Isolation and characterization of aclacinomycin A-non-producing *Streptomyces galilaeus* (ATCC 31615) mutants

Kristiina Ylihonko,1 Juha Hakala,2 Jarmo Niemi,1 Juhani Lundell3 and Pekka Mäntsälä1

Author for correspondence: Kristiina Ylihonko. Tel: +358 21 6338057. Fax: +358 21 6338050.

Twelve mutants of *Streptomyces galilaeus* (ATCC 31615) blocked in the production of aclacinomycin A, an anthracycline antibiotic with significant antitumour activity, accumulated intermediates of the biosynthesis of aclacinomycins and several anthracyclines with variant sugar moieties. Three of these aklavinone glycosides have not been described before. Mutant strains H028, H061 and H036 were blocked before the formation of aklavinone, a common intermediate for most anthracyclines. Strain H039 accumulated aklavinone and H026, H035, H038 and H054 had mutations that changed glycosylation of aklavinone. Characterization of the mutants and their products is described.

Keywords: *Streptomyces galilaeus*, aclacinomycin A, anthracycline antibiotics, antibiotic biosynthesis

INTRODUCTION

Anthracyclines are polyketide antibiotics produced especially by *Streptomyces* species. Since the isolation of the first anthracycline, rhodomycin, by Brockmann & Bauer (1950) about 200 natural anthracyclines have been isolated. Anthracyclines consist of an aglycone skeleton (7,8,9,10-tetrahydro-5,12-naphthacene quinone) and a sugar moiety (Fig. 1). The significant antitumour activity of anthracyclines makes them commercially valuable. The demand for new anthracyclines among other antibiotics and the limitation of the traditionally used methods have led to the development of new methods. Hopwood et al. (1985) were the first to produce new antibiotics, so called hybrid antibiotics, by interspecies cloning. Subsequently, the hybrid technique has been successfully applied to producing modified products in other *Streptomyces* species (McAlpine et al., 1987; Epp et al., 1989; Strohl et al., 1989).

*Streptomyces galilaeus* produces a complex of aclacinomycin (Acm) A, B and Y, first described by Oki et al. (1975). Aklavinone (Akn), the aglycone of aclacinomycins, is a common precursor for many anthracyclines, including also the daunorubicin and rhodomycin families. Two invariant sugar components in the aclacinomycin complex are rhodosamine (Rhn) and 2-deoxyfucose (dF) (Fig. 1). The third sugar residue is either cinerulose A (CinA), l-aculose (Acu) or cinerulose B (CinB) (Fig. 1). Of the aclacinomycins, AcmA is available for clinical use. Studies on production (Oki et al., 1979a), structure (Oki et al., 1979b) and biosynthesis (Kitamura et al., 1981) of aclacinomycins have been reported.

Our interests are to study the biosynthesis and production of anthracycline antibiotics and, as the final goal, to create novel hybrid anthracycline molecules. An essential requirement for such work is a set of bacterial strains with mutations at various steps of antibiotic biosynthesis. These blocked mutants are needed to identify the genes encoding the enzymes in the pathways and also for bioconversion experiments. In this paper we describe the isolation and characterization of several mutants of the aclacinomycin pathway and mutants producing anthracyclines with variant sugar moieties in *S. galilaeus*. Also, a new aspect of the biosynthetic pathway of aklavinone is suggested.

METHODS

**Antibiotics.** Aclacinomycin A was kindly supplied by Leiras Oy, Turku, Finland.

**Microbial strains.** Aclacinomycin-producing *Streptomyces*
**RESULTS**

**Isolation of mutants from *Streptomyces galilaeus***

To examine the biosynthesis of anthracyclines we isolated a series of *S. galilaeus* mutants blocked in aclacinomycin biosynthesis. The proposed biosynthetic pathway of aclacinomycins is shown in Fig. 2. Colonies differing from the parent strains in colour were picked from ISP4 agar plates after NTG treatment. These colonies were used to inoculate production medium and the production profile of each mutant was measured by TLC; based on these results, eight mutant classes were observed. Characteristic
Aclacinomycin A non-producing mutants

1 Propionate + 9 acetates

<table>
<thead>
<tr>
<th>Step</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Decaketide formation</td>
</tr>
<tr>
<td>2</td>
<td>2-OH-Aklanonic acid formation</td>
</tr>
<tr>
<td>3</td>
<td>Aklanonic acid methyl ester formation</td>
</tr>
<tr>
<td>4</td>
<td>Neutral glycosides formation</td>
</tr>
<tr>
<td>5</td>
<td>Aminoglycosides formation</td>
</tr>
</tbody>
</table>

Fig. 2. Proposed biosynthetic pathway of aclacinomycins. The proposed blocks in mutants of *S. galilaeus* (ATCC 31615) are also shown. The steps are as follows: 1, condensation; 2, cyclization, aromatization and 2-dehydroxylation; 3, 10-methylation; 4, ring closure and reduction; 5-7, transferring a glycosyl; 8-9, oxidoreduction; 10, reduction; 11, oxidation. The biosynthetic pathway of aklavinone was described by Strohl et al. (1989) and the glycosylation of aklavinone by Oki et al. (1980).

Abstract

Members are described in Table 1. A pedigree of these mutants is shown in Fig. 3.

Six of the mutants (H028, H036, H061, H068, H069 and H070) were blocked before the formation of aklavinone. A typical colony of the wild-type *S. galilaeus* on ISP4 agar was light yellow with a grey spore pigment. The mutant strains H028 and H070 produced no pigment on ISP4 agar or in the E1 medium. H036, H068 and H069 were picked as red colonies, whereas H069 had a brownish colour in its aerial mycelium. None of these strains produced any detectable aclacinomycins or aklavinone. Feeding of the blocked mutants with aklavinone gave aklavinone glycosides as the strains from which they were derived.

Characterization of the mutants

Mutants blocked before aklavinone. For characterizing the mutants unable to produce aklavinone, the coloured products were identified and bioconversion and cosynthesis experiments were performed. The mutant blocked earlier in the biosynthetic pathway should be able to convert later intermediates to aclacinomycins.

The colourless mutant H028 converted the products of H036 to aclacinomycins but failed to convert the products of H061 (the reason for this is discussed in more detail below). Cosynthesis of these strains blocked before aklavinone gave aclacinomycins. These results suggested that H028 is blocked at an early step of the aclacinomycin pathway.

According to the MS spectrum, H061 produced 2-OH-aklanonic acid as the main product. This mutant showed a product profile similar to the previously described *S. galilaeus* strain (ATCC 31671), which is a producer of 2-OH-aklavinone analogues (Matsuzawa et al., 1981). The product from a culture of H036 was extracted with toluene at pH 7, and a yellow compound isolated from the culture medium was identified as aklanonic acid methyl ester. (Authentic samples of aklanonic acid and aklanonic acid methyl ester were kindly supplied by Dr K. Eckardt, Zentralinstitut für Mikrobiologie und experimentelle Mikrobiologie.)
Table 1. Summary of aclacinomycin A-non-producing *S. galilaeus* mutants and characterization of their mutations

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Colour</th>
<th>Main products</th>
<th>TLC (Rf value)</th>
<th>Comments/ reference*</th>
<th>Characterization of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>H028</td>
<td>Light</td>
<td>No coloured compound</td>
<td></td>
<td>-</td>
<td>Mutation before first stable intermediate</td>
</tr>
<tr>
<td>H070</td>
<td>Light</td>
<td>No coloured compound</td>
<td></td>
<td>-</td>
<td>Mutation before first stable intermediate</td>
</tr>
<tr>
<td>H061</td>
<td>Brownish yellow</td>
<td>2-OH-Aklanonic acid</td>
<td>0.36</td>
<td>Known (1)</td>
<td>2-dehydroxylase deficient mutant</td>
</tr>
<tr>
<td>H036</td>
<td>Red</td>
<td>Aklanonic acid methyl ester</td>
<td>0.72</td>
<td>Known (2)</td>
<td>Mutation in ring closure</td>
</tr>
<tr>
<td>H068</td>
<td>Red</td>
<td>Aklanonic acid methyl ester</td>
<td>0.72</td>
<td>Known (2)</td>
<td>Mutation in ring closure</td>
</tr>
<tr>
<td>H069</td>
<td>Red</td>
<td>Aklanonic acid methyl ester</td>
<td>0.72</td>
<td>Known (2)</td>
<td>Mutation in ring closure</td>
</tr>
<tr>
<td>H039</td>
<td>Bright yellow</td>
<td>(1) Aklinovine (2) Akn-Rho-Rho</td>
<td>0.83</td>
<td>Known (3)</td>
<td>Loss of amino sugar</td>
</tr>
<tr>
<td>H038</td>
<td>Yellow</td>
<td>Akn-Rhn</td>
<td>0.74</td>
<td>New</td>
<td>Unknown mutation in glycosylation</td>
</tr>
<tr>
<td>H026</td>
<td>Yellow</td>
<td>Akn-Rhn-dF-Rho</td>
<td>0.11</td>
<td>Known (5)</td>
<td>Oxidoreductase deficient</td>
</tr>
<tr>
<td>H035</td>
<td>Yellow</td>
<td>Not yet identified</td>
<td>0.04</td>
<td>Unknown</td>
<td>Unknown mutation in glycosylation</td>
</tr>
<tr>
<td>H054</td>
<td>Bright yellow</td>
<td>(1) Akn-Rho-dF-CinA (2) Akn-dF-dF-CinA (3) Akn-dF-Rho-Rho (4) Akn-Rho-dF (5) Akn-dF-dF</td>
<td>0.69 0.63 0.58 0.54 0.43</td>
<td>New Known (1) Known (1) Known (1) New Known (1)</td>
<td>Loss of amino sugar *</td>
</tr>
<tr>
<td>H042</td>
<td>Yellow</td>
<td>AcmA</td>
<td>0.14</td>
<td>Known (6)</td>
<td>Overproducer of AcmB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AcmY</td>
<td>0.18</td>
<td>Known (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AcmB</td>
<td>0.28</td>
<td>Known (6)</td>
<td></td>
</tr>
</tbody>
</table>

* (1) Matsuzawa et al. (1981); (2) Eckardt et al. (1985a); (3) Gordon et al. (1960); (4) Kumar et al. (1977); (5) Oki et al. (1977); (6) Oki et al. (1975).

Fig. 3. A pedigree of mutants obtained by mutagenization of *S. galilaeus* (ATCC 31615).

ATCC 31615

H026 — H070

H028 H035 H036 H038 H039

H069 H068 H070

H042

1362 products of aklanonic acid methyl ester. Aklanonic acid has been described as an early intermediate in the biosynthesis of anthracyclines (Eckardt et al., 1985a).

H028 effectively converted aklanonic acid methyl ester isolated from H036 to aclacinomycins. Also, H061 converted the product of H036 to aclacinomycins. Cosynthesis of H028 and H036 or H061 and H036 gave aclacinomycins. From the toluene extracts of the co-cultures, only the products of H036 were converted to aclacinomycins.

Based on the products of H036, co-culture experiments and bioconversion behaviour, we conclude that H036 is defective in the structural gene encoding closure of the fourth ring in aklavinone. The probability that this mutation occurred in a single structural gene is supported by the finding of two similar mutants, H068 and H069 by a single mutagenization treatment of two mutant strains, H035 and H038, which produce a variety of different aklavinone glycosides.
Table 2. MS data of the aklavinone neutral glycosides obtained from S. galiileaus mutants H039 and H054

<table>
<thead>
<tr>
<th>Compound</th>
<th>MS data</th>
</tr>
</thead>
<tbody>
<tr>
<td>H039-1 Akn-Rho-Rho (641); Akn-Rho (526); Akn (412); -(H2O) (376)</td>
<td>M+-(H2O) (376) M+ (768) M+-H2O (753)</td>
</tr>
<tr>
<td>H054-1 Akn-Rho-dF-CinA (768); Akn-Rho (526); Akn-(H2O) (376)</td>
<td>M+ (543) M+-H2O (376)</td>
</tr>
<tr>
<td>H054-2 Akn-dF-dF-CinA (784); -(H2O) (760); Akn-dF (543); Akn-(H2O) (376)</td>
<td>M+-(H2O) (376) M+ (412) Akn-(H2O) (376)</td>
</tr>
<tr>
<td>H054-3 Akn-dF-Rho-Rho (770); Akn-dF-Rho (656); Akn-dF (542); Akn-(H2O) (376)</td>
<td>M+ (412) Akn-Rho (656); Akn-Rho (526); Akn (412)</td>
</tr>
<tr>
<td>H054-4 Akn-Rho-dF (656); Akn-Rho (526); Akn (412); -(H2O) (376)</td>
<td>M+ (768) M+-H2O (376)</td>
</tr>
<tr>
<td>H054-5 Akn-dF-dF (672); Akn-dF (542); Akn-(H2O) (376)</td>
<td>M+ (768) M+-H2O (376)</td>
</tr>
</tbody>
</table>

Mutants producing neutral glycosides. H039 accumulated unglycosylated aklavinone as the main product. When cultivation was continued for more than 5–7 d, a glycosylated compound was also obtained. Acid hydrolysis gave aklavinone as an aglycone. According to the MS spectrum, the molecular mass of this compound was 641 Da (Table 2). Based on hydrolysis, molecular mass and fragmentation analysis, the structure of this compound is Akn-Rho-Rho.

H054 grew as a bright yellow colony on ISP4 agar. This mutant produced five main products, which gave aklavinone after hydrolysis. Partial hydrolysis of the mutant produced five main products, which gave mass peaks at M+ (789), M+-(H2O) (771), and M+-(Akn+H2O) (376). A similar aklavinone dimer was prepared from AcmA by hydrogenolysis or by reductive cleavage with NADPH (Oki et al., 1979b).

DISCUSSION

Three mutants blocked before aklavinone were found in these screening programmes. Two of the possible classes of mutants blocked before aklavinone and producing coloured intermediates according to the proposed pathway were not obtained. One of the missing mutants could be a producer of aklanonic acid (Eckardt et al., 1985a) and the other a producer of aklaviketone (Eckardt et al., 1988). Aklaviketone is the intermediate that is methylated to form aklanonic acid methyl ester and a cyclization product is aklavinone, which is the last intermediate before aklavinone.

Based on their product profile, cosynthesis and biotransformation behaviour of the mutants H028 and H036 were as expected. Unexpectedly, the coloured products of H061 remained unchanged in the experiments performed. An inability of 2-OH-aklanonic acid to enter the cells is unlikely, because Eckardt et al. (1985b) and Wagner et al. (1984) succeeded in converting aklanonic acid to aglycones by biotransformation.

We propose that 2-OH-aklanonic acid is a shunt product; the true intermediate in the aklavinone pathway produced by H061 could be an uncoloured and perhaps unstable compound.

Although the products of H061 were similar to those of S. galilaeus (ATCC 31671), we failed to find 2-OH-aklanonic acid or any glycosylated compounds from highly concentrated extracts of H061 culture broths. However, exogenously added aklavinone was converted to aclacinomycins. Yoshimoto et al. (1981) succeeded in converting 2-OH-aklanonic acid to 2-OH-aclacinomycin A by blocked mutants. By fusing protoplasts from a mutant producing...
2-OH-aklavinone and from an early blocked mutant, it has also been possible to produce 2-OH-aclacinomycins (Yoshimoto et al., 1984). In these cases glycosylation was successful, and glycosyltransferase accepted 2-OH-aklavinone as substrate. We suggest that 2-OH-aklavinone, which was not present in cultures of HO61, is also a shunt product and that the enzyme responsible for ring closure to make the aglycone skeleton has a high specificity, closure failing if a hydroxyl group is attached to position 2. However, different culture conditions could favour the formation of 2-OH-aklavinone either enzymically or spontaneously, or the mutants derived from \textit{S. galilaeus} (ATCC 31133), albeit having the same product profile as HO61 in conditions described in Methods, have a different enzyme specificity.

H039 and H054 produce aklavinone glycosides with rhodinose as the first sugar residue, the compounds which were not described before. Further mutagenesis of H054 gave a mutant, H070, which was similar to H028. Feeding of this mutant with aklavinone gave neutral glycosides, as H054. This blocked mutant may be used as a converter strain for different aglycones to produce novel aglycone glycosides.

Further mutagenization of H035 and H038 gave aklanonic acid methyl ester producers, H069 and H068, respectively. Feeding of these mutants with aklavinone provided similar glycosylation profiles as their parent strain. These mutants are possible converter strains for aglycones to synthesize a variety of anthracyclines because their own products were easily separated from aminoglycosides.

We noticed that H026 is an industrially interesting mutant. Conversion of AcmB to AcmA was detected in the culture broth of H026. The same activity is also present in cultures of wild-type \textit{S. galilaeus}, but it is masked by the activity of oxidoreductase (Hoshino et al., 1983). The fact that H026 converted AcmB to AcmA but did not catalyse the reverse reaction (AcmA to AcmY to AcmB) could be advantageous for industrial production of the valuable product, AcmA. We obtained more AcmB than AcmA from over-producing mutant cultures (e.g. H042), but failed to find any stable strain producing large amounts of AcmA. To increase the yield of AcmA, a H026-type strain could be used as a biocatalyst in an AcmB-over-producing mutant culture.

The exact nature of the mutations in H039, H054, H035 and H038 was not clear. The metabolites of H039 and H054 contained no amino sugar. It is possible that the mutation occurred in the genes encoding glycosyltransferase in H039 that prevents glycosylation. In contrast, alterations in carbohydrate metabolism (for example shortage of rhodamine) could result in addition of different sugar moieties to the aglycone. In any case these two strains have a different mutation in the glycosylation system.

H026 could be classified as an oxidoreductase-deficient mutant. Other mutations in various strains producing aminoglycosides may be concerned with conversion of sugars or in the glycosyltransferase reaction. In the case of H038, the addition of deoxyfucose to the glycoside residue of aklavin is prevented in an unknown way. A shortage of deoxyfucose or inactivity of the glycosyltransferase may be responsible for this failure.

**ACKNOWLEDGEMENTS**

This research was supported by the Finnish Academy of Sciences. We thank Dr K. Lampi (Leiras Oy, Finland) for valuable advice and helpful information, Drs M. Reunanen (Aabo Akademi, Finland), H. Nikander (Leiras Oy, Finland) and J. Vepsäläinen (University of Kuopio, Finland) for recording the spectrum data, and Professor D. A. Hopwood (John Innes Institute, Norwich, UK) for critical reading of the manuscript.

**REFERENCES**


Aclacinomycin A non-producing mutants


Received 29 September 1993; revised 6 December 1993; accepted 15 December 1993.