Characterization of mutations in aclacinomycin A-non-producing *Streptomyces galilaeus* strains with altered glycosylation patterns

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In this study a set of *Streptomyces galilaeus* ATCC 31615 mutants was characterized, which are incapable of synthesizing some or all of the deoxyhexose sugars of aclacinomycin A. Complementation experiments with the mutant strains H026, H038, H039, H054, H063, H065 and H075 were carried out with glycosylation genes previously derived from the wild-type *S. galilaeus*. Mutations in strains H038, H063 and H075 were complemented with single PCR-amplified genes. Furthermore, amplification and sequencing of the corresponding genes from the mutant strains revealed single point mutations in the sequences. First, in H038 a transition mutation in *aknQ*, encoding a putative dTDP-hexose 3-ketoreductase, causes an amino acid substitution from glycine to aspartate, suppressing the biosynthesis of both 2-deoxyfucose and rhodinosose and thus leading to the accumulation of aclacinomycin T with rhodosamine as its only sugar. Second, in H063, which accumulates aklavinone without a sugar moiety, amino acid substitution occurs, with threonine being substituted by isoleucine in dTDP-glucose synthase, the first enzyme participating in deoxyhexose biosynthesis, encoded by *aknY*. Third, a nonsense mutation in *aknP* leads to truncated dTDP-hexose 3-dehydratase in H075, which is incapable of synthesizing rhodinosose. In addition, mutants H054 and H065, which accumulate aclacinomycins without aminosugars, were complemented by a gene for an aminotransferase, *aknZ*. Characterization of the nature of the mutations adds to the usefulness and value of the mutants in the analysis of gene function and in the creation of novel compounds by combinatorial biosynthesis. Furthermore, these results strengthen the assignments of *akn* gene products and enlighten the biosynthetic pathway for deoxyhexoses.

Keywords: anthracycline, complementation, deoxyhexose pathway

INTRODUCTION

Aclacinomycins are a group of anthracycline antibiotics. Along with several other anthracyclines, aclacinomycins exhibit powerful cytotoxic and antibacterial properties. A representative member, aclacinomycin A (aclarubicin)

(Fig. 1), is in clinical use to a limited extent as an anticancer agent for haematological malignancies. Aclarubicin was first described in 1975 as a product of *Streptomyces galilaeus* by Oki *et al.* (1975). The aglycone of aclarubicin is aklavinone (Akv), a common intermediate in the biosynthesis of several anthracyclines, and the attached trisugar moiety consists of rhodosamine (Rhn)-2-deoxyfucose (dF)-cinerulose A. A number of other aclacinomycins, with varying sugar moieties, have been described in the course of the intense search for novel anticancer agents (Fujii & Ebizuka, 1997; Torkkell *et al.*, 2001; Ylihonko *et al.*, 1994). The mixture of aclacinomycins found in *S. galilaeus* ATCC

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Abbreviations: Akv, aklavinone; dF, 2-deoxyfucose; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PKS, polyketide synthase; Rho, rhodinosose; Rhn, rhodosamine.

Fig. 1. Structure of aclacinomycin A.

31615 consists of three forms: aclacinomycin A, B and Y (AcmA, AcmB and AcmY) (Fig. 2a) that differ from each other in the third sugar residue.

Akv is synthesized via a polyketide pathway catalysed by a type II polyketide synthase (PKS), whereas the trisugar moiety is formed by stepwise addition of sugar residues generated by modification of a glucose molecule in a deoxyhexose pathway. Earlier methods, such as feeding of labelled precursors (Casey *et al.*, 1978; Kitamura *et al.*, 1981; Paulick *et al.*, 1976), analysis of products from blocked mutants generated by UV irradiation or chemical mutagenesis (see reviews by Fujii & Ebizuka, 1997; Grein, 1987; Hutchinson, 1997; Strohl *et al.*, 1997) and biotransformations of late biosynthetic intermediates (Eckardt *et al.*, 1985; Schumann *et al.*, 1986; Wagner *et al.*, 1984) have created a basis for understanding the biosynthetic steps involved. However, more detailed understanding was gained from molecular genetic studies on anthracyclines as well as other aromatic polyketides (see reviews by Fujii & Ebizuka, 1997; Hutchinson, 1997; Strohl *et al.*, 1997).

The biosynthetic pathway of Akv starts by the condensation of one propionate and nine acetates in a reaction series catalysed by the minimal PKS (Fig. 2a) (see reviews by Hutchinson, 1997; Strohl *et al.*, 1997). Subsequently, ketoreduction, cyclization and oxygenation reactions in the PKS complex lead to a stable intermediate, aklanonic acid. Post-polyketide reactions, such as methylation of the carboxylic acid, cyclization and reduction, result in Akv, which is ready for glycosylation. Three deoxysugars synthesized in *S. galilaeus* are attached to position C-7 of Akv in the following sequence: Rhn, dF and rhodnose (Rho) (resulting in AcmN). The attached Rho is rapidly converted by an extracellular oxidoreductase to cinerulose A (to form AcmA) and further to aculose (to form AcmY) (Yoshimoto *et al.*, 1979). The third sugar residue

is further converted to cinerulose B to form AcmB, which is then taken into the bacterial cell and converted back to AcmA (Gräfe *et al.*, 1988).

We have previously described an *S. galilaeus* ATCC 31615 mutant series blocked at different steps of the production of aclacinomycin A by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) mutagenization (Ylihonko *et al.*, 1994). The mutants were characterized on the basis of their products and biotransformations. Cloning of the genes for aclacinomycins (Rätý *et al.*, 2000) and nogalamycin, another anthracycline (Torkkell *et al.*, 2001, 1997; Ylihonko *et al.*, 1996a, b), facilitated complementation of the mutants. Knowledge of the nature of the mutations clarifies the steps of the biosynthetic pathway for anthracyclines, provides tools for the analysis of gene functions and makes it possible to use the mutants rationally in combinatorial biosynthesis to create novel molecules.

Deoxysugars are in many cases essential for the activity of antibiotics and, thus, knowledge of their biosynthesis is important for rational design of novel active molecules. Therefore, in this study we focused on *S. galilaeus* strains with altered glycosylation patterns, aiming to identify the mutations leading to a deficient sugar moiety in aclacinomycins. Four strains included in previously described mutant series (Ylihonko *et al.*, 1994), H026, H038, H039 and H054, and three additional mutants, H063, H065 (Rätý *et al.*, 2000) and H075 (DSM 11638) generated recently by NTG mutagenization, were investigated (Table 1, Fig. 2a). Complementation experiments with DNA fragments derived from *S. galilaeus* and other anthracycline producers have already given an idea of the biosynthetic genes blocked in strains H039, H054, H063 and H065 (Rätý *et al.*, 2000; Torkkell *et al.*, 2001, 1997). Here, we report on a more thorough characterization of the *S. galilaeus* mutants to clarify the sequence of biosynthetic reactions of the sugar moiety. Mutated genes were revealed by complementation of the mutants with DNA fragments derived from *S. galilaeus* and further with PCR-amplified single genes. Moreover, corresponding genes from the mutants were amplified to demonstrate the mutation based on the DNA sequence. Finally, these results together with previous ones were used to confirm the mutations and to elucidate the biosynthetic pathway for aclacinomycin sugars, Rhn, dF and Rho.

METHODS

Bacterial strains and plasmids. *Escherichia coli* XL-1 Blue MRF' (Stratagene), *E. coli* TOP10 (Invitrogen) and *Streptomyces lividans* TK24 (Kieser *et al.*, 2000) were used as cloning hosts. *S. galilaeus* glycosylation mutants derived from *S. galilaeus* ATCC 31615 are described in Table 1 and *Streptomyces* plasmids used in this work are listed in Table 2.

Culture conditions and cloning procedures. For *E. coli* and *Streptomyces* strains the general culture conditions were as described by Sambrook *et al.* (1989) and Hopwood *et al.* (1985), respectively. For anthracycline production, the *Streptomyces* cultures were grown in E1 medium consisting of 20 g

Fig. 2. (a) Proposed biosynthetic pathway of aclacinomycins A, Y and B (AcmA, AcmY and AcmB) produced in *S. galilaeus* ATCC 31615. The steps are as follows: 1, condensation; 2, ketoreduction, aromatization, cyclizations, oxygenation; 3, 10-methylation, cyclization; 4, reduction; 5–7, glycosylation; 8–9, oxidoreduction; 10, reduction; 11, oxidoreduction. Products of *S. galilaeus* mutants with altered glycosylation patterns (H026, H038, H039, H054, H063, H065 and H075) are also presented. (b) Organization of the Sg9 gene cluster from *S. galilaeus* and the fragments used in the construction of expression plasmids. The putative functions of the gene products are described in Table 4. (c) Proposed pathways for dTDP-Rhn, dTDP-2-dF and dTDP-Rho in *S. galilaeus*. Probable enzymically catalysed steps are: (1) dehydration, (2) ketoreduction, (3) and (4) C-3 deoxygenation, (5) epimerization, (6) ketoreduction, (7) transamination and (8) aminomethylation. Genes from the Sg9 DNA fragment are presented beside the corresponding biosynthetic steps. Furthermore, mutants with altered glycosylation patterns are shown beside the mutated genes. Complemented mutants and complementing genes are indicated with boxes – black boxes indicate complete complementation and white boxes partial complementation.

glucose, 20 g soluble starch, 5 g Pharmamedia (Traders protein), 2.5 g yeast extract, 1 g K_2HPO_4 , 1 g $MgSO_4 \cdot 7H_2O$, 3 g NaCl and 3 g $CaCO_3$ in 1 litre of tap water (pH 7.5) (Ylihonko *et al.*, 1994). For other purposes ISP4 agar plates (Difco) and TSB medium (Tryptone Soya Broth, Oxoid) were used. The plasmid-carrying strains were grown in the presence of 10 μ g thiostrepton ml^{-1} in liquid medium and 50 μ g ml^{-1} in solid medium.

DNA isolation and manipulation in *E. coli* and *Streptomyces* strains were carried out by standard procedures (Hopwood *et al.*,

1985; Sambrook *et al.*, 1989). All *Streptomyces* strains were transformed by standard methods (Hopwood *et al.*, 1985) with minor modifications (Ylihonko *et al.*, 1996a). The DNA propagated in *E. coli* was cloned in pIJE486 and introduced into *S. lividans* TK24 and further into H039, which is easier to transform than the other *S. galilaeus* strains. Subsequently, DNA isolated from H039 was introduced into the other *S. galilaeus* mutant strains in question.

PCR. For expression constructs, genes *aknP*, *aknQ*, *aknY* and *aknX2* from *S. galilaeus* were amplified separately by PCR,

Table 1. *S. galilaeus* mutants used in this work, plasmids which complement them and deduced mutated genes

Mutant	Characteristic products	Reference	Complementing plasmids	Reference	Mutated gene
H026	Akv-Rhn-dF-Rho	Ylihonko <i>et al.</i> (1994)	–		Oxidoreductase
H038	Akv-Rhn	Ylihonko <i>et al.</i> (1994)	pSgs9, pSgs5, pSgsQ	This work	dTDP-hexose 3-ketoreductase (<i>aknQ</i>)
H039	Akv-(Rho) ₂₋₃	Ylihonko <i>et al.</i> (1994)	pSYE36*†	Torkkell <i>et al.</i> (1997)	dTDP-4-keto-6-deoxyhexose reductase
H054	Akv-Rho-dF-(CinA) ₀₋₁ Akv-dF-dF-(CinA) ₀₋₁ Akv-dF-Rho-Rho	Ylihonko <i>et al.</i> (1994)	pSgs4*, pSgs44* pSY61‡ pSgs9*, pSgs45*	Rätty <i>et al.</i> (2000) Torkkell (2001) This work	Aminotransferase (<i>aknZ</i>)
H063	Akv	Rätty <i>et al.</i> (2000)	pSgs4, pSgs44 pSgs9, pSgsY	Rätty <i>et al.</i> (2000) This work	dTDP-glucose 1-synthase (<i>aknY</i>)
H065	Akv with neutral glycosides	Rätty <i>et al.</i> (2000)	pSgs4*, pSgs44* pSY61‡ pSgs9*, pSgs45*	Rätty <i>et al.</i> (2000) Torkkell (2001) This work	Aminotransferase (<i>aknZ</i>)
H075	Akv-Rhn-dF-dF, Akv-dF-dF-dF	Ylihonko <i>et al.</i> (1999)	pSgs9, pSgs5, pSgsP	This work	dTDP-hexose 3-dehydratase (<i>aknP</i>)

* Partial complementation.

† Genes for dTDP-hexose 3,5-epimerase (*snogF*) and dTDP-4-keto-6-deoxyhexose reductase (*snogG*) from *S. nogalater* nogalamycin cluster.

‡ Genes for an aminomethylase (*snogA*), a dTDP-glucose-synthase (*snogJ*) and an aminotransferase (*snogI*) from *S. nogalater* nogalamycin cluster.

Table 2. *Streptomyces* plasmids used in this work

Plasmid	Characteristics*	Source or reference
pIJ486	<i>Streptomyces</i> cloning vector	Ward <i>et al.</i> (1986)
pIJE486	<i>ermE</i> promoter (Bibb <i>et al.</i> , 1985) cloned into the polylinker of pIJ486	Ylihonko <i>et al.</i> (1996b)
pSgs45	Sg45 fragment from <i>S. galilaeus</i> cloned into pIJE486; genes <i>aknY</i> and (<i>Z</i>)	Räty <i>et al.</i> (2000)
pSgs5	Sg5 fragment from <i>S. galilaeus</i> (Räty <i>et al.</i> , 2000) cloned into <i>XbaI/HindIII</i> -digested pIJE486; genes <i>akn(N)</i> , <i>O</i> , <i>P</i> , <i>Q</i> and <i>R</i>	This work
pSgs9	Sg9 fragment from <i>S. galilaeus</i> cloned into <i>XbaI/HindIII</i> -digested pIJE486; genes <i>akn(N)</i> , <i>O</i> , <i>P</i> , <i>Q</i> , <i>R</i> , <i>S</i> , <i>T</i> , <i>U</i> , <i>V</i> , <i>W</i> , <i>X2</i> , <i>Y</i> , (<i>Z</i>)	This work
pSgsP	PCR-amplified <i>aknP</i> in fragment SgP from <i>S. galilaeus</i> cloned into <i>XbaI/HindIII</i> -digested pIJE486	This work
pSgsQ	PCR-amplified <i>aknQ</i> in fragment SgQ from <i>S. galilaeus</i> cloned into <i>XbaI/HindIII</i> -digested pIJE486	This work
pSgsX2	PCR-amplified <i>aknX2</i> in fragment SgX2 from <i>S. galilaeus</i> cloned into <i>XbaI/HindIII</i> -digested pIJE486	This work
pSgsY	PCR-amplified <i>aknY</i> in fragment SgY from <i>S. galilaeus</i> cloned into <i>XbaI/HindIII</i> -digested pIJE486	This work

* Putative functions of the gene products are described in Table 4.

Table 3. PCR primers used in amplification of genes *aknP*, *aknQ*, *aknY* and *aknX2*

Gene	Sequence (5'–3')	Portion of the gene	RE site*
<i>aknP</i>	<u>TCTAG</u> ATCCTTACGGAGGGACGGC	5'	<i>XbaI</i>
	AAGCTT GTGTTTCGACCGGATGATC	3'	<i>HindIII</i>
<i>aknQ</i>	<u>TCTA</u> GACCTGCCGTGGCTAGCGTG	5'	<i>XbaI</i>
	AAGCTT CCTTCTCTCGTCGTGCGG	3'	<i>HindIII</i>
<i>aknY</i>	<u>TCTAGA</u> TTGCTGCCGAGCGAATCC	5'	<i>XbaI</i>
	AAGCTT CATATG CGCGGTCTTTTCGTGCGGTGTTTCGC	3'	<i>HindIII</i> , <i>NdeI</i>
<i>aknX2</i>	<u>TCTAGA</u> GCCGACGGAGGGAACAGAC	5'	<i>XbaI</i>
	AAGCTT ACCTTCCTCCGGCTCAGC	3'	<i>HindIII</i>

* Restriction endonuclease recognition site (underlined) inserted within the primer sequence.

using the primers shown in Table 3. Furthermore, the postulated mutated gene was amplified from the corresponding mutant strain. PCR was carried out with 25 pmol each oligonucleotide primer, 10–15 ng of the strain's chromosomal DNA, 0.2 mM each dNTP, 2% DMSO and 0.7 U DNA polymerase DyNAzyme EXT (Finnzymes) or Pfu (Promega). The template was initially denatured by heating at 96 °C for 3 min, followed by 30 cycles of amplification, i.e. denaturation at 94 °C for 30 s, annealing at 60–64 °C for 1 min and extension at 73 °C for 1 min 15 s (with DyNAzyme EXT) or for 2 min 45 s (with Pfu). The reaction was completed with extension for 8.5 min. When Pfu was used, the 3' A-overhangs were generated by additional extension at 73 °C for 10 min with 0.7 U Dynazyme II DNA polymerase (Finnzymes). The PCR products obtained were cloned in *E. coli*, using the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions.

Sequencing and sequence analysis. Amplified genes were verified by sequencing. Plasmids were isolated by alkaline lysis and further purified by the Qiagen gel extraction kit. DNA sequencing was performed by an automatic ABI DNA sequencer (Perkin-Elmer) according to the manufacturer's instructions. Sequence analysis was carried out using the GCG

(Version 8; Genetics Computer Group, Madison, Wisconsin, USA) and Vector NTI Suite (Version 6.0) sequence analysis software packages. The deduced gene products were analysed by using the network service programs BLASTP and CD-search provided by the US National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>).

Expression constructs and complementations. Overlapping *S. galilaeus* DNA fragments Sg4 and Sg5 from plasmids pSgc4 and pSgc5 (Räty *et al.*, 2000) were combined to get the contiguous DNA fragment Sg9 (Table 4, Fig. 2b). First, *XhoI/NotI*-digested Sg5 was cloned into pSL1190 to give pSgc5SL. Second, the *NotI-BglII* portion of Sg4 from *NotI/PstI*-digested pSgc4 was cloned into pSgc5SL to give Sg9 in pSgc9SL. Subsequently, the Sg9 fragment from *XbaI/HindIII*-digested pSgc9SL was cloned into pIJE486 and the plasmid was designated pSgs9. Also, *XbaI/HindIII*-digested Sg5 from pSgc5SL was cloned in pIJE486 to give expression construct pSgs5. Both pSgs5 and pSgs9 were first introduced into *S. lividans* TK24 and further into *S. galilaeus* H039, and subsequently into all other *S. galilaeus* mutants with altered glycosylation patterns: H026, H038, H054, H063, H065 and H075.

Genes *aknP*, *aknQ*, *aknY* and *aknX2* in DNA fragments SgP,

Table 4. Putative functions of Sg9 gene products (Rätty *et al.*, 2000)

Gene product	Amino acids	Function
AknN	> 661	Unknown
AknO	272	Activator
AknP	434	dTDP-hexose 3-dehydratase
AknQ	329	dTDP-hexose 3-ketoreductase
AknR	323	dTDP-glucose 4,6-dehydratase
AknS	443	Glycosyltransferase
AknT	443	Unknown
AknU	267	Aklaviketone reductase
AknV	144	Unknown
AknW	259	Cyclase
AknX2	238	Aminomethylase
AknY	291	dTDP-glucose 1-synthase
AknZ	> 340	Aminotransferase

SgQ, SgY and SgX2, respectively, were amplified by PCR from wild-type *S. galilaeus* and cloned from pCR2.1-TOPO vectors in pIJE486 under the control of the *ermE* promoter using *Xba*I and *Hind*III restriction sites inserted within primer sequences. Plasmids pSgsP, pSgsQ, pSgsY and pSgsX2 were then introduced, in addition to TK24 and H039, into *S. galilaeus* mutants thought to contain mutations in the corresponding genes: pSgsP into H075, pSgsQ into H038, pSgsY into H063 and pSgsX2 into H054 and H065.

Detection of anthracycline metabolites. The strains were cultivated in 30 ml E1 medium for 4–5 days to determine the nature and amounts of anthracycline metabolites. A 500 µl sample of E1 culture was adjusted to pH 7.0 by addition of 500 µl 1 M potassium phosphate buffer and cells were subsequently extracted with 250 µl methanol and 250 µl toluene. The toluene layer was concentrated and spotted in 10 µl toluene on a precoated Kieselgel 60 F₂₅₄ glass plate (Merck) developed with toluene/ethyl acetate/methanol/formic acid (50:50:15:3, by vol.). Whenever necessary, metabolites were also detected by HPLC on a Hewlett Packard 1100 series chromatograph equipped with a LiChroCART RP-18e column (Purospher, 3 µm, 4.0 × 55 mm) and a diode array detector. The compounds were separated using 0.1% formic acid and 0.1% formic acid in MeCN with a gradient from 70 to 2% formic acid. The flow rate was 1 ml min⁻¹, and detection was done at 254 and 430 nm. The structures of the anthracyclines were deduced from *R_f* values, retention times and UV/VIS-spectra by comparing with references.

RESULTS

DNA fragment Sg9 (Fig. 2b, Table 4), which contains genes for the sugar moiety of aclacinomycins, was introduced into pSgs9 into *S. galilaeus* mutants H026, H038, H039, H054, H063, H065 and H075 with altered glycosylation patterns (Fig. 2a, Table 1) to study its effect on anthracycline metabolites of the mutants. Complemented strains were further studied by introducing expression constructs containing single genes. The production profiles of the wild-type *S. galilaeus*, the investigated mutant and the plasmid-carrying mutant

were analysed on TLC and further by HPLC to note any differences. Mutation in the gene that causes the mutant phenotype was further demonstrated by identifying the error in the DNA sequence. Deduced mutations of all the strains together with their products and plasmids complementing them are presented in Table 1. Furthermore, a hypothetical biosynthetic pathway for aclacinomycin sugars (Fig. 2c) was deduced from the data obtained in this work and previous results (Rätty *et al.*, 2000).

Complementation of the mutations in *S. galilaeus* strains with a set of genes for aclacinomycins (Sg9)

Fragments Sg4 and Sg5, obtained from *S. galilaeus* gene libraries, were demonstrated previously to contain genes for the deoxyhexose pathway for aclacinomycins (Rätty *et al.*, 2000). Here, these two fragments were combined to give pSgs9 in pIJE486, carrying a 14.8 kb contiguous DNA sequence for aclacinomycin biosynthesis. Plasmid pSgs9 was introduced into *S. galilaeus* mutants. Genes in the Sg9 fragment complemented the mutation in H063 completely and in H054 and H065 partially, as expected, because the mutations were earlier complemented similarly by the introduction of pSgs4 (Rätty *et al.*, 2000). In addition, mutations in H038 and H075 were fully complemented by the introduction of plasmid pSgs9 to restore the wild-type production profile. In contrast, introduction of pSgs9 into mutants H026 and H039 did not have any effect on anthracycline metabolites of the strains.

Mutations in *S. galilaeus* strains

Mutations in strains H038, H054, H063, H065 and H075, complemented by the introduction of plasmid pSgs9, were studied more closely. The gene that was concluded to be responsible for the complementation of the corresponding mutant, as deduced from the putative gene product and production profile of the mutant, was amplified by PCR from the wild-type *S. galilaeus*, sequenced and expressed in the mutant. Furthermore, the corresponding gene was amplified by PCR from the mutant strain and its mutation was analysed by comparing its DNA sequence to that of the wild-type *S. galilaeus*.

H038. The endogenous product of H038 is AcnT, an intermediate of aclacinomycin A biosynthesis, with Rhn as its only sugar. The mutation in H038 was thus concluded to be in a gene which participates in the biosynthesis or transfer of both dF and Rho. In addition to pSgs9, introduction of its subclone pSgs5 into H038 restored wild-type production in the mutant. Thus, based on the proposed hypothetical pathway, *aknQ*, encoding dTDP-hexose 3-ketoreductase, was the most probable candidate for the mutated gene. Consistently, expression of *aknQ* in H038 restored wild-type production in the mutant.

AknQ has significant amino acid homology to several dTDP-hexose 3-ketoreductases from antibiotic clusters

containing 2-deoxyhexose sugars. Recently, *in vitro* studies of AknQ homologues Gra Orf26 (identity/similarity 48/61%) from the *Streptomyces violaceoruber* Tü22 granaticin cluster (Ichinose *et al.*, 1998) and Tü99 Orf11 (51/64%) from the *Streptomyces antibioticus* Tü99 oleandomycin cluster (Draeger *et al.*, 1999) illustrated their participation in the 2-deoxygenation step (Draeger *et al.*, 1999). These enzymes were shown to operate in concert with dTDP-4-keto-deoxyglucose 2,3-dehydratases by stabilizing their labile 2-deoxygenated product through 3-ketoreduction.

Sequencing of the mutated *aknQ* from H038 revealed a G-to-A transition at nucleotide 524, causing a glycine-to-aspartate substitution at amino acid 175. This glycine is conserved in the C-terminal α/β domain of the GFO_IDH_MocA oxidoreductase family (pfam02894) and, furthermore, it is the first in a set of four identical amino acids, GGAL, in AknQ and its closest homologues. This missense mutation prevents the biosynthesis of both Rho and dF in H038 and was complemented by the expression of *aknQ*.

H063. Mutant strain H063 accumulates Akv. This indicates the mutation to be in a gene catalysing one of the first steps in the biosynthetic pathway of deoxyhexoses or in a glycosyltransferase. Recently, the mutation in H063 was complemented with pSgs44, carrying genes only for cyclase (*aknW*), aminomethylase (*aknX2*), dTDP-glucose-1-synthase (*aknY*) and partial aminotransferase (*aknZ*) (Räty *et al.*, 2000). Since dTDP-glucose-1-synthase catalyses the well-established first step of the deoxyhexose pathway, the mutation was suggested to be in gene *aknY*. Here, as expected, expression of PCR-amplified *aknY* in H063 complemented the mutation in the strain.

The DNA sequence of *aknY* in *S. galilaeus* H063 demonstrated a C-to-T transition mutation at position 53, resulting in a threonine-to-isoleucine change at amino acid 18 in the conserved nucleotidyl transferase domain (pfam00483). The mutation prevents the biosynthesis of deoxyhexoses completely, because no detectable amount of glycosidic metabolites was recovered from the culture broth of H063, but complementation with *aknY* was complete.

H075. In addition to its main product Akv-Rhn-dF-dF, H075 produces minor amounts of Akv-dF-dF-dF. Thus, the mutation in H075 would be predicted to be in a gene taking part in the biosynthesis or transfer of Rho. Very recently, dTDP-hexose 3-dehydratase, encoded by *rdml*, from rhodomycin-producing *Streptomyces purpurascens* ATCC 25489, was revealed to complement the mutation in strain H075 (data not shown). Here, plasmid pSgs5, which includes *aknP* for dTDP-hexose 3-dehydratase, also complemented mutation in H075. Consistently, expression of *aknP* restored the wild-type production profile in H075.

AknP, a polypeptide of 434 aa, resembles several putative 3-dehydratases involved in the C-3 deoxygenation step in the deoxysugar biosynthesis of antibiotics. The closest homologues are UrdQ (identity/similarity

72/81%) from the *Streptomyces fradiae* Tü2717 urdamycin biosynthetic gene cluster (Hoffmeister *et al.*, 2000), LanQ (71/81%) from the *Streptomyces cyano-genus* S136 landomycin cluster (Westrich *et al.*, 1999) and the *gra*-ORF23 product (71/82%) from the *Streptomyces violaceoruber* Tü22 granaticin cluster (Ichinose *et al.*, 1998; Tornus & Floss, 2001). Moreover, AknP is similar to CDP-4-keto-6-deoxyglucose-3-dehydratases RfbH and AscC (E₁) participating in the deoxysugar biosynthesis of the O-antigen in *Yersinia pseudotuberculosis* (Kessler *et al.*, 1993; Lei *et al.*, 1995). AscC and RfbH are pyridoxamine 5'-phosphate (PMP)-dependent iron-sulphur-containing enzymes, which catalyse the C–O bond cleavage at C-3 of the substrate, leading to the formation of 3,6-dideoxyhexose. All above-mentioned 3-dehydratases share the Gx₃Dx₇Ax₈EDx₁₀Gx₃Gx₁₃Hx₄GEGGx₁₉Gx₂Cx₁Cx₇C motif, which is similar to the secondary metabolic aminotransferase family (SMAT) (Piepersberg, 1994). However, 3-dehydratases differ from their proposed ancestors, pyridoxal 5'-phosphate-dependent aminotransferases, in having a conserved histidine residue at the active site, instead of lysine, indicating dependence of 3-dehydratases on PMP (Lei *et al.*, 1995). In addition, iron-sulphur-binding cysteine residues are not found in the aminotransferases.

Sequencing of the mutated *aknP* from H075 revealed a G-to-A transition at codon 245 that changes tryptophan (TGG) to a stop codon (TGA). The mutation precedes the last conserved glycine in the 3-dehydratase motif. As is typical of nonsense mutations, 3-dehydratase enzyme activity is fully missing in H075, resulting in products without Rho.

H054 and H065. Both H054 and H065, whose production profiles are not the same but are very similar, accumulate anthracyclines consisting of only neutral sugars, thus being deficient in the biosynthesis or transfer of the aminosugar Rhn. Instead, a different combination of Rho, dF and cinerulose A, either as di- or trisugar moieties, is produced. In an earlier work, mutations in H054 and H065 were partially complemented with plasmid pSgs44, carrying genes for a cyclase (*aknW*), an aminomethylase (*aknX2*), a dTDP-glucose-1-synthase (*aknY*) and a part of an aminotransferase lacking the C terminus (*aknZ*) (Räty *et al.*, 2000). Of these genes, *aknX2* and *aknZ* participate in the biosynthesis of Rhn solely. The incompleteness of gene *aknZ* led us to the misassumption that the mutated gene in strains H054 and H065 is the aminomethylase (*aknX2*). In this study, however, the introduction of plasmid pSgsX2 carrying *aknX2* into mutants H054 and H065 did not alter their production profiles. Furthermore, there were no nucleotide changes in the sequence of *aknX2* in either mutant when compared to that of the wild-type gene.

According to our results, the complementing gene has to be the one encoding aminotransferase, although it is only partial in plasmid pSgs44. Therefore, the expression construct pSgs45 (Räty *et al.*, 2000) (Table 2, Fig. 2b), containing partial *aknZ* with *aknY*, which encodes dTDP-glucose-1-synthase, was introduced into H054 and H065. Both mutants were partly complemented, as

was noted with pSgs44. Thus, the truncated AknZ seems to be capable of partial complementation of the mutations in H054 and H065. The length of the product of partially cloned *aknZ* is 340 aa, whereas a corresponding gene, *aclZ*, from another aclacinomycin-producing *S. galilaeus* (mutant strain 3AR-33, which is derived from strain ATCC 31133) encodes a polypeptide of 369 aa (96/97%; GenBank, accession no. AB008466). The lack of about 30 aa in AknZ leads to weakened, but not abolished, aminotransferase activity, enabling partial complementation of mutations in H054 and H065.

DISCUSSION

Because of its trisugar moiety with different sugar residues attached to an Akv core, aclacinomycin is an ideal target for studies on deoxyhexose biosynthesis. Mutations in deoxyhexose genes result in loss of one or more of the naturally synthesized deoxysugars. Here, single point mutations were shown to be responsible for blocking the biosynthesis of aclacinomycin sugars in mutants with altered glycosylation patterns. Knowledge of the nature of the mutations will facilitate rational use of these mutants in future and elucidate the proposed hypothetical biosynthetic pathway (Fig. 2c) for the deoxysugars of aclacinomycins (Rätty *et al.*, 2000).

Strain H063, accumulating Akv, was shown to contain a missense mutation in the putative dTDP-glucose-1-synthase gene *aknY*. dTDP-glucose-1-synthase adds a deoxynucleotide to a phosphorylated glucose, catalysing the common and well-established first step in the deoxyhexose pathway. Deficiency in this enzyme explains the lack of any deoxysugars in H063 products. In addition, the complementation of H063 with *aknY* strengthens the previous assignment of AknY function.

In strain H038, a missense mutation in the dTDP-hexose 3-ketoreductase gene *aknQ* was seen to result in accumulation of AcMT, with Rhn as its only sugar. This strengthens the previous assumption that biosynthetic routes of Rho and dF diverge from that of Rhn in the 2-deoxygenation step (Rätty *et al.*, 2000). *In vitro* studies with purified enzymes have clarified the mechanism of the 2-deoxygenation step of neutral deoxyhexoses (Chen *et al.*, 1999; Draeger *et al.*, 1999). It was illustrated with enzymes derived from the granaticin and oleandomycin clusters that 2,3-dehydratase in concert with 3-ketoreductase converts dTDP-4-keto-6-deoxy-D-glucose into dTDP-4-keto-2,6-dideoxy-D-glucose (Draeger *et al.*, 1999). We assume that 3-ketoreduction in the deoxy-sugar biosynthetic pathway of aclacinomycin results similarly in dTDP-4-keto-2,6-dideoxy-D-glucose with the equatorial (3R) hydroxyl, although 3-ketoreduction in mycarose biosynthesis has been shown to lead to opposite stereochemistry (Chen *et al.*, 1999). This is because AknQ is more similar to 3-ketoreductases Gra Orf26 and Tü99 Orf11 derived from the granaticin and oleandomycin biosynthetic clusters, respectively. Furthermore, both AknQ and Gra Orf26 are involved in the biosynthesis of Rho.

The mutation in H075, which accumulates aclacinomycins without Rho or its derivatives, was shown to be a nonsense mutation in the gene encoding dTDP-hexose 3-dehydratase, *aknP*. This is in accordance with the previous assumption that the biosynthesis of Rho differs from that of dF in the 3-deoxygenation step (Rätty *et al.*, 2000).

Mutants H054 and H065, which accumulate only aclacinomycins with neutral sugars, were shown to be deficient in the aminotransferase AknZ. Also, complementation experiments with genes derived from the nogalamycin biosynthetic cluster support this conclusion (Torkkell, 2001). Mutations in H054 and H065 were complemented by *snogI*, encoding a 370 aa aminotransferase for nogalamine, a sugar moiety of nogalamycin. The absence of approximately 30 aa in AknZ does not abolish all of its catalytic activity, whilst it explains why the mutations were only partially complemented. As discussed above, the biosynthetic pathway of Rhn differs from that of Rho and dF in the 2-deoxygenation step. Instead of 3-ketoreductase, the product of dTDP-4-keto-6-deoxyglucose 2,3-dehydratase is probably stabilized by an aminotransferase (AknZ), as has been suggested for other aminosugars (Draeger *et al.*, 1999; Olano *et al.*, 1999).

Apart from the above-mentioned mutants complemented with *akn* genes, the mutation in strain H039 has been complemented partially with a dTDP-4-keto-6-deoxyhexose reductase gene, *snogG*, derived from the nogalamycin biosynthetic cluster (Torkkell *et al.*, 2001, 1997). Since the sugar moieties of aclacinomycins produced by H039 consist of Rho residues only, inactivation of 4-ketoreductase seems to prevent the biosynthesis of both Rhn and dF. Thus, according to the proposed pathway, the same 4-ketoreductase catalyses the reduction of both dF and Rhn. Very recently, a homologue of *snogG*, *aknM*, was also found in the *S. galilaeus* aclacinomycin cluster (Rätty *et al.*, 2002). However, *aknM* is only partially in the cloned region and did not complement the mutation in strain H039 (data not shown). Expression of another dTDP-4-keto-6-deoxyhexose reductase gene from the nogalamycin biosynthetic cluster, *snogC*, producing the inverted (4S) configuration, resulted in the production of Akv-4'-epi-dF in H039 (Torkkell *et al.*, 2001). SnogC probably acts on the 2-deoxygenated sugar dTDP-4-keto-2,6-dideoxy-D-glucose in aclacinomycin deoxyhexose biosynthesis, preventing the 3-deoxygenation step in the biosynthesis of Rho, but allowing epimerization of the dF route. Epimerization might also precede 3-deoxygenation and would thus be a common step in the biosynthesis of Rho and dF. However, the synthesis of D-Rho in *S. fradiae* mutant RN-435, which produces urdamycin M, implies that in Rho biosynthesis 3-deoxygenation occurs prior to 5-epimerization (Hoffmeister *et al.*, 2000).

The mutation in H026 has not yet been complemented. However, the production of Akv-Rhn-dF-Rho strongly implies that H026 is deficient in an oxidoreductase

which converts attached Rho into cinerulose A (resulting in AcmA) and further into L-aculose (AcmY) (Yoshimoto *et al.*, 1979). This conclusion is supported by the capability of H026 to convert fed AcMB into AcmA, but not further into AcmY (Ylihonko *et al.*, 1994).

The diversity of aclacinomycin glycosylation in *S. galilaeus* mutants implies loose substrate specificity of glycosyltransferases. Although Rhn appears only as the first sugar in aclacinomycins produced by the mutants, dF and Rho can be found in different combinations as first, second or third sugar. Furthermore, 4'-epi-dF was attached to Akv when the dTDP-4-keto-6-deoxyhexose reductase gene from the nogalamycin biosynthetic cluster, *snogC*, was expressed in the H039 mutant (Torkkell *et al.*, 2001). Recently, relaxed specificity for both sugar co-substrates and alcohol substrates has been described for various glycosyltransferases (Aguirrezabalaga *et al.*, 2000; Blanco *et al.*, 2001; Hoffmeister *et al.*, 2000; Tang & McDaniel, 2001; Trefzer *et al.*, 2001; Zhao *et al.*, 1999). So far, two glycosyltransferases, AknK and AknS, have been cloned from the *S. galilaeus* aclacinomycin biosynthetic cluster (Räty *et al.*, 2000, 2002). However, their individual function in sugar attachment has not been assigned, and since the whole biosynthetic cluster for aclacinomycins has not yet been cloned, we do not know whether they are involved in the transfer of one or more deoxysugars.

In the present work, we studied more closely the mutations behind the deficient glycosylation of aclacinomycins in *S. galilaeus* mutants. In each characterized mutant, a single point mutation in a deoxyhexose biosynthetic gene was responsible for abolished enzyme activity leading to the accumulation of products missing one or more of the inherently synthesized deoxysugars. Exact knowledge of the nature of the mutations enhances the usage and value of these mutants in the analysis of gene function and creation of novel compounds. In addition, the results strengthen the assignments of the *akn* gene products and enlighten the biosynthetic pathway for deoxyhexoses. These results also show that chemical mutagenization, despite its randomness, still offers a convenient tool to create single point mutations affecting only a specific biosynthetic gene, and that it can be used together with molecular biology to study the biosynthesis of different compounds and to learn more about features important to the catalytic activity of the enzymes.

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