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.

¹ Department of Biochemistry and Food Chemistry, University of Turku, Biocity, Tykistökatu 6, FIN-20520 Turku, Finland

² Centre for Biotechnology, University of Turku, Tykistökatu 6, BioCity, FIN-20520 Turku, Finland

³ Leiras Oy, PO Box 415, FIN-20101, Turku, Finland

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*

Abbreviations: AcmA, -B, -N, -T, -Y, aclacinomycin A, B, N, T (aklavin), Y; Acu, L-aculose; Akn, aklavinone; CinA, -B, cinerulose A, B; dF, 2-deoxyfucose; Rhn, rhodosamine; Rho, rhodinosine.

Fig. 1. Chemical structures of the sugar components in aclacinomycins and in aklavinone glycosides.

galilaus (ATCC 31615) was used as a parent strain from which blocked mutants or variants were induced by mutagenesis. The strains were maintained on solid agar at 4 °C and as spore suspensions at -20 °C or as mycelia stocks at -70 °C.

Media. ISP4 or ISP2 (Difco) agar media were used for plating mutagenized cell suspensions. Cells were grown in E1-medium, containing glucose (2%, w/v), soluble starch (2%, w/v), Farmamedia (Trader's Mill Protein Co.) (0.5%), yeast extract (0.25%), K_2HPO_4 (0.1%), $MgSO_4 \cdot 7H_2O$ (0.1%), NaCl (0.3%) and $CaCO_3$ (0.3%) in tap water (pH 7.5). This medium was also used for production of anthracyclines. TSB medium (Oxoid Tryptone Soya Broth powder, Difco) was used for mutagenization.

Mutagenesis treatment. NTG was used as a mutagen. TSB medium (50 ml) was inoculated from agar slants or spore suspensions and cultivated by shaking (330 r.p.m.) for 2 d at 28 °C. Homogenized (Sorvall Omni Mixer Homogenizer) and sonicated (MSE 100W Ultrasonic Disintegrator, 75 W, 5 s) culture (20 ml) was diluted to 60 ml with TSB and adjusted to pH 8.5. Half of this suspension was used for NTG treatment. The NTG concentration was 0.5 mg ml⁻¹ and incubation was for 0.5 h at 37 °C with shaking. This treatment gave a killing frequency of about 90%. Immediately after treatment, the NTG medium was removed by centrifugation, the cells were resuspended in 50 ml TSB and cultivated overnight at 28 °C. After sonication (75 W, 5 s) the culture was plated on ISP4 agar and incubated for 5–10 d. Colonies differing from the parent strain in colour were tested for antibiotic production.

Fermentation and product isolation. Selected colonies were

inoculated in 60 ml of the E1 medium in 250 ml Erlenmeyer flasks. Cultivation was at 28 °C for 5–8 d in a rotary shaker at 330 r.p.m. The culture was adjusted to pH 7 in a test tube and then extracted with an equal volume of toluene:methanol (1:1, v/v). The solvent layer (10–20 µl) was spotted on Silica Gel 60F-254-plates (Riedel-de Haen, 0.25 mm) and developed with toluene:ethyl acetate:methanol:formic acid (50:50:15:10, by vol.). Thicker plates (0.5 mm) were used for preparative separation of the compounds. AcmA and Akn were used as references, with R_F values of 0.14 and 0.83, respectively. Chloroform:methanol (10:1, v/v) was used for preparative separation of aglycones. An R_F value of 0.46 was obtained for Akn in this solvent mixture.

HPLC analysis. The purity of the products was determined by HPLC using a Merck's Hitachi apparatus with a Hibar RT 250–40000 reverse phase analytical column. Acetonitrile:water (25:75, v/v) was used as the mobile phase, at a flow rate of 0.5 ml min⁻¹. Samples were dissolved in acetonitrile:water (1:1, v/v) and 20 µl of solution was loaded. Detection was at A_{254} and A_{436} nm using a L-4250 UV-VIS-detector.

Hydrolysis and partial hydrolysis of anthracycline glycosides.

A solution of about 2 mg anthracycline in 1 ml 0.5 M HCl was incubated for 20 min on a boiling water bath. The aglycones were extracted with an equal volume of toluene:methanol (1:1, v/v). Partial hydrolysis was done in 1 ml 0.05 M HCl for 30 min at 55 °C. After incubation, the mixture was neutralized by adding 0.1 ml sodium phosphate buffer, pH 7. The glycosides were extracted with 2 ml dichloromethane. The products were purified by TLC as described above.

Glycosylation of Akn. Mutants defective in Akn or Akn glycoside production were tested for their ability to glycosylate exogenously added Akn. Saturated Akn (1 ml) in methanol was added to each culture on the third day of growth and growth was continued for about 24 h, after which the anthracyclines were extracted and the Akn glycosides were determined by TLC as described above.

Oxidoreductase activity. Conversion of AcmA to AcmY was followed by the oxidoreductase method of Yoshimoto *et al.* (1979). The amount of AcmY formed was measured by TLC.

Cosynthesis. Mutants were cultured in a rotary shaker at 28 °C as described, and after 2 d the cultures were combined and growth was continued for an extra day. The products were extracted with toluene:methanol (1:1, v/v) and measured by TLC as described above.

General methods. ¹HNMR was recorded by a Bruker AM-200 at 200 MHz, and MS by a VG Analytical 7070E Organic Mass Spectrometer.

RESULTS

Isolation of mutants from *Streptomyces galilaus*

To examine the biosynthesis of anthracyclines we isolated a series of *S. galilaus* 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

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). * Alternative route for synthesis. Abbreviations: AcmA, -B, -N, -T, -Y, aclacinomycin A, B, N, T (aklavin), Y; Acu, L-aculose; Akn, aklavinone; CinA, -B, cinerulose A, B; dF, 2-deoxyfucose; Rhn, rhodosamine; Rho, rhodinosose.

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 H061 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 converted to aclacinomycins by H061, but the products from H061 were unchanged when H061 was cultured together with H036, although aclacinomycins were still obtained. The mutation in H061 is proposed to occur in the gene encoding the 2-dehydroxylation step in the pathway (step 2, Fig. 2).

The products of H036 were 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

Table 1. Summary of aclacinomycin A-non-producing *S. galilaeus* mutants and characterization of their mutations

Mutant	Colour	Main products	TLC (R_F value)	Comments/reference*	Characterization of mutation
H028	Light	No coloured compound	—	—	Mutation before first stable intermediate
H070	Light	No coloured compound	—	—	Mutation before first stable intermediate
H061	Brownish yellow	2-OH-Aklanonic acid	0.36	Known (1)	2-dehydroxylase deficient mutant
H036	Red	Aklanonic acid methyl ester	0.72	Known (2)	Mutation in ring closure
H068	Red	Aklanonic acid methyl ester	0.72	Known (2)	Mutation in ring closure
H069	Red	Aklanonic acid methyl ester	0.72	Known (2)	Mutation in ring closure
H039	Bright yellow	(1) Aklavinone (2) Akn-Rho-Rho	0.83 0.74	Known (3) New	Loss of amino sugar
H038	Yellow	Akn-Rhn	0.13	Known (4)	Unknown mutation in glycosylation
H026	Yellow	Akn-Rhn-dF-Rho	0.11	Known (5)	Oxidoreductase deficient
H035	Yellow	Not yet identified	0.04 0.07 0.11	Unknown	Unknown mutation in glycosylation
H054	Bright yellow	(1) Akn-Rho-dF-CinA (2) Akn-dF-dF-CinA (3) Akn-dF-Rho-Rho (4) Akn-Rho-dF (5) Akn-dF-dF	0.69 0.63 0.58 0.54 0.43	New Known (1) Known (1) New Known (1)	Loss of amino sugar
H042	Yellow	AcmA AcmY AcmB	0.14 0.18 0.28	Known (6) Known (6) Known (6)	Overproducer of AcmB

* (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).

Therapie, Jena, Germany.) In addition to aklanonic acid methyl ester at least five yellow or red products were isolated from the culture of H036. On the basis of MS spectra, these compounds were identified as condensation

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. galilaeus* mutants H039 and H054

Compound	MS data
H039-1	M ⁺ Akn-Rho-Rho (641); Akn-Rho (526); Akn (412); -(H ₂ O) (376)
H054-1	M ⁺ Akn-Rho-dF-CinA (768); Akn-Rho (526); Akn-(H ₂ O) (376)
H054-2	M ⁺ Akn-dF-dF-CinA (784); -(H ₂ O) (766); Akn-dF (543); Akn-(H ₂ O) (376)
H054-3	M ⁺ Akn-dF-Rho-Rho (770); Akn-dF-Rho (656); Akn-dF (542); Akn-(H ₂ O) (376)
H054-4	M ⁺ Akn-Rho-dF (656); Akn-Rho (526); Akn (412); -(H ₂ O) (376)
H054-5	M ⁺ Akn-dF-dF (672); Akn-dF (542); Akn-(H ₂ O) (376)

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 products was not successful, but also yielded aklavinone. Extraction by toluene over a wide pH range and the R_F values obtained (0.69, 0.63, 0.58, 0.54, 0.43) suggest that these compounds have neutral sugars instead of amino sugars which are characteristic of the products in the wild-type strain. The structures of the products were determined by mass spectroscopy (Table 2). The stereochemistry of the sugar moiety was deduced by analogy to aminoglycosides. The main metabolites are as follows: H054-1, Akn-Rho-dF-CinA; H054-2, Akn-dF-dF-CinA; H054-3, Akn-dF-Rho-Rho; H054-4, Akn-Rho-dF; H054-5, Akn-dF-dF.

Mutants producing aminoglycosides. Stepwise glycosylation of aklavinone to aclacinomycins was described by Oki *et al.* (1980). The model of glycosylation based on the mutants obtained by screening (Fig. 2) correlates well with the glycosylation pathway of aklavinone proposed by Oki *et al.* (1980). The product of H038 was identified as AcnT, also known as aklavin (Kumar *et al.*, 1977), by ¹HNMR. AcnT is the first glycosylated intermediate in the pathway. Acid hydrolysis of aklavin gave aklavinone as an aglycone and rhodosamine as a sugar residue. H038 also exhibits oxidoreductase activity, converting AcnA to AcnY.

H026 produced an anthracycline AcnN, whose structure was described by Oki *et al.* (1979b). A specific oxidoreductase is responsible for conversion of AcnN to AcnA and further to AcnY (Yoshimoto *et al.*, 1979).

Neither the cells nor the culture supernatant of H026 converted AcnA to AcnY, but with the supernatant of the blocked mutant (H028) AcnN from H026 was converted to AcnY and AcnA, suggesting that AcnN is a true intermediate in AcnA biosynthesis. It is probable that H026 has a mutation in the structural gene encoding the oxidoreductase.

H035 showed a different product profile from the wild-type (ATCC 31615) in TLC analysis: R_F values of 0.04, 0.07 and 0.11 for the main compounds were obtained. Acid hydrolysis of these compounds gave aklavinone as an aglycone and partial hydrolysis of products from H035 gave AcnT. The R_F values of the hydrolysed, partially hydrolysed and non-hydrolysed products suggest that the synthesis of these three products occurs by a branch from the AcnA pathway in step 6 (Fig. 2). However, possible additional sugar moieties have not yet been identified.

Besides these unidentified aminoglycosides, H035 produced a yellow compound which in hydrolysis and in extraction behaved as an aglycone. Based on MS data the structure was a dimer of aklavinone. The dimer gave mass peaks at M⁺(789), M⁺-H₂O(771), and M⁺-(Akn+H₂O)(376). A similar aklavinone dimer was prepared from AcnA 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 aklanononic acid (Eckardt *et al.*, 1985a) and the other a producer of aklaviketone (Eckardt *et al.*, 1988). Aklanononic acid is the intermediate that is methylated to form aklanononic acid methyl ester and a cyclization product is aklaviketone, 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 aklanononic 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-aklavinone 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-aklavinone 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 H061, 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 *S. galilaensis* (ATCC 31133), albeit having the same product profile as H061 in conditions described in Methods, have a different enzyme specificity.

H039 and H054 produce aklavinone glycosides with rhodnose 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 *S. galilaensis*, 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 rhodosamine) 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 aklavinone 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 (Åbo 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.

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