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Research Paper

Two sequence elements of glycosyltransferases involved in urdamycin biosynthesis are responsible for substrate specificity and enzymatic activity

Dirk Hoffmeister [a], Koji Ichinose [b], Andreas Bechthold [a,*]

[a] *Albert-Ludwigs-Universität Freiburg, Pharmazeutische Biologie, Stefan-Meier-Strasse 19, D-79104 Freiburg, Germany*
[b] *The University of Tokyo, Graduate School of Pharmaceutical Sciences, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

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Abstract

Background: Two deoxysugar glycosyltransferases (GTs), UrdGT1b and UrdGT1c, involved in urdamycin biosynthesis share 91% identical amino acids. However, the two GTs show different specificities for both nucleotide sugar and acceptor substrate. Generally, it is proposed that GTs are two-domain proteins with a nucleotide binding domain and an acceptor substrate site with the catalytic center in an interface cleft between these domains. Our work aimed at finding out the region responsible for determination of substrate specificities of these two urdamycin GTs.

Results: A series of 10 chimeric GT genes were constructed consisting of differently sized and positioned portions of *urdGT1b* and *urdGT1c*. Gene expression experiments in host strains *Streptomyces fradiae* Ax and XTC show that nine of 10 chimeric GTs are still functional, with either UrdGT1b- or UrdGT1c-like activity. A 31 amino acid region (aa 52–82) located close to the N-terminus of these enzymes, which differs in 18 residues, was identified to control both sugar donor and acceptor substrate specificity. Only one chimeric gene product of the 10 was not functional. Targeted stepwise alterations of glycine 226 (G226R, G226S, G226SR) were made to reintroduce residues conserved among streptomycete GTs. Alterations G226S and G226R restored a weak activity, whereas G226SR showed an activity comparable with other functional chimeras.

Conclusions: A nucleotide sugar binding motif is present in the C-terminal moiety of UrdGT1b and UrdGT1c from *S. fradiae*. We could demonstrate that it is an N-terminal section that determines specificity for the nucleotide sugar and also the acceptor substrate. This finding directs the way towards engineering this class of streptomycete enzymes for antibiotic derivatization applications. Amino acids 226 and 227, located outside the putative substrate binding site, might be part of a larger protein structure, perhaps a solvent channel to the catalytic center. Therefore, they could play a role in substrate accessibility to it.

Keywords: Chimera; Glycosyltransferase; *Streptomyces*; D-Olivose; L-Rhodinose

1. Introduction

The biosynthesis of polysaccharides, complex carbohydrates and other glycosylated cellular metabolites is of major importance, since these molecules are directly involved in numerous biological and cellular processes [1]. Glycobiology ranges from storage of photosynthetic products, cell–cell recognition and adhesion as well as glycosylation of proteins. Vaccinations and pathogenicity are also glycosylation-based processes. Within the pharmaceutical sciences, glycosylated secondary metabolites and especially polyketides have attracted attention since they exhibit potent antibacterial activity. The biosynthesis of oligo- and polysaccharides is catalyzed by glycosyltransferases (GTs), which transfer sugar residues from a donor substrate, usually a nucleotide sugar, to an aglycone or a growing carbohydrate chain. Donor and acceptor substrate specificities are the key to the structural diversity of saccharides produced by a cell [2,3].

So far, a large number of GTs from both prokaryotic and eukaryotic sources has been characterized, reflecting

* Correspondence: Andreas Bechthold;
E-mail: andreas.bechthold@uni-freiburg.de

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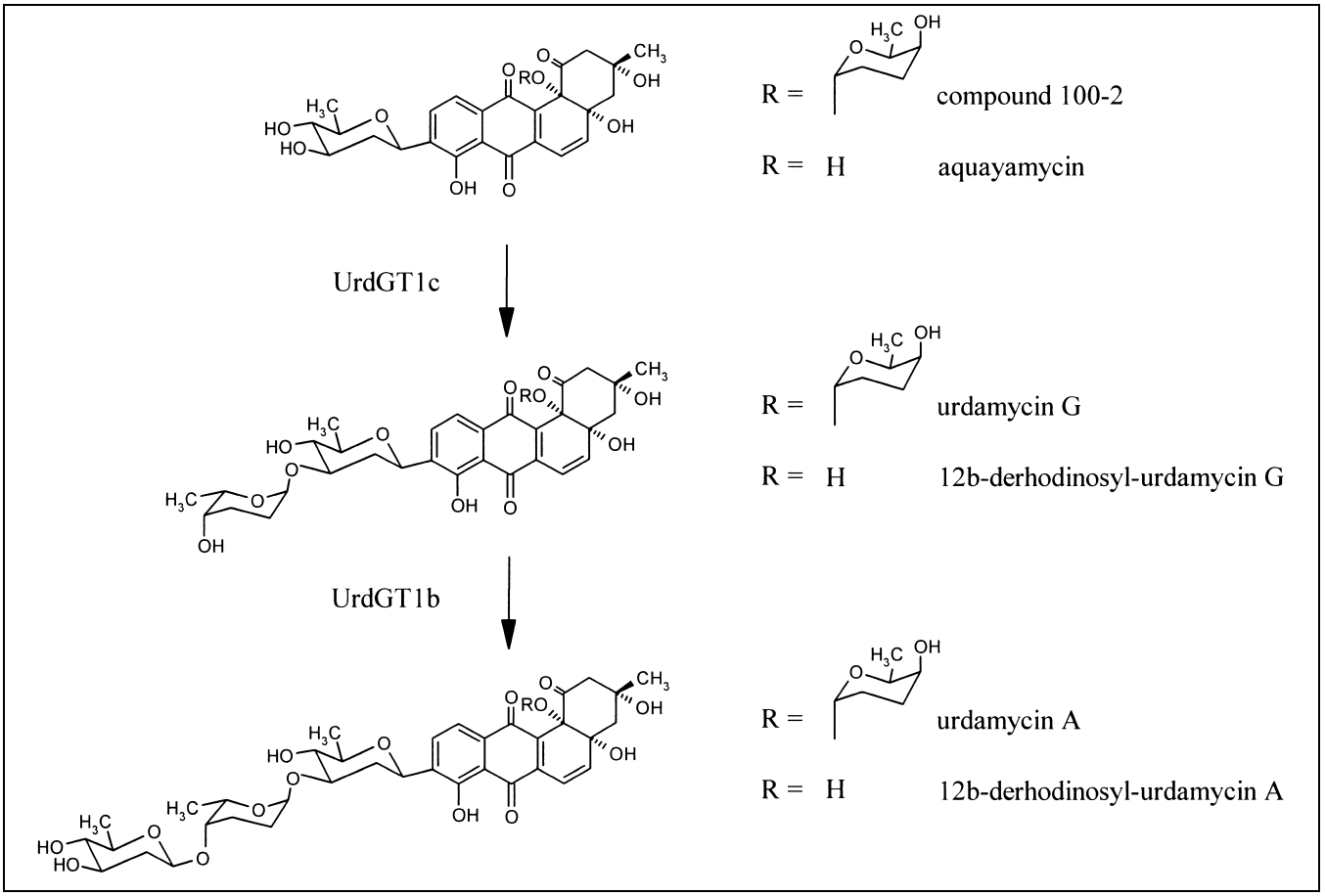

Fig. 1. Urdamycin A and its precursors and derivatives. Arrows indicate the biosynthetic steps catalyzed by the GTs UrdGT1b and UrdGT1c.

the wide range of substrates. Mechanistically, GTs can be categorized into two main subgroups, inverting and retaining enzymes, since they form glycosidic bonds by either inverting or retaining the anomeric configuration. Sequence comparison shows that GTs are divergent in their primary structure, but frequently share common motifs [3]. Based on these findings a sophisticated approach for classification is the CAZy system, by now expanded to 51 GT families [4]. Following this classification, the enzymes UrdGT1b and UrdGT1c described in this publication, along with other GTs coded in streptomycete natural product biosynthetic gene clusters, belong to CAZy family 1. It contains inverting GTs also of viral, bacterial, plant or fungal origin as well as invertebrate and vertebrate GTs including mammalian and human glucuronosyltransferase families. Widespread among many families is the so-called DXD motif [5–11] of two aspartic acid residues, flanked N-terminally by apolar residues. A common nucleotide binding motif is a sequence covering about 40 amino acids with some highly conserved residues [12–14]. In the context of streptomycete antibiotic biosynthesis, CAZy family 1 streptomycete enzymes have been characterized [15–23], among them UrdGT1b and UrdGT1c of the urdamycin biosynthetic gene cluster. Urdamycin A (Fig. 1), produced by *Streptomyces fradiae* Tü2717, is an angucycline-type antibiotic and bears a trisaccharide unit (D-olivose-L-rhodinose-D-olivose) attached C-glycosidically to the polyketide core at C9, and an O-glycosidically appended single L-rhodinose at C12b [24]. Four GT genes, *urdGT1a*, *urdGT1b*, *urdGT1c* (GenBank accession number AF164961), and *urdGT2* (GenBank accession number AF164960), have been detected within the urdamycin biosynthetic gene cluster, and a specific function during urdamycin biosynthesis could be assigned to each gene product [21,22]. UrdGT1a and UrdGT2 are involved in the formation of compound 100-2 (Fig. 1), and UrdGT1c transfers dNDP-L-rhodinose to compound 100-2 forming an α-(1,3)-glycosidic bond. UrdGT1b completes urdamycin A biosynthesis by attaching a dNDP-D-olivose to urdamycin G (Fig. 1) via a β-(1,4)-glycosidic bond. Both enzymes follow an inverting mechanism. It has been demonstrated that UrdGT1c and UrdGT1b both accept intermediates as substrates which lack the L-rhodinose at position 12b: aquayamycin (Fig. 1) or urdamycinone B [25] in the case of UrdGT1c, and 12b-derhodinosyl-urdamycin G (Fig. 1) or compound 100-1 [25] in the case of UrdGT1b. Interestingly, despite different functions and substrate specificities UrdGT1b and UrdGT1c are strikingly highly homolo-

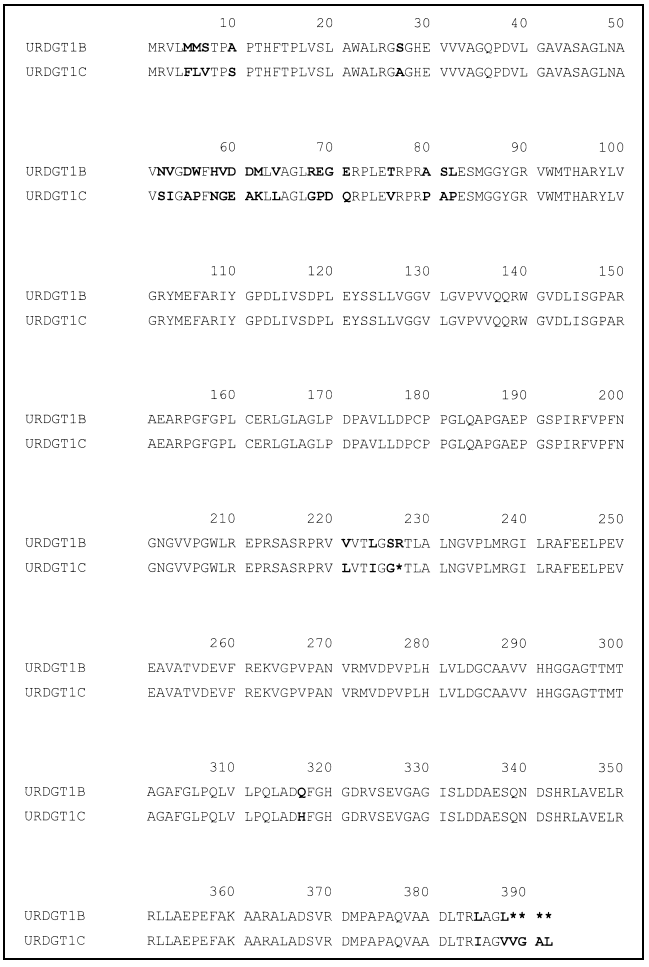

Fig. 2. Comparison of amino acid sequences of the GTs UrdGT1b (upper line) and UrdGT1c (lower line). Non-identical amino acids are shown in bold, asterisks indicate lacking amino acids. Region I: aa 1–31; region IIa: aa 32–83; region IIb: aa 84–184; region III: aa 185–240 in UrdGT1b and aa 185–239 in UrdGT1c; region IV: aa 241–388 in UrdGT1b and aa 240–391 in UrdGT1c.

gous, with 91% identity at the amino acid level. Consequently, we harnessed this enzyme pair UrdGT1b/UrdGT1c to locate and confine regions that determine the specificities of these enzymes. Recently, we described the generation of a mutant of *S. fradiae* lacking both *urdGT1b* and *urdGT1c* but containing instead on its chromosome a chimeric gene consisting of 715 nucleotides from *urdGT1b* and 458 nucleotides from *urdGT1c*. The gene product, UrdGT1b-1c, in this contribution referred to as chimera 6, which differs from UrdGT1b in six amino acids in the C-terminal moiety, was active and catalyzed the same reaction as UrdGT1b [22]. We now describe the construction of a set of chimeric GT genes. To test the catalytic activities of the functional gene products these genes were expressed in different GT mutants of *S. fradiae* Tü2717. We identified a constricted region of 31 aa within UrdGT1b and UrdGT1c (aa 52–82, differing in 18 positions) which controls the substrate specificity of both enzymes.

2. Results and discussion

2.1. Sequence comparison of UrdGT1b and UrdGT1c, and construction of chimeric genes

The urdamycin biosynthetic gene cluster has been cloned and sequenced, and numerous genes have been identified [21,22,26,27]. To determine the function of four GT genes, inactivation and expression experiments were performed previously [22]. Sequence comparison of UrdGT1b (dNDP-L-rhodinose:urdamycin G glycosyltransferase) and UrdGT1c (dNDP-D-olivose:100-2 glycosyltransferase) revealed that both enzymes share 91% identical amino acids (Fig. 2). To determine which region of these two GTs would govern specificity we constructed a set of chimeric GT genes in which differently sized and positioned elements of one parental gene (*urdGT1c*) had been replaced by the equivalent of the second parental gene (*urdGT1b*) or vice versa. The regions to be swapped among the two genes are defined by restriction sites common to both genes (*Pfl*MI, nucleotides 84–94; *Pst*I, nucleotides 547–552; *Bsm*I, nucleotides 716–721 in *urdGT1b* and 713–718 in *urdGT1c*) thus making it possible to clone sequence regions I (nucleotides 1–94, amino acids 1–31), II (nucleotides 95–552, amino acids 32–184), III (nucleotides 553–721 in *urdGT1b* and 553–718 in *urdGT1c*, amino acids 185–240 in UrdGT1b and 185–239 in UrdGT1c) and IV (nucleotides 722–1167 in *urdGT1b* and 719–1176 in *urdGT1c*, amino acids 241–388 in UrdGT1b and 240–391 in UrdGT1c). Region II was subdivided into region IIa (nucleotides 95–249, amino acids 32–83) with 18 out of 50 amino acids different, and region IIb (nucleotides 250–552, amino acids 84–184) with perfect amino acid identity. Ten chimeric genes were constructed representing different combinations of regions of *urdGT1b* and *urdGT1c* (Fig. 3). Additionally, one of the chimeras (chimera 4) was further edited by three different pinpoint mutations leading to targeted amino acid substitutions.

2.2. Generation of a suitable host for testing for UrdGT1b-like activity

UrdGT1c-like activity of chimeric GTs was tested by expressing each individual chimeric gene in mutant Ax [22]. This mutant, deficient for *urdGT1a*, *urdGT1b* and *urdGT1c*, predominantly accumulates aquayamycin and, less prominently, urdamycinone B. The latter is converted to compound 100-1, and aquayamycin to 12b-derhodinosyl-urdamycin G, when *urdGT1c* is expressed in this mutant. To test UrdGT1b-like activity, we generated mutant XTC. This mutant lacks *urdGT1a* and *urdGT1b* but contains *urdGT1c* under the control of the *ermE* promoter integrated back into the chromosome of mutant Ax. Expected integration and gene expression were confirmed by the production of mainly 12b-derhodinosyl-urdamycin G. In lower amounts, the previously described urdamycin N

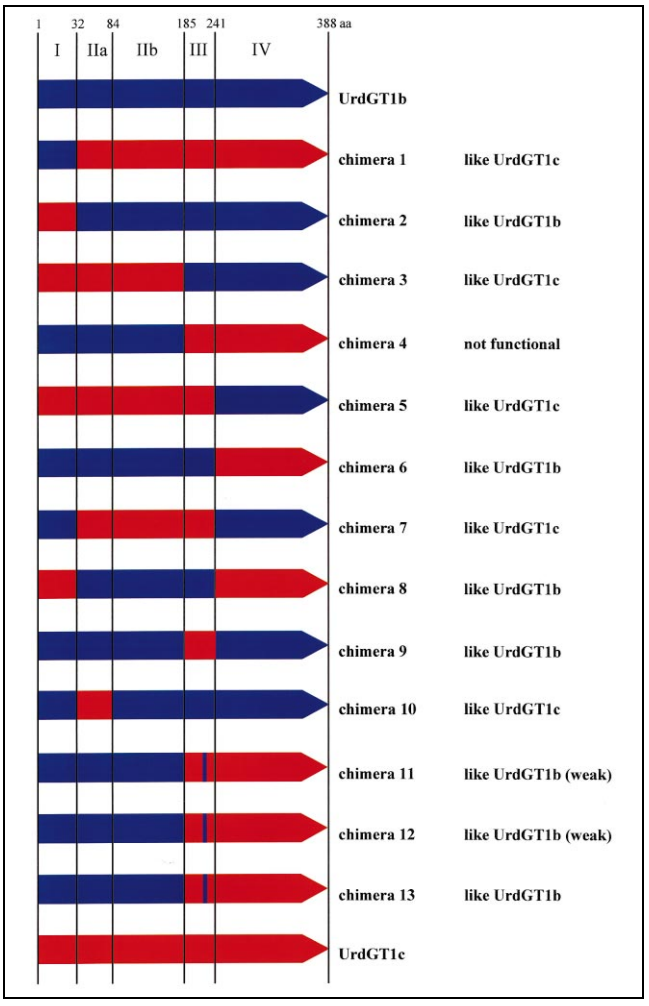

Fig. 3. Comparison of chimeric and wild-type GTs constructed during this study. Protein regions are indicated by roman numerals, vertical lines represent the borders, numbers on top indicate the corresponding amino acid residues referred to UrdGT1b wild-type sequence.

was also present [27]. When the wild-type gene *urdGT1b* was expressed in mutant XTC 12b-derhodinosyl-urdamycin A was the major product (Fig. 1, HPLC profiles shown in Fig. 4).

2.3. *Expression of the chimeric genes in mutant Ax: conversion of UrdGT1b into UrdGT1c*

Mutant Ax was transformed with chimeras 1–5 and 7–10. No conversion of aquayamycin and urdamycinone B was observed when chimeras 2, 4, 8 and 9 were expressed indicating that products of these chimeric genes either acted like UrdGT1b or were not active at all. In contrast, expression of chimeras 1, 3, 5, 7 and 10 led to the production of 12b-derhodinosyl-urdamycin G and compound 100-1 indicating that they were functional like UrdGT1c (Fig. 4). These results revealed that changes in regions I, III and IV did not affect substrate specificity of UrdGT1c. When regions I and II of UrdGT1b were simultaneously replaced by their equivalents from UrdGT1c (chimera 3), UrdGT1b activity switched over to UrdGT1c activity. The same observation was made with chimera 7, where regions II and III of UrdGT1b were replaced by those of UrdGT1c. In addition, chimera 10, which is UrdGT1b engineered such that it carries merely region IIa of UrdGT1c origin, shows UrdGT1c-like activity as well. These findings demonstrate that region IIa located within the N-terminal region of the enzyme governs both nucleotide sugar and acceptor substrate specificity in UrdGT1c, and that functionality is maintained regardless of where the other regions originate from. However, a putative nucleotide binding motif was found to reside within the C-terminal region of UrdGT1b and UrdGT1c. This motif is found among human UDP-glucuronosyltransferases, with a smaller degree of conservation it is also present in the flavonol *O*(3)-glucosyltransferase from *Zea mays*, *Streptomyces lividans* macrolide GT, other CAZy family 1 members and other families [12,13]. Data from the crystal structure of MurG, an *Escherichia coli* peptidoglycan GT of CAZy family 28, confirm this motif to be involved in nucleotide binding [14]. Other crystallographic studies have been published on phage T4 β-glucosyltransferase [28,29], SpsA from *Bacillus subtilis* [30], the bovine β-1,4-galactosyltransferase T1 [31], and the rabbit *N*-acetylglucosaminyltransferase I, [9], as well as the structure of amylomaltase from *Thermus aquaticus* [32] and an engineered cyclodextrin glucanotransferase from *Bacillus* sp. [33], to reveal structure–mechanism relationships and also confirmed the notion of the two-domain character of these enzymes.

For the T4 β-glucosyltransferase and the topologically identical and structurally similar enzyme MurG of *E. coli*, the sugar within the enzyme could not be detected but its position was modelled. MurG binds the UDP-*N*-acetylglucosamine into a pocket in the C-domain, and for some conserved residues a possible role was suggested. It was predicted, for example, that glutamine 289 of MurG is positioned in hydrogen bonding distance and interacts with the hydroxyl group at C4 of the sugar, which could be the reason why UDP-glucosamine is accepted as a substrate, but not the isomeric UDP-galactosamine [14]. Analogously, we speculate that in the case of UrdGT1c amino acids of region IIa serve as possible sterical sensors towards the donor sugar substrate, which would require an interaction of the N- and C-terminal moieties with the nucleotide-bound sugar before catalysis is initiated. Based on the modelling of the sugar binding site of T4 β-glucosyltransferase, the glucose resides in a pocket, surrounded by both nucleotide moiety and amino acid residues of both domains [29]. Additional support for our assumption of an N-terminal domain interacting with the sugar is lent by earlier studies on the human UDP-glucuronosyltransferase 2B4. It was demonstrated that the nucleotide diphosphate binds at the C-terminal moiety, whereas the sugar interacts with the amino-terminal part of the enzyme [13,34]. Another motif which is highly conserved among many

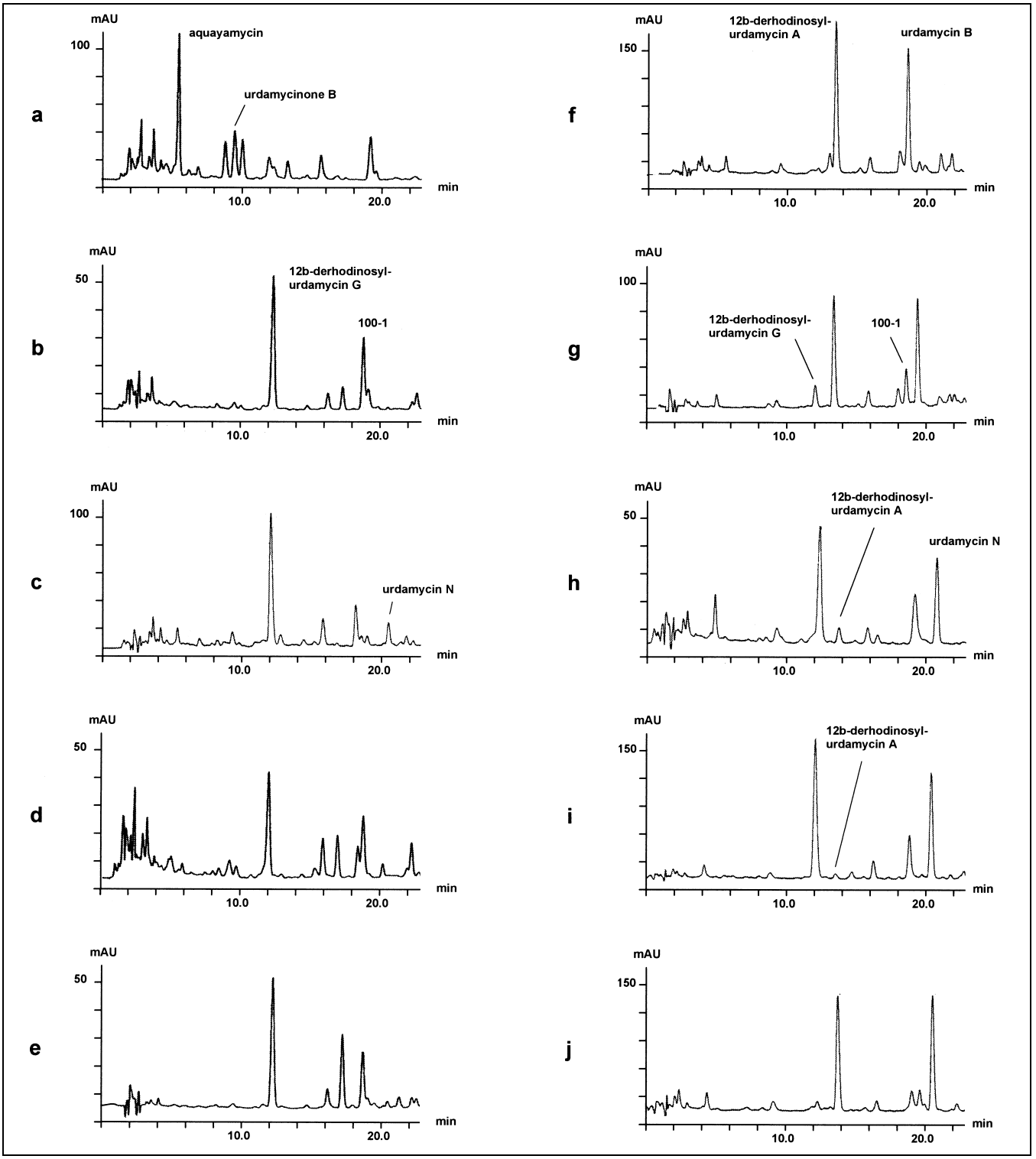

Fig. 4. Selected HPLC chromatograms. (a) *S. fradiae* strain Ax. (b) Ax expressing *urdGT1c*. (c) *S. fradiae* strain XTC. (d) Ax expressing chimera 7. Identical profiles were obtained with chimeras 1, 3, 5, and 10. (e) XTC expressing non-functional chimera 4. (f) XTC expressing *urdGT1b*. (g) XTC expressing chimera 2. Identical profiles were obtained with chimeras 8 and 9. (h) XTC expressing chimera 11. (i) XTC expressing chimera 12. (j) XTC expressing chimera 13. Except urdamycin N [27] no shunt or follow-up products are indicated.

GTs is the DXD motif. The consensus is described as hhhhDxDxh, with 'h' denoting a hydrophobic residue. It has been shown that the first aspartic acid is necessary to bind the donor sugar, the second highly conserved aspartic acid participates in coordination of a divalent metal cation, necessary to counter the negative charge of the β-phosphate [9–11]. In UrdGT1b and UrdGT1c this motif is present as LxD (^{165}GLAGLPDPA^{173}) which is consis-

tent with the finding that the first residue of DXD may vary, whereas the second is strongly conserved. However, for ExoM, involved in exopolysaccharide synthesis of *Sinorhizobium meliloti*, which also possesses the DXD motif, it was shown in vitro that only the first, but not the second aspartic acid is crucial for enzymatic activity [8].

2.4. Expression of the chimeric genes in mutant XTC: conversion of UrdGT1c into UrdGT1b

Chimeras 1–5 and 7–10 were also used to transform mutant XTC to screen for UrdGT1b-like activity. Expression of chimeras 1, 3, 5, 7, and 10 did not alter the spectrum of urdamycin-type metabolites in the host strain. We anticipated this finding as the products of these chimeric genes have been shown to act like UrdGT1c (see above). However, 12b-derhodinosyl-urdamycin A was the major compound produced by mutant XTC expressing either chimera 2, 8, or 9 (Fig. 4). This indicates that these gene products possess UrdGT1b-like features. The product of chimera 4 did not show any UrdGT1b-like activity (Fig. 4) nor did it act like UrdGT1c. Thus we had to assume that this particular enzyme is not functional. In contrast, chimera 9, which is very similar to chimera 4 but contains region IV of UrdGT1b instead of UrdGT1c, was functional and acted as UrdGT1b. The UrdGT1b-like activity of chimera 6 has been reported earlier [22]. These results demonstrate that region IIa also determines substrate specificity for nucleotide sugar and acceptor in the case of UrdGT1b. In contrast to UrdGT1c-like enzymes, UrdGT1b-like enzymes additionally need region III (chimera 8) or IV (chimera 9) of UrdGT1b origin for enzymatic functionality. Therefore, UrdGT1b seems to be less tolerant of sequence alterations than UrdGT1c.

After having shown the individual specificity of each chimeric GT we were interested to determine their catalytic activity compared with the wild-type parental enzymes. HPLC profiles of the metabolites allowed us to estimate whether acceptor substrates (aquayamycin and urdamycinone B for UrdGT1c, 12b-derhodinosyl-urdamycin G and compound 100-1 for UrdGT1b) would disappear due to quantitative enzymatic conversion. We found that the wild-type enzyme UrdGT1c and all chimeras specific like UrdGT1c quantitatively converted aquayamycin and urdamycinone B to 12b-derhodinosyl-urdamycin G and compound 100-1, respectively (Fig. 4). In contrast, none of the UrdGT1b-like chimeras was able to totally convert its substrates into 12b-derhodinosyl-urdamycin A and urdamycin B whereas the wild-type enzyme UrdGT1b was (Fig. 4). Again, UrdGT1b appears to be more sensitive to engineering than UrdGT1c.

2.5. Conversion of the non-functional chimera 4 into functional chimeras 10, 11 and 12

The gene product of chimera 4 acted neither like UrdGT1b nor like UrdGT1c indicating that it was not functional, whereas the product of chimera 6 showed UrdGT1b-like activity. Both gene products differ in three amino acid positions of region III: leucine versus valine (aa 221); isoleucine versus leucine (aa 224); glycine versus serine-arginine (aa 226 and 226–227). To explore the possibility of refunctionalization, we edited chimera 4 in three different ways such that the codon for glycine was replaced by codons for serine, arginine, or both, resulting in chimera 11 (G226S), chimera 12 (G226R) and chimera 13 (G226SR). We focused on these particular amino acids since numerous deoxysugar GTs possess, following a conserved glycine, at least either a serine (rarely a threonine) or an arginine or even both amino acids in the positions occupied by a second glycine in UrdGT1c or by serine and arginine in UrdGT1b. Among these GTs are LanGT1, LanGT2 and LanGT3 of the landomycin cluster [35], those for erythromycin [36,37], megalomicin [38], mithramycin [39,40] and oleandomycin biosynthesis [41], GraOrf14 of the granaticin cluster [42] and DnrS/DnmS of the daunorubicin biosynthetic pathway [15,43]. When expressed in XTC, the product of chimeras 11 and 12 showed a weak UrdGT1b-like activity yielding low amounts of 12b-derhodinosyl-urdamycin A (Fig. 4). It was present at a maximum 1:9 ratio of product to acceptor substrate (12b-derhodinosyl-urdamycin G). However, when chimera 13 was expressed in XTC, we found that refunctionalization was fully accomplished (Fig. 4). Chimera 13 is highly and comparably active like the UrdGT1b-type chimeras 2, 6, 8, and 9. Obviously, the region in which the edited amino acid positions are located is crucial, not for specificity itself, but for activity in concert with region IIa. For full olivosyltransferase activity both amino acids in question, serine and arginine, are required. Conversely, chimera 9 acts fully like UrdGT1b without these amino acids but having instead a glycine in position 226. In addition to the importance of region IIa mentioned earlier, a possible role of region III of UrdGT1b and UrdGT1c, particularly of the serine and arginine residues, can be explained assuming a larger internal structure, e.g. a solvent channel. In the rabbit *N*-acetylglucosaminyltransferase I [9] and T4 β-glucosyltransferase [28,29] amino acids building up a channel structure are known. Such a channel might have been disturbed or completely destroyed in chimera 4. An extra hydroxyl group of D-olivose, compared with L-rhodinose, could require hydrogen bonding, or coordination of water molecules to fit the binding site appropriately. Arginine 227 may be crucial for a conformational change upon substrate binding to initiate interdomain salt bridges which cannot be set up adequately under the combination of regions III and IV while IIa is of different origin. The conformational conversion of a GT has been studied in detail, e.g. for T4 β-glucosyltransferase [28]: three arginine residues were shown to be involved in hydrogen bonding of nucleotide phosphate groups, with two of them also

implicated in interdomain salt bridges. Thus, substrate binding and conformational change are synchronized [29].

The region swapping technique was previously shown to be successful for similar enzyme pairs to assign features to a restricted enzymatic region. This includes e.g. locating functional domains in terpene cyclases [44] or triterpene synthases of plant origin [45], xylanases from prokaryotic sources [46] as well as identifying a dimerization domain in dehalogenases [47] and substrate specificity conferring domains in dioxygenases for degradation of aromatic substances [48]. Furthermore, with this approach enzyme properties such as thermostability, pH optima or chaperone-like activity [49–51] were enhanced after region swapping experiments. In the field of modularly organized enzymes, this technique was used e.g. to create artificial non-ribosomal peptide synthetases [52]. In our work we applied the region swapping strategy to *Streptomyces* genes involved in antibiotic biosynthesis. It was demonstrated that only one region (IIa) with 18 amino acids variable among UrdGT1b and UrdGT1c is responsible for enzymatic specificity. A second region (III) has been identified as crucial for activity. Thus, future pinpoint mutation work in this system UrdGT1b/UrdGT1c, especially within region IIa, should shed light on the question which amino acids act as determinants or sensors for substrate specificity. For human GTs involved in formation of the histo-blood group ABO(H) antigens it has already been shown that one single amino acid decides donor sugar specificity [53]. With nine of 10 active, the ratio of functional chimeras was very high, and each one showed either one or the other parental specificity. When it switched over, we always observed an all-or-nothing exchange. None of the chimeras possesses a dual or relaxed specificity. The only non-functional chimera (chimera 4) resumed working after slightly editing its primary sequence. GTs are important tools in combinatorial biosynthesis approaches since deoxysugars frequently play a crucial role for bioactivity of natural products, such as antibiotics and anticancer agents. Several cases of GTs with bifunctional or relaxed activity, although involved in very specific glycosylation processes, have been described [27,54,55]. Therefore, design of GTs with an altered or broader specificity seems to be a feasible task to generate a library of molecules with greater structural diversity, leading to the development of new drugs.

3. Significance

Glycosyl transfer is a cellular process of major importance. A combined crystallographic and computational approach identified GTs as two-domain enzymes with an acceptor substrate binding site and a nucleotide sugar binding motif. The present work aimed to further explore the genetic basis of urdamycin production in *S. fradiae* Tü2717. Two highly homologous GT genes, yet encoding enzymes of different specificities, were used as source genes to generate and express a series of chimeric GT genes. We could demonstrate that it is an N-terminally located region of 31 amino acids that confers both acceptor substrate and donor nucleotide sugar specificity. This finding provides novel opportunities for future engineering of GTs as tailoring enzymes to eventually generate novel pharmacologically active metabolites in streptomycete hosts. In addition, a serine-arginine motif (^{226}SR^{227} in UrdGT1b) conserved among streptomycete GTs was identified as the residues responsible for D-olivose transfer. The only non-functional chimera, in which this motif was obscured, regained olivosyltransferase activity by engineering either Ser or Arg into the primary sequence. Full activity was restored when both amino acids were introduced.

4. Materials and methods

4.1. Strains, growth conditions, media and vectors

For standard purposes, *S. fradiae* Tü2717 (wild-type) and mutant strains Ax and XTC were grown on 1% malt extract, 0.4% yeast extract, 0.4% glucose and 1 mM $CaCl_2$, pH adjusted to 7.2 (HA medium), prepared as solid or liquid medium, at 28°C. For maintenance of mutant XTC, apramycin was added to a final concentration of 25 μg/ml. For urdamycin production NL 111V liquid medium (20 ml in a single baffled 100-ml Erlenmeyer flask) was used. Incubation was done at 28°C and 160 rpm for 5–6 days. DNA manipulation was carried out in *E. coli* XL-1 Blue MRF′ (Stratagene). Before transforming *S. fradiae* mutants Ax and XTC plasmids were propagated in *E. coli* ET 12567 (*dam*$^-$, *dcm*$^-$, *hsd*S, Cm^R) [56] to obtain unmethylated DNA. *E. coli* strains were grown on Luria–Bertani (LB) agar or liquid medium containing the appropriate antibiotic for selection. Vector pBluescript SK$^-$ (pBSK$^-$) was from Stratagene, pUC19 was from New England Biolabs. For expression of chimeric GT genes, vector pUWL201 (courtesy of Prof. W. Piepersberg and Dr. U. Wehmeyer, University of Wuppertal, Germany) conferring thiostrepton and ampicillin resistance was employed. For the gene integration procedure to generate mutant XTC, pSET152 was used, which confers resistance to apramycin [57].

4.2. General techniques, DNA sequencing and genetic manipulation of Streptomyces

Standard molecular biology procedures were performed as described [58]. Isolation of plasmid DNA from *E. coli* and DNA restriction/ligation were performed following the protocols of the manufacturers of kits, enzymes, and reagents (Amersham Pharmacia, Macherey-Nagel, New England Biolabs, Promega). *Streptomyces* protoplast preparation, transformation, and protoplast regeneration were performed as described [59]. Nucleotide sequences of the chimeric genes were determined by the dideoxy chain termination method using an automatic laser fluorescence

DNA sequencer (Molecular Dynamics Vistra 725). Sequencing reactions were done using a thermosequenase cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia) and standard primers (M13 universal and reverse, T3, T7). PCR reactions were done on a Perkin Elmer GeneAmp 2400 thermal cycler.

4.3. Construction of S. fradiae XTC mutant

pUWLurdGT1c [22] was subjected to *Kpn*I/*Xba*I restriction digestion to obtain a 1.6-kb fragment that covers the complete reading frame *urdGT1c* as well as the *ermE* promoter sequence. This fragment was inserted in the multiple cloning site of pUC19 cleaved with the same enzymes. This plasmid was named pUC19-1cerm. Then, *urdGT1c* and *ermE* promoters were excised by *Eco*RI restriction and ligated into pSET152 to create gene integration plasmid pSET-1cerm. It was deployed to transform protoplasts of *S. fradiae* Ax mutant. The transformation procedure resulted in about 30 apramycin-resistant colonies per μg DNA. One clone, now referred to as mutant XTC, was chosen for further experiments.

4.4. Construction of chimeric GT genes

Plasmids pUWLurdGT1b and pUWLurdGT1c are pUWL201-based constructs containing the GT genes *urdGT1b* and *urdGT1c*, respectively, and have been described [22]. After cloning in pBSK$^-$, all chimeric genes mentioned below were sequenced to confirm the intended construction.

4.5. Construction of chimera 1 and chimera 2

Plasmids pUWLurdGT1b (pUWLurdGT1c) each possess two restriction sites for *Pfl*MI. Restriction of these plasmids with this enzyme resulted in 1.1- and 6.7-kb fragments. Along vector DNA the 1.1-kb fragment represents the first 31 codons of the GT gene *urdGT1b* (*urdGT1c*), whereas codons 32–388 of *urdGT1b* (32–391 of *urdGT1c*) are located on the 6.7-kb fragment. The 1.1-kb *Pfl*MI fragment of pUWLurdGT1b (pUWLurdGT1c) was ligated to the 6.7-kb *Pfl*MI fragment of pUWLurdGT1c (pUWLurdGT1b) to generate chimera 1 (chimera 2). The *Hin*dIII/*Xba*I fragment containing the chimeric gene was ligated into pBSK$^-$ to generate chimera 1b (chimera 2b).

4.6. Construction of chimera 3 and chimera 4

pUWLurdGT1b (pUWLurdGT1c) was co-restricted by *Pst*I/*Bgl*II to create a 0.65-kb *Pst*I-*Bgl*II fragment comprising codons 185–388 of *urdGT1b* (185–391 of *urdGT1c*) and a 7.15-kb *Bgl*II-*Pst*I fragment which represents the vector pUWL201 moiety and codons 1–184 of *urdGT1b* (1–184 of *urdGT1c*). The 0.65-kb *Pst*I-*Bgl*II fragment of pUWLurdGT1b (pUWLurdGT1c) was then ligated to the 7.15-kb *Bgl*II-*Pst*I fragment carrying the portion of *urdGT1c* (*urdGT1b*) to reconstitute complete plasmids but carrying a hybrid reading frame now referred to as chimera 3 (chimera 4). The *Hin*dIII/*Xba*I fragment containing the chimeric gene was ligated into pBSK$^-$ to generate chimera 3b (chimera 4b).

4.7. Construction of chimera 5

pUWLurdGT1c was restricted by *Bsm*I/*Xba*I digestion to obtain the 7.15-kb fragment that contains codons 1–239 of *urdGT1c*. pUWLurdGT1b was restricted equally but now the 0.65-kb fragment representing codons 241–388 of *urdGT1b* was used for the further cloning procedure. It was ligated to the 7.15-kb *Bsm*I/*Xba*I fragment to generate chimera 5. The *Hin*dIII/*Xba*I fragment containing the chimeric gene was ligated into pBSK$^-$ to generate chimera 5b.

4.8. Construction of chimera 6

The construction of the chromosomal mutant 16-14, here referred to as chimera 6, has been described [22]. This mutant lacks both *urdGT1b* and *urdGT1c* but contains a chromosomally located chimeric gene consisting of 715 nucleotides from *urdGT1b* and 458 nucleotides from *urdGT1c*.

4.9. Construction of chimera 7 and chimera 8

Chimera 1 (chimera 2) was restricted with *Bsm*I/*Xba*I to create a 7.15-kb *Bsm*I-*Xba*I fragment which represents the vector pUWL201 moiety and codons 1–238 of chimera 1 (1–239 of chimera 2). Subsequently, pUWLurdGT1b (pUWLurdGT1c) was restricted with *Bsm*I/*Xba*I to create a 0.65-kb fragment comprising codons 240–388 of *urdGT1b* (240–391 of *urdGT1c*). The 0.65-kb *Bsm*I/*Xba*I fragment of pUWLurdGT1b (pUWLurdGT1c) was then ligated to the 7.15-kb *Bsm*I/*Xba*I fragment of chimera 1 (chimera 2) to generate chimera 7 (chimera 8), which possesses aa 1–31 and 239–388 of UrdGT1b, and aa 32–238 of UrdGT1c (aa 1–31 and 239–391 of UrdGT1c, and 32–239 of UrdGT1b). The *Hin*dIII/*Xba*I fragment containing the chimeric gene was ligated into pBSK$^-$ to generate chimera 7b (chimera 8b).

4.10. Construction of chimera 9

Chimera 3 and chimera 4 were restricted by *Xba*I/*Bsm*I digestion to obtain a 7.15-kb fragment of chimera 3 and a 0.65-kb fragment of chimera 4. The 7.15-kb fragment contains codons 1–238 of chimera 3 and the 0.65-kb fragment codons 240–388 of chimera 4. Ligation of these two DNA fragments created a chimeric GT gene whose codons 1–184 and 240–388 originate from *urdGT1b*, whereas codons 185–239 are derived from *urdGT1c*. The *Hin*dIII/*Xba*I fragment containing the chimeric gene was ligated into pBSK$^-$ to generate chimera 9b.

4.11. Construction of chimera 10

Chimera 7 (pUWLurdGT1b) was co-restricted with *Pst*I/*Bgl*II to obtain a 7.15-kb fragment representing the vector pUWL201 and codons 1–184 of chimera 7 (a 0.65-kb fragment of *urdGT1b* with the codons 185–388). The fragments were ligated to each other to create chimera 10, the *Hin*dIII/*Xba*I fragment containing the chimeric gene was ligated into pBSK$^-$ to generate chimera 10b.

4.12. Construction of chimeras 11, 12 and 13 by pinpoint mutations in chimera 4

To replace glycine 226 of the gene product of chimera 4 by a serine, an arginine, or both amino acids, oligonucleotide primers LHS-1 (5′-CGCCCACAGAATGCCCCGCATCAACGGCACCCCGTTCAACGCCAACGT**ACT**ACCGATGGTGAC-3′), LHS-2 (5′-CGCCCACAGAATGCCCCGCATCAACGGCACCCCGTTCAACGCCAACGT**CCG**ACCGATGGTGAC-3′) and LHS-3 (5′-CGCCCACAGAATGCCCCGCATCAACGGCACCCCGTTCAACGCCAACGT**CCGACT**ACCGATGGTGAC-3′) were designed such that this particular codon is altered from GGC (Gly) to AGT (Ser) by primer LHS-1, CGG (Arg) by primer LHS-2 and AGTCGG (Ser-Arg) by primer LHS-3. The *Bsm*I site used for the cloning procedure is underlined, the altered nucleotides are shown in bold. Using chimera 4b as template 0.75-kb fragments were amplified by PCR using *Taq* DNA polymerase and primers LHS-1, LHS-2 or LHS-3 and primer T7 (Stratagene). PCR conditions were as follows: initial denaturing 97°C for 7 min, denaturing at 95°C for 90 s, primer annealing at 49°C for 60 s, polymerization at 72°C for 60 s, 30 cycles, afterwards an additional 10 min at 72°C. The *Bsm*I/*Hin*dIII restricted PCR products were then used to replace the equivalent section in chimera 4b generating chimera 11b (containing the serine codon), chimera 12b (containing the arginine codon) and chimera 13b (containing both the serine and arginine codons). Proper ligation and codon alteration were confirmed by DNA sequencing. Chimeras 11, 12 and 13 were generated by subcloning the *Hin*dIII/*Xba*I fragments containing the chimeric genes into pUWL201.

4.13. Chemical analysis of urdamycin derivatives

Aliquots (3 ml) of production cultures of *S. fradiae* strains Ax and XTC expressing the different chimeric GT genes were extracted with an equal volume of ethyl acetate. The solvent was evaporated to dryness in a speedvac centrifuge, then the dried extracts were redissolved in methanol. TLC analysis was carried out on silica gel plates (silica gel 60 F_{254}, Merck) with methylene chloride/methanol/ethyl acetate (8:1:1, v/v) as solvent. HPLC analysis was performed on a Tosoh SC-8020 liquid chromatograph with a photodiode array detector and a Tosoh TSK gel ODS-80TM (150×4.6 mm). The detection wavelength range was 250–500 nm, the gradient was: solvent A: 0.5% AcOH in H_2O, solvent B: 0.5% AcOH in CH_3CN, non-linear gradient, 0–95% B within 30 min at a flow rate of 0.8 ml/min. LC/MS was performed on a Thermoquest LCQ equipped with Hewlett Packard HP1000 series LC under the identical conditions described for HPLC, by atmospheric pressure chemical ionization and detection in the positive and negative mode. Urdamycins were identified by HPLC-UV and mass spectrometry.

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