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

Kaj Räty,1 Anne Hautala,2 Sirke Torkkell,1 Jaana Kantola,2
Pekka Mäntsälä,1 Juha Hakala2† and Kristiina Ylihonko2

Author for correspondence: Kaj Räty. Tel: +358 2 333 6856. Fax: +358 2 333 6860.
e-mail: kaj.raty@utu.fi

In this study a set of *Streptomyces galilaeus* ATCC 31615 mutants was
categorized, which are incapable of synthesizing some or all of the
deoxyhexose sugars of aclacinomycin A. Complementation experiments with
the mutants 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 rhodinose 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 rhodinose. 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 rhodo-
samine (Rhn)-2-deoxyfucose (dF)-cinerulose A. A num-
ber of other aclacinomycins, with varying sugar moi-
eties, 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

1 Department of
Biochemistry and Food
Chemistry, University of
Turku, Vatselankatu 2,
FIN-20014, Turku, Finland
2 Galilaeus Oy, PO Box 113,
FIN-20781, Kaarina,
Finland

† Present address: Lividans Oy, Lemminkäinenkatu 30, FIN-20520 Turku,
Finland.

Abbreviations: Akv, aklavinone; dF, 2-deoxyfucose; NTG, N-methyl-N'-
nitro-N-nitrosoguanidine; PKS, polyketide synthase; Rho, rhodinose; Rhn,
rhodosamine.
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 rhodinose (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 (Räty 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 (Räty 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 (Räty 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...
Characterization of *S. galilaeus* mutants

**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 K₂HPO₄, 1 g MgSO₄·7H₂O, 3 g NaCl and 3 g CaCO₃ 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 μg thioestreptone ml⁻¹ in liquid medium and 50 μg 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 pJE486 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 *aknXZ* 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

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Characteristic products</th>
<th>Reference</th>
<th>Complementing plasmids</th>
<th>Reference</th>
<th>Mutated gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>H026 Akv-Rhn-dF-Rho</td>
<td></td>
<td>Ylihonko et al. (1994)</td>
<td>pSgs9, pSgs5, pSgsQ</td>
<td>This work</td>
<td>Oxidoreductase</td>
</tr>
<tr>
<td>H038 Akv-Rhn</td>
<td></td>
<td>Ylihonko et al. (1994)</td>
<td></td>
<td>pSgs9, pSgs5, pSgsQ</td>
<td>dTDP-hexose 3-ketoreductase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(aknQ)</td>
</tr>
<tr>
<td>H039 Akv-(Rho)$_{2-3}$</td>
<td></td>
<td>Ylihonko et al. (1994)</td>
<td>pSYE36$^+$</td>
<td>Torkkell et al. (1997)</td>
<td>dTDP-4-keto-6-deoxyhexose</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>reductase</td>
</tr>
<tr>
<td>H054 Akv-Rho-dF-(CinA)$_{0-1}$</td>
<td></td>
<td>Ylihonko et al. (1994)</td>
<td>pSgs4$, pSgs44$</td>
<td>Räty et al. (2000)</td>
<td>Aminotransferase (aknZ)</td>
</tr>
<tr>
<td>Akv-dF-dF-(CinA)$_{0-1}$</td>
<td></td>
<td></td>
<td>pSgs9$, pSgs45$</td>
<td>Torkkell (2001)</td>
<td></td>
</tr>
<tr>
<td>Akv-dF-Rho-Rho</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H063 Akv</td>
<td></td>
<td>Räty et al. (2000)</td>
<td>pSgs4, pSgs44</td>
<td>This work</td>
<td>dTDP-glucose 1-synthase (aknY)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pSgs9, pSgsY</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pSgs9$, pSgs45$</td>
<td>Torkkell (2001)</td>
<td></td>
</tr>
<tr>
<td>H075 Akv-Rhn-dF, Akv-dF-dF</td>
<td></td>
<td>Ylihonko et al. (1999)</td>
<td>pSgs9, pSgs5, pSgsP</td>
<td>This work</td>
<td>dTDP-hexose 3-dehydratase (aknP)</td>
</tr>
</tbody>
</table>

$^*$ 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.
Amplified genes were sequenced and sequence analysis. For 2 min 45 s (with Pfu). The reaction was completed with extension at 73 °C. DNA, 0 moligonucleotide primer, 10–15 ng of the strain’s chromosomal ing mutant strain. PCR was carried out with 25 pmol each using the primers shown in Table 3. Furthermore, the *Restriction endonuclease recognition site (underlined) inserted within the primer sequence.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5′–3′)</th>
<th>Portion of the gene</th>
<th>RE site*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>aknP</em></td>
<td>TCTAGATCCCTACGGGAGGACGACGC</td>
<td>5′</td>
<td>XbaI</td>
</tr>
<tr>
<td></td>
<td>AAGCTTTGTTGACGGAGTAAGTC</td>
<td>3′</td>
<td>HindIII</td>
</tr>
<tr>
<td><em>aknQ</em></td>
<td>TCTAGAGCCTCGGCTGGTACGCGT</td>
<td>5′</td>
<td>XbaI</td>
</tr>
<tr>
<td></td>
<td>AAGCTTTCCCTCTCGTCGGTCCG</td>
<td>3′</td>
<td>HindIII</td>
</tr>
<tr>
<td><em>aknY</em></td>
<td>TCTAGAGCTCTGCCGAGGAAATCC</td>
<td>5′</td>
<td>XbaI</td>
</tr>
<tr>
<td></td>
<td>AAGCTTTCCATATTGGGGTCTTTGTCGGTGGT</td>
<td>3′</td>
<td>HindIII, NdeI</td>
</tr>
<tr>
<td><em>aknX2</em></td>
<td>TCTAGAGGCGACGGAGGAAACGACC</td>
<td>5′</td>
<td>XbaI</td>
</tr>
<tr>
<td></td>
<td>AAGCTTTACCTGGCTCGGATGCG</td>
<td>3′</td>
<td>HindIII</td>
</tr>
</tbody>
</table>

* 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 (Ráty et al., 2000) were combined to get the contiguous DNA fragment Sg9 (Table 4, Fig. 2b). First, *xbo*I/NotI-digested Sg5 was cloned into pSL1910 to give pSgc5SL. Second, the NotI–BglII portion of Sg4 from *notI*/ *psrI*-digested pSgc4 was cloned into pSgc5SL to give Sg9 in pSgc59SL. Subsequently, the Sg9 fragment from *xbo*I/HindIII-digested pSgc9SL was cloned into pJE846 and the plasmid was designated pSgs9. Also, *xbo*I/HindIII-digested Sg9 from pSgc59SL was cloned in pJE846 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 (Raty et al., 2000)

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

SgQ, SgY and SgX2, respectively, were amplified by PCR from wild-type *S. galilaeus* and cloned from pCR2.1-TOPO vectors in pJJE486 under the control of the ermE promoter using XbaI and HindIII 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 the culture was adjusted to pH 7.0 by addition of 0.1 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 F 254 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 x 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 Rp 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 (Raty 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 (Raty et al., 2000). Here, these two fragments were combined to give pSgs9 in pJJE486, carrying a 148 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 (Raty 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 AcmT, 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, 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 Tu22 granaticin cluster (Ichinose et al., 1998) and Tu99 Orf11 (51/64%) from the Streptomyces antibioticus Tu99 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-deoxyxygenated 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 glycolicid 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 rdm1, 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 deoxy suger biosynthesis of antibiotics. The closest homologues are UrdQ (identity/similarity 72/81%) from the Streptomyces fradiae Tu2717 urdamycin biosynthetic gene cluster (Hoffmeister et al., 2000), LanQ (71/81%) from the Streptomyces cyano genus 5136 landomycin cluster (Westrich et al., 1999) and the gra-ORF23 product (71/82%) from the Streptomyces antibioticus Tu22 granaticin cluster (Ichinose et al., 1998; Tornus & Floss, 2001). Moreover, AknP is similar to CDP-4-keto-6-deoxyglucose-3-dehydratases RifH and AscC (E1) participating in the deoxy suger biosynthesis of the O-antigen in Yersinia pseudotuberculosis (Kessler et al., 1993; Lei et al., 1995). AscC and RifH 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 Gx Dx Ax Ex Dx Gx Cx Cx C 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 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 (aknZ) participating in the deoxysugar 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

3381
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 (Räty 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 assignation of AknY function.

In strain H038, a missense mutation in the dTDP-hexose 3-ketoreductase gene aknQ was seen to result in accumulation of AcnT, 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-deoxyxygenation step (Räty et al., 2000). In vitro studies with purified enzymes have clarified the mechanism of the 2-deoxyxigenation 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 deoxysugar 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 TuU99 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-deoxyxigenation step (Räty 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 snogl, 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-deoxyxigenation 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 (Räty 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-deoxyxigenated sugar dTDP-4-keto-2,6-dideoxy-d-glucose in aclacinomycin deoxyhexose biosynthesis, preventing the 3-deoxyxigenation step in the biosynthesis of Rho, but allowing epimerization of the dF route. Epimerization might also precede 3-deoxyxigenation 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-deoxyxigenation occurs prior to 5-epimerization (Hoffmeister et al., 2000).
which converts attached Rho into cinerulose A (resulting in AcmA) and further into l-aculose (AcmY) (Yoshimoto \textit{et al.}, 1979). This conclusion is supported by the capability of H026 to convert fed AcmB into AcmA, but not further into AcmY (Ylihonko \textit{et al.}, 1994).

The diversity of aclacinomycin glycosylation in \textit{S. galilaeus} mutants implies loose substrate specificity of glycosyltransferases. Although Rnh 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, \textit{snogC}, was expressed in the H039 mutant (Torkkell \textit{et al.}, 2001). Recently, relaxed specificity for both sugar co-substrates and alcohol substrates has been described for various glycosyltransferases (Aguirrezabalaga \textit{et al.}, 2000; Blanco \textit{et al.}, 2001; Hoffmeister \textit{et al.}, 2000; Tang & McDaniel, 2001; Trefzer \textit{et al.}, 2001; Zhao \textit{et al.}, 1999). So far, two glycosyltransferases, AkmK and AkmS, have been cloned from the \textit{S. galilaeus} aclacinomycin biosynthetic cluster (Räty \textit{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 deoxy sugars.

In the present work, we studied more closely the mutations behind the deficient glycosylation of aclacinomycins in \textit{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 deoxy sugars. 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 \textit{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.

**ACKNOWLEDGEMENTS**

This research was supported by the Academy of Finland and the National Technology Agency, Finland (TEKES). We thank Hilkka Salmen for comments on the English language in this paper.

**REFERENCES**


Tornus, D. & Floss, H. G. (2001). Identification of four genes from the gramicidin biosynthetic gene cluster of Streptomyces vi-