid compounds. These compounds contain two 1,4-connected D-olivose units, as known from the landomycin hexasaccharide. Thus, these compounds combine structural elements of both landomycins and urdamycins and were therefore designated ladamycins. The lower production level of ladamycin A_2 compared to ladamycin A_3 might be attributed to a slow glycosylation rate of aquayamycin by LanGT1, while the second glycosylation step, the attachment of the L-rhodinose, occurs faster. Although ladamycins A_2 and A_3 were only recognized through HPLC-MS, sufficient NMR data exist for ladamycins B_3 and D_3, the latter being a follow-up product of ladamycin A_3. These NMR

Table 7
HMBC correlations of ladamycin B_3, ladamycin D_3, 5-hydroxy-100-1, 12b-derhodinosyl-urdamycin G and lourdamycin

Position	Ladamycin B_3	Ladamycin D_3	5-Hydroxy-100-1	12b-Derhodinosyl-urdamycin G	Lourdamycin
2-H_{eq}	1, 2, 4	1, 3, 4, 12b	1, 3, 3-CH_3, 4, 12b	1, 3, 4	1, 3, 4
2-H_{ax}	1, 3	1, 3	1, 3, 3-CH_3	1, 3, 3-CH_3	1, 3
3-CH_3	2, 3, 4	1, 2, 3, 4	1, 2, 3, 4	1, 2, 3, 4	2, 3, 4
4-H_{eq}	2, 3, 4a, 5, 12b	–	2, 3, 3-CH_3, 4a, 5, 12b	2, 3, 3-CH_3, 4a	2, 3, 4a, 5
4-H_{ax}	3, 4a, 12b	–	3, 3-CH_3, 4a, 5, 12b	3, 3-CH_3, 4a	4a, 5
5-H	4, 6a, 12b	6a, 12b	–	6a, 12b	–
5-OH	–	–	–	–	4a, 5, 5a
6-H	4a, 7, 12a	4a, 7, 12a	4a, 5, 6a, 7, 12a	4a, 7, 12a	–
7-H	–	–	–	–	6, 9, 10a
8-H	–	–	–	–	1′, 6a, 10,
10-H	1′, 8, 11a	1′, 8, 9, 11a, 3″	1′, 8, 11a	1′, 8, 11a	–
10-OH	–	–	–	–	9, 10, 10a
11-H	7a, 9, 12	–	7a, 9, 12	7a, 9, 12	–
12-H	–	–	–	–	1, 4a, 5a, 11
5″-H	–	3″, 4″, 6a″, 10a″	–	–	–
7″-H	–	10a″, 9″	–	–	–
8″-H	–	6a″, 10″	–	–	–
9″-H	–	7″, 10a″	–	–	–
10″-H	–	6a″, 8″, 4″	–	–	–
1′-H	8, 9, 10	9	9, 10	8, 9, 10, 2′, 3′, 5′	8, 9
2′-H_{eq}	3′, 4′	3′, 4′	3′, 4′	3′, 4′	3′, 4′
2′-H_{ax}	–	–	9, 1′	9, 1′, 3′,	1′
3′-H	–	–	–	1A, 4′	–
4′-H	1A, 3′, 5′, 6′	1A, 3′, 5′, 6′	3′, 5′, 6′	3′, 5′, 6′	–
5′-H	–	–	–	–	–
6′-CH_3	3′, 4′, 5′	4′, 5′	3′, 5′	4′, 5′	3′, 4′, 5′
1A-H	4′	4′	3′, 3A, 5A	3′, 3A, 5A	5A
2A-H_{eq}	1A, 3A, 4A	1A, 3A, 4A	1A, 4A	–	–
2A-H_{ax}	1A, 3A	1A, 3A	–	–	–
3A-H_{eq}	–	–	–	–	–
3A-H_{ax}	–	–	–	1A	–
4A-H	3A	3A, 5A, 6A	4A, 6A	–	–
5A-H	–	–	–	1A, 4A, 6A	–
6A-CH_3	4A, 5A	4A, 5A	4A, 5A	4A, 5A	4A, 5A
1B-H	3A, 3B, 5B	3A, 5B	–	–	–
2B-H_{eq}	–	–	–	–	–
2B-H_{ax}	–	–	–	–	–
3B-H_{eq}	–	–	–	–	–
3B-H_{ax}	–	–	–	–	–
4B-H	–	–	–	–	–
5B-H	4B, 6B	4B, 6B	–	–	–
6B-CH_3	4B, 5B	4B, 5B	–	–	–

data (HMBC, ^{13}C chemical shifts) clearly reveal the 1,4-linkage of the two D-olivose building blocks.

From these results we conclude that LanGT1 is a D-olivosyltransferase, which links a second D-olivose to the 4-OH group of another D-olivose, whereas LanGT4 is an L-rhodinosyltransferase, which attaches an L-rhodinose to the 3-OH group of a D-olivose. These functions are consistent with the prediction from the sequence comparison of the landomycin with the urdamycin glycosyltransferases and their proposed involvement in the hexasaccharide biosynthesis of landomycin. Especially LanGT4 displayed relaxed substrate specificity and was able to glycosylate various alcohol substrates at reasonable rates, showing the potential of glycosyltransferases to design and create new oligosaccharide structures.

4. Significance

Glycosyltransfer is an important step in the biosynthesis of antibiotics and is often needed to confer biological activity to a compound. We used two glycosyltransferase gene mutant strains derived from the urdamycin producer *S. fradiae* Tü2717 as hosts to analyze the expression of glycosyltransferase genes from the landomycin biosynthetic gene cluster of *S. cyanogenus* S136. A versatile new cloning system was implemented to facilitate the construction of gene cassettes. The production of several novel metabolites at high efficiency proves again that glycosyltransferases are useful tools in combinatorial biosynthesis due to their substrate flexibility. As shown by the production of ladamycin B_3 this technique is not restricted

to the addition of single sugar moieties but can be used to design and generate larger deoxysugar moieties. Combined with the recently gained knowledge on deoxysugar biosynthetic genes, this will further extend the possibilities of combinatorial biosynthesis.

5. Materials and methods

5.1. Bacterial strains and culture conditions

S. fradiae Tü2717 was obtained from the Department of Microbiology, University of Tübingen [12], Germany, *S. fradiae* BF-1-1 was obtained from B. Faust, Pharmaceutical Biology, University of Tübingen, Germany [15]. Conditions of growth were as described [16]. *E. coli* XL1 blue MRF′ from Stratagene was used as host for plasmid propagation. Prior to transformation of *S. fradiae* plasmids were passed through *E. coli* ET12567 (*dam*-, *dcm*-, *hsds*-, *Cm*+) to generate demethylated DNA [42].

5.2. General genetic manipulation, DNA sequencing/sequence analysis and PCR

Routine methods were performed as described [43]. Transformation of *S. fradiae* strains was performed according to standard procedures [44]. Southern hybridization, sequencing and enzymatic manipulation of DNA were carried out according to manufacturer's directions (Amersham-Pharmacia Biotech, Roche Diagnostics, Stratagene, Promega). Oligonucleotide primers were purchased from Amersham-Pharmacia Biotech. PCR was performed using a GeneAmp PCR System 2400 (Applied Biosystems) with similar conditions as described [16]. For amplification of the different glycosyltransferase genes together with their own ribosomal binding site suitable cosmids or plasmids from the urdamycin or landomycin gene clusters were used as templates. Primer sequences are given in Table 3. The amplified fragments were cloned into pMUNII except for *lanGT2*, which was cloned into pMUNI. Nucleotide sequences were determined by the dideoxy chain-termination method using automatic laser fluorescence sequencers (Vistra 725, Amersham-Pharmacia Biotech). Sequencing reactions were done using a thermosequenase cycle sequencing kit with 7-deaza-dGTP (Amersham-Pharmacia Biotech) and standard primers (M13 universal and reverse, T3, T7). Computer-aided sequence analysis was done with the DNASIS software package (version 2.1, 1995; Hitachi Software Engineering), database searches were performed with the BLAST 2.0 program [45] on the server of the National Center for Biotechnology Information, Bethesda, MD, USA.

5.3. Construction of pMUN vectors

pBluescript SK− obtained from Stratagene was used for the construction of pMUNI. Introduction of new restriction sites into the multiple cloning site was performed by ligating double-stranded oligonucleotides into suitable cut plasmids (Table 4). Double-stranded oligonucleotides were obtained by annealing two complementary single-stranded oligonucleotides. For this purpose the two single-stranded oligonucleotides were denatured by heating (98°C, 10 min) and then gradually cooled down to 45°C (non-linear, 30 min). To obtain an overhang for ligating to the appropriately cut plasmid, the single-stranded oligonucleotides were extended at the 5′-end. The sequence of new plasmids was confirmed by sequencing. For the construction of pMUNI restriction sites *Bam*HI, *Sma*I, *Pst*I, *Eco*RI, *Eco*RV and *Hin*dIII in pBluescript were replaced by *Bam*HI, *Nsi*I, *Eco*RI, *Eco*RV, *Mun*I and *Hin*dIII. For the generation of pMUNII the *Bam*HI, *Nsi*I, *Eco*RI, *Eco*RV and *Mun*I sites of pMUNI were exchanged for *Eco*RI, *Bgl*II, *Nde*I, *Nsi*I, *Nco*I and *Mun*I while deleting the *Bam*HI site. pMUNIII is similar to pMUNII but retains the *Bam*HI site from pMUNI (Table 1).

5.4. Plasmids

The bifunctional plasmid pUWL201 [17] was a kind gift of U. Wehmeier and W. Piepersberg, University of Wuppertal, Germany, and used for expression of single genes and gene cassettes in *S. fradiae*. The construction of the gene inactivation plasmid pKC-12-B2 and of plasmids pMUNurdGT1a, pMUNurdGT1b and pMUNurdGT1c has been described [16].

5.5. Production, purification, structure elucidation and chemical analysis of urdamycins and novel hybrid compounds

Production, purification, TLC conditions, and HPLC-UV/Vis conditions for urdamycin derivatives were essentially as described previously [16]. For HPLC-electrospray ionization (ESI)-MS analysis separation was performed on an HP1110 (Hewlett-Packard) with a HP-ODS-Hypersil (100×2.1 mm; 5 μm) at a flow rate of 0.1 ml/min, detection at 250 nm and the following gradient: 0–5 min 20% B, 5–50 min linear to 55% B, 50–60 min linear to 90% B (solvent A: 90% H_2O, 9.95% acetonitrile, 0.05% acetic acid; solvent B: 90% acetonitrile, 9.95% H_2O, 0.05% acetic acid). Mass spectra were recorded on a Bruker Esquire LC 1.6n mass spectrometer (Bruker Daltronik) equipped with an electrospray ion source (positive ion mode) (ESI) at a scan range of 50–1200 *m/z* with nominal mass resolution.

The structures of ladamycins B_2, B_3, D_3, 5-hydroxy-100-1, urdamycin Q and the rearranged lourdamycin were identified mainly by NMR spectroscopy, all these structures were also supported by MS. The instrumentation used was essentially the same as described previously [6,46], except the Varian NMR instrument, which was recently upgraded into an INOVA 400. For details, see text and Tables 5–7. The FAB mass spectra and the high resolutions were recorded at the University of South Carolina Department of Biochemistry and Chemistry facilities in Columbia, SC, USA, using a VG70SQ double focussing magnetic sector MS instrument. The APCI mass spectra were recorded at the Medical University of South Carolina regional mass spectroscopy center using a Finnigan MAT LCQ.

The structures of ladamycins A_2 and A_3 were determined by HPLC-MS (see text).

The known urdamycins mentioned here were identified by comparison with authentic samples using TLC, HPLC-UV/Vis, HPLC-MS and ^1H NMR.

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