ACARBOSE ACB CLUSTER FROM *ACTINOPLANES* SP. SE 50/110.

The present invention relates to the isolation of further acarbose biosynthesis and metabolism genes from actinomycetes, mainly from *Actinoplanes* sp. SE 50/110 and its mutants, which genes are found together with the previously known biosynthesis genes in a common gene cluster, to the use of these genes for preparing acarbose and acarbose homologues using *Actinoplanes* sp. and other producers of acarbose-related natural substances (pseudooligosaccharides), to the use of these genes for optimizing the process with the aid of biochemical/molecular biological engineering methods, and to the heterologous expression of these genes in other microorganisms.

The recognition that a number of actinomycetes, in particular the Actinoplanaceae, form oligosaccharide-like inhibitors of glycoside hydrolases, especially carbohydrate-cleaving enzymes of the digestive tract, forms part of the subject-matter of previous patent applications (e.g. DE 20 64 092 and DE 22 09 834). The compound O-4,6-dideoxy-4-[[1S-(1S,4R,5S,6S)-4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl]-amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucopyranose, known as acarbose, is reported to be the most potent inhibitor of this group [DE 23 47 782].

Acarbose is a potent inhibitor of α-glucosidase and is employed, under the trade name Glucobay®, as an oral antidiabetic agent for the therapy of diabetes mellitus. The secondary metabolite acarbose is formed by *Actinoplanes* sp. SE 50 (CBS No. 791.96) and by a natural variant of this strain, SE 50/110 (CBS 793.96) [DE 22 09 834], as well as by their selectants and mutants. The said Patent Applications describe, for example in Examples 1 to 4 of the said German Patent Application P 22 09 834, the isolation of an α-glucosidase inhibitor of this type.

Molecular biological methods can be used to isolate particular genes directly from an uncharacterized genome using gene probes, for example ^{32}P-labelled DNA fragments, which bind specifically to the sought-after DNA sequence.

It has furthermore been disclosed that, in actinomycetes, in particular streptomycetes, the biosynthesis genes are arranged side by side in a cluster on the chromosome, less frequently on a plasmid, in the secondary metabolite producers which have so far been investigated [Hershberger C.L., et al. (1989)]. Gene probes can therefore be used to isolate adjacent, previously unknown biosynthesis genes whose importance for the desired biosynthesis can then be elucidated. The gene probes can likewise be used to detect the corresponding biosynthesis genes in other microorganisms.

From the structure of acarbose, it was to be presumed that the deoxyglucose moiety of the acarbose molecule is formed in accordance with the biosynthesis of the 6-deoxy sugar residues of various antibiotics (for example aminoglycosides, such as streptomycin and kasugamycin; macrolides, such as erythromycin and tylosin; polyenes, such as amphotericin A, and B, and nystatin; anthracyclines, such as daunorubicin; and glycopeptides, such as vancomycin). For this reason, a gene probe, and PCR primer pairs which it was possible to employ heterologously, were derived from highly conserved regions of known dTDP-glucose dehydratase enzyme proteins.

In the case of *Actinoplanes* sp. SE 50/110, use of the above-described techniques led initially to the isolation and sequencing of a 2.2 kb *Bam*HI DNA fragment containing the complete DNA sequence of *acbB* (encoding dTDP-glucose 8 dehydratase) and the partial DNA sequences of *acbA* (encoding dTDP-glucose synthase) and *acbC* (encoding a cyclase) [EP A 0 730 029/DE 19507214]. Other enzymes which have been reported to be involved in acarbose biosynthesis are acarviosyl transferase from *Actinoplanes* sp. SE 50/110 and its mutants (encoded by *acbD*) [DE 196 26 269.5] and acarbose 7-phosphotransferase (encoded by *acbK*) [Goeke, K. et al. (1996); Drepper, A., et al. (1996)]. Acarviosyl transferase has been reported to have the ability to replace acarviosin with other sugars in pseudooligosaccharides which contain this particular sugar residue. It is possible that acarbose 7-phosphotransferase (acarbose kinase) is involved in preparing a form of acarbose which enables it to be transported out of the cell. In addition to this, acarbose 7-phosphotransferase is regarded as being part of a self-defence mechanism:

while acarbose strongly inhibits the cytoplasmic α-glucosidase of the production strain, the compound which has been phosphorylated by acarbose 7-phosphotransferase does not, and as a consequence the cell-specific substrate metabolism is not disrupted. Many producers of aminocyclitol antibiotics have been reported to possess protective mechanisms of this nature.

The present patent application describes the cluster of the biosynthesis genes and other genes of acarbose metabolism in an 18 kb segment in *Actinoplanes* sp. SE 50/110 (see Figs. 1-3). The isolation of further genes of acarbose metabolism demonstrated, surprisingly, that the previously known biosynthesis genes, *acbABC* [EP A 0 730 029/ DE 19507214], are located in a common gene cluster together with genes of acarbose modification (acarviosyl transfer and phosphorylation, i.e. genes *acbD* and *acbK*), of extracellular and cytoplasmic maltodextrin and glucose metabolism (enzymes of the α-amylase and 4-α-glucanotransferase and amylomaltase families, respectively) and of binding protein-dependent sugar transport (uptake of maltodextrin or disaccharide into the cytoplasm). This finding is of substantial importance for the biotechnological production of acarbose with regard to optimizing the process in a targeted manner. The reason for this is that this finding makes available important parts of acarbose metabolism in its entirety for the methods of biochemical/molecular biological engineering giving a novel viewpoint which widens the possibilities which were highlighted in previous patents. Thus, it is now also possible to influence the provision, which is evidently important for acarbose synthesis, of α-1,4-glucan precursors by way of starch/maltodextrin degradation, the uptake/release, and also the cytoplasmic alteration, of oligosaccharides up to the malto stage, the multiplicity of the product spectrum and the modifications (e.g. acarbose phosphorylation) which are possibly of importance for secreting acarbose and/or liberating it outside the cell.

The invention consequently relates to the isolation of further biosynthesis genes from *Actinoplanes* sp. SE 50/110, and to their use for isolating the adjoining DNA regions, for the purpose of further elucidating the acarbose gene cluster.

Elucidation of the acarbose gene cluster, involving isolation and characterization of the

acarbose biosynthesis genes, is essential for improving the production process in a targeted manner, for example by

- Increasing the ability of *Actinoplanes* to synthesise acarbose by amplifying the genes for bottleneck enzymes, using more effective promotors, and abolishing or amplifying regulatory events.

- Improving the provision of precursors, especially from sugar metabolism, together with optimizing the transport mechanisms such as the transport of substrates into the cell and the secretion of acarbose or modified compounds.

- Restricting the product spectrum in *Actinoplanes* to the desired main product acarbose by switching off unwanted pathways for biosynthesizing minor components or by switching off unwanted enzymic degradation reactions.

- Expressing in heterologous host strains
  for the purpose of increasing production by means of an improved space/time yield,
  for the purpose of simplifying the working-up procedure, and
  for the purpose of restricting the product spectrum in a specific manner.

- Using individual acarbose biosynthesis genes, or several acarbose biosynthesis genes, for synthesizing acarbose, or compounds of this substance class, *in vitro* using precursors which have been prepared synthetically or microbially.

The invention therefore discloses:

A recombinant DNA molecule which comprises genes for the biosynthesis of acarbose and acarbose homologues, which genes are arranged in the gene cluster depicted in Figure 2.

A recombinant DNA molecule which has the restriction enzyme cleavage pattern

depicted in Figure 1.

The complete DNA sequence of the 18 kb fragment containing the genes listed in Tab. 1, as depicted in Fig. 3, and the amino acid sequences resulting therefrom.

Tab. 1: Properties of the *acb* genes and the Acb gene products

Gene		Gene product[b]	
Designation	from-to[a]	AA	postulated function, related proteins
acbA	8914-9838	307	dTDP-glucose synthase; 58.1% sequence identity in 217 AA overlap with *Shigella flexneri* (L14842) RfbA
acbB	8844-7818	341	dTDP-glucose 4,6-dehydratase; 63% sequence identity in 327 AA overlap with *Streptomyces argillaceus* (Y10907) MtmE
acbC	6628-7529	381	C7-sugar cyclase, similar to 3-dehydroquinic acid synthases; 26.8% identity in 340 AA overlap with *Mycobacterium tuberculosis* (X59509) AroB
acbD	13373-15548	724	Acarviosyl transferase, similar to other oligodextrin to polydextrin transferases or hydrolases; greatest similarity to the cyclomaltodextrin glucotransferase family; 41.1% identity in 734 AA overlap with *Bacillus circulans* (X68326) CdgT protein
acbE	12385-9320	1021	α-amylase; 45.0% identity in 897 AA overlap with an α-amylase from *Streptomyces lividans* (X70255)

acbF	17515-16537	325	MalF-like membrane protein of a binding protein-dependent ABC transporter for lactose; 29.9% identity in 251 AA overlap with *Synechocystis* sp. PCC6803 (D90905) LacF protein
acbG	16541-15119	252	MalG-like membrane protein of a binding protein-dependent ABC transporter; 27.8% identity in 234 AA overlap with ORF2 protein from the thermophilic bacterium RT8.B4 (L18965)
acbH	(18482-17511)	322	MalE-like binding protein of a binding protein-dependent ABC transporter; 22.2% identity in 279 AA overlap with *Streptococcus mutans* (M77351) MsME protein
acbK	1991-2894	300	Acarbose 7-kinase, similar to sugar kinases and adenosine kinases; 57.9% identity in 297 AA overlap with *Streptomyces* sp. (U08602) Urf2 protein, whose gene is located adjacent to a gene for an α-amylase
acbL	(3966-4988)	340	Oxidoreductase, similar to sorbitol dehydrogenases; 26.6% identity in 214 AA overlap with *Haemophilus influenzae* (L42023) HI0053 protein
acbM	2890-3970	359	Unknown function (no significant similarity)
(acbN)	(5049-5823)	258	Oxidoreductase

acbQ	1-1960	527	Maltodextrin glucotransferase; MalQ-like protein having 33.0% identity in 339 AA overlap with *Haemophilus influenzae* (L45989) MalQ protein
acb/O	5819-6627	230	Unknown function (no significant similarity)

[a] the position numbers relate to the base pairs in the BglII-SstI fragment in Fig. 3. Sequence information which is either still incomplete or relates to uncertain reading frames is placed in brackets;

[b] the accession Nos. of the database entries are indicated in brackets;

AA = amino acids;

In this context, the genes *acbA* and *acbB* very probably encode enzymes of acarbose biosynthesis, since their encoded proteins AcbA and AcbB, respectively, exhibit a high degree of sequence identity with known bacterial dTDP-glucose synthases and dTDP-glucose 4,6-dehydratases, respectively. The similarity of the protein sequences to the representative of the two enzyme families which is the nearest known representative in each case (cf. Fig. 3) is much greater than that of other arbitrary pairs of functionally identical proteins from the two groups. In this context, it is surprising that while the nearest relatives of AcbA are to be found among streptomycetes proteins, AcbB is more closely related to various RfbB proteins in Gram-negative bacteria. However, this phenomenon is also demonstrated by other corresponding streptomycetes proteins, for example TylA1 and TylA2 from the tylosin producer *Streptomyces fradiae* [Merson-Davies and Cundliffe (1994)], which are likewise encoded by adjacent genes in a common gene cluster.

The gene acbC encodes an enzyme which is probably involved in acarbose biosynthesis since the enzyme AcbC is related to AroB proteins, i.e. dehydroquinic acid synthases, and, following overexpression in *Streptomyces lividans*, exhibits an enzyme activity which, as to be expected, converts heptulose phosphates (e.g. sedoheptulose-7-phosphate) into products which possess similar properties to those of valienone and

valiolone (possible precursors of acarbose biosynthesis) but which are not identical to these compounds.

The genes *acbK* (acarbose 7-kinase), *acbL* (ketosugar or sugar alcohol oxidoreductase) and *acbM* (unknown function), and also the *acbN*-gene (direct link to the *acbL* and *acbC* reading frames is indicated by overlapping start/stop codons) probably encode enzymes of acarbose biosynthesis since they all, together with *acbC* and possibly *acbQ* as well, form a probable operon (transcription unit) and are probably read in a translationally linked manner. The functions of a specific, cytoplasmically located acarbose kinase (AcbK) and of a possible dehydrogenase (AcbL) also provide support for direct involvement in acarbose metabolism within the cell; in this context, the sugar dehydrogenase, AcbL, might be involved in the synthesis of the C-7 cyclitol or 6-deoxyhexose precursors, or their condensation.

The two genes *acbD* (acarviosyl transferase) [DE 196 25 269.5; Goeke, K., et al. (1996); Drepper, A., et al. (1996)] and *acbE* (α-amylase), which lie in opposite directions with a common promoter region in the *Actinoplanes* sp. SE 50/110 cluster and which both encode enzymes of the amylase family, suggest that the regulation of starch degradation and the production of acarbose are closely interrelated. This is confirmed by the finding that, when *Actinoplanes* sp. is grown on starch as the C source, the two enzymes are the most strongly expressed extracellular proteins in the culture supernatant. This also applies to the expression of *acbE* under the control of its own promoter even in *Streptomyces lividans* (see Examples).

The genes *acbH, F* and *G* encode what is probably an extracellular sugar-binding protein, AcbH, and two typical membrane components AcbF and AcbG, of a bacterial sugar transporter of the ABC importer type. They probably participate in the metabolism of acarbose by taking up oligo-maltodextrins or recycle acarbose, as transport vehicles for short oligo-α-1,4-glucans (higher homologues of acarbose), by taking it up into the cell. The amylomaltase-like gene product (AcbQ) of the *acbQ* gene could also be involved in a process of this nature.

The invention furthermore discloses:

A process for isolating acarbose biosynthesis genes from actinomycetes, in particular from *Actinoplanes*, characterized in that gene probes are used which are derived from a 2.2 kb *Bam*HI fragment. The 2.2 kb *Bam*HI fragment, which was derived from highly conserved regions of known dTDP-glucose dehydratase enzyme proteins with the aid of a gene probe which was isolated using PCR primers, is described in Patent Application [EP A 0 730 029/DE 19507214].

A process for isolating genes for biosynthesizing acarbose-related natural substances in actinomycetes (e.g. for validamycin, oligostatins (trestatin) and adiposins).

A process for increasing the ability of *Actinoplanes* to synthesize acarbose by means of

- increasing the dosage of genes for velocity-determining biosynthetic enzymes,
- more effective promoters for velocity-determining biosynthetic enzymes, and
- eliminating undesirable regulatory steps.

A process for increasing the ability of *Actinoplanes* to synthesize acarbose by means of protein engineering in association with the biosynthetic steps which limit acarbose synthesis, or for avoiding degradation products which arise due to undesirable reverse reactions of biosynthetic enzymes.

A process for restricting the product spectrum in *Actinoplanes* to the desired main product by means of eliminating unwanted pathways for biosynthesizing minor components or by means of eliminating unwanted enzymic degradation reactions, for example by means of inactivating the *acbD* gene.

A process for altering transport mechanisms with a view to improving the transport of substrates into the cell or more effectively secreting acarbose out of the cell.

A process for carrying out expression in heterologous host strains (e.g. pseudo-oligosaccharide-forming streptomycetes and in other streptomycetes such as *Streptomyces lividans*, in rapidly growing bacteria such as *E.coli*, or in yeasts and fungi),

- in order to increase production by improving the space/time yield,
- in order to simplify the working-up procedure, and
- in order to restrict the product spectrum in a targeted manner.

A process for using the acarbose biosynthesis genes for the *in-vitro* synthesis of acarbose or compounds of this substance class, proceeding from precursors which are prepared synthetically or microbially.

The invention is described in detail below. In addition, the invention is defined by the content of the claims.

Unless otherwise indicated, all the genetic manipulation methods were carried out as described in Sambrook et al. (1989).

Three different gene probes were used for the screening. They were isolated from plasmids pAS2, p/AS5/7.3 and pAS6/3. Plasmid pAS2 was prepared from *E.coli* DH5α by means of the "boiling method" or by means of alkaline lysis and hydrolysed with the restriction endonuclease *Bam*HI. The resulting 2.2 kb *Bam*HI fragment was isolated and labelled with ^{32}P-labelled deoxynucleotides by means of so-called nick translation. This radioactively labelled fragment was employed as a gene probe for isolating acarbose biosynthesis genes and is designated acb probe II below. The second gene probe was isolated from plasmid pAS5/7.3. The *Sph*I-*Sst*I fragment was isolated and radioactively labelled as described above. This gene probe is designated acb probe III below. The third gene probe was isolated from plasmid pAS6/3. The *Bam*HI fragment was isolated and radioactively labelled as described above. This probe was given the designation acb probe IV.

Acarbose biosynthesis genes were isolated in two different ways, as follows:

1) Chromosomal DNA from *Actinoplanes* sp. was hydrolysed with the restriction enzymes *Ssf*I, *Bgl*/I and *Pst*I, and the restriction fragments were separated by gel chromatography and screened for a homologous DNA sequence by means of Southern hybridization using acb probe II (*Sst*I and *Bgl*II hydrolysis) or acb probe III (*Pst*I hydrolysis). The *Sst*I fragment which hybridized with the gene probe had a size of approx. 10.7 kb and the *Bg/*II fragment had a size of approx. 10.2 kb. The 10.7 kb *Sst*I fragment and the 12 kb *Bg/*II fragment were eluted from the gel and ligated into the vectors pUC18 and pBluescript II KS, respectively, and cloned into *E.coli* DH5α. The resulting plasmids were given the designations pAS5 (*Sst*I fragment) and pAS6 (*Bg/*II fragment). A 2.8 kb *Pst*II fragment, which overlapped with the *Sst*I fragment and which hybridized with the acb probe III gene probe, was cloned into the pUC18 vector, with the recombinant plasmid being designated pMJ1.

2) A GEM12 page library of *Actinoplanes* sp. genomic DNA was screened with probes acb probe III and acb probe IV by means of plaque hybridization. In all, 15 phages were isolated using acb probe III and two phages were isolated using acb probe IV, which phages comprise a total of approx. 38.5 kb of colinear *Actinoplanes* sp. DNA containing the acarbose biosynthesis genes. The phages which were characterized in more detail carry the designations 10/3 and 5/4. Plasmid pMJ1 was obtained from phage 10/3 by hydrolysing with the restriction enzyme *Pst*I and cloning a 2.8 kb *Pst*I DNA fragment into plasmid pUC18. Plasmid pMJ9 (N 6.3 kb fragment) was obtained from phage 5/4 by means of hydrolysis with the restriction enzyme *Sst*I and subsequent cloning into pUC18 (*Sst*I-hydrolysed).

In order to determine the DNA sequence of the *Actinoplanes* sp. 10.7 kb *Sst*I fragment (pAS5), the following recombinant plasmids were constructed, proceeding from the pUC18 vector, and the sequences of the inserted DNA fragments were analysed:

pAS5 10.7 kb *Sst*I fragment from *Actinoplanes* chromosomal DNA

pAS2 2.2 kb *Bam*HI fragment from *Actinoplanes* chromosomal DNA (see

Patent Application DE 195 07214)

pAS5/15 3.8 kb *Hind*III/*Sst*I fragment from pAS5

(see Patent Application DE 196 25 269.5)

pAS5/15.1 =2.6kb *Hind*III/*Pst*I fragment from pAS5

pAS5/15.2 =0.75 kb *Sal*I fragment from pAS5/15.1

pAS5/15.3 =0.5 kb *Sal*I fragment from pAS5/15.1

pAS5/15.4 =0.4 kb *Sal*I fragment from pAS5/15.1

pAS5/15.5 =0.35 kb *Sal*I fragment from pAS5/15.1

pAS5/15.6 =1.25 kb *Pvu*II fragment from pAS5/15.1

pAS5/15.7 =0.7 kb *Pvu*II/HindIII fragment from pAS5/15.1

pAS5/15.9 =0.1 kb *Pvu*II fragment from pAS5/15.1

pAS5/15.11 =1.1 kb *Knp*I/*Nco*I fragment from pAS5/15

pAS5/15.12 =0.9 kb *Kpn*I/*Nco*I fragment from pAS5/15

Three DNA regions were amplified using the PCR method and the corresponding fragments were cloned and sequenced:

pAS5/17 =0.46 kb PCR fragment

pAS5/18 =0.26 kb PCR fragment

pAS5/19 =0.27 kb PCR fragment

pAS5/6 5.4 kb *Pst*I fragment from plasmid pAS5

Clones which were prepared using the enzymes exonuclease III and S1 nuclease, proceeding from plasmid pAS5/6 and following hydrolysis with *Xho*I and *Sst*I:

pAS5/6.3-15 = 5.1 kb of insert DNA

pAS5/6.12-4 = 4.7 kb of insert DNA

pAS5/6.3-18 = 4.3 kb of insert DNA

pAS5/6.6-3 = 4.2 kb of insert DNA

pAS5/6.9-2 = 3.8 kb of insert DNA

pAS5/6.9-6 = 3.8 kb of insert DNA

pAS5/6.12-6 = 3.2 kb of insert DNA

pAS5/6.3-6 = 3.0 kb of insert DNA
pAS5/6.15-1 = 2.8 kb of insert DNA
pAS5/6.3-16 = 2.3 kb of insert DNA
pAS5/6.9-1 = 1.8 kb of insert DNA
pAS5/6.9-3 = 1.2 kb of insert DNA
pAS5/6.6-1 = 0.9 kb of insert DNA
pAS5/6.12-3 = 0.47 kb of insert DNA
pAS5/6.12-2 = 0.17 kb of insert DNA

pAS5/3 1.4 kb *Bam*HI fragment from plasmid pAS5
pAS5/3.1 = 0.35 kb *Sph*I/*Fsp*I fragment from pAS5/3
pAS5/3.2 = 0.85 kb *Sph*I/*Bam*HI fragment from pAS5/3
pAS5/3.3 = 0.55 kb *Sph*I/*Bam*HI fragment from pAS5/3

pAS5/4 1.2 kb *Bam*HI fragment from plasmid pAS5
pAS5/5 0.48 kb *Sst*I/*Bam*HI fragment from plasmid pAS5
pAS5/7 1.2 kb *Pst*I/*Sst*I fragment from plasmid pAS5
pAS5/7.1 0.64 kb *Pvu*II/*Acc*I fragment from plasmid pAS5/7
pAS5/7.2 0.54 kb *Pst*I/*Sph*I fragment from plasmid pAS5/7
pAS5/7.3 0.67 kb *Sph*I/*Sst*I fragment from plasmid pAS5/7
pAS5/11 0.68 kb *Bgl*II/*Hind*III fragment from plasmid pAS5
pAS5/12 0.63 kb *Bgl*II/*Pst*I fragment from plasmid pAS5
pAS5/13 4.8 kb *Bam*HI/*Sst*I fragment from plasmid pAS5
pAS5/16 0.5 kb *Bam*HI fragment from plasmid pAS5

Plasmids which were constructed for determining the DNA sequence and which contain DNA fragments containing *Actinoplanes* sp. acarbose biosynthesis genes from plasmid pAS6 (cf. Example 6). The DNA cloned in plasmid pAS6 possesses a *Bgl*II/*Sst*I fragment, containing acarbose biosynthesis genes, which is also possessed by plasmid PAS5. The following recombinant plasmids were constructed in the pUC18 vector for the purpose of sequencing the 5.9 kb *Bgl*II/*Sst*I fragment which adjoins plasmid pAS5 (Fig. 1).

pMJ6/6 5.9 kb *Bgl*II/*Sst*I fragment from plasmid pAS6

pMJ6/4.2 0.5 kb *Bam*HI/*Pst*I fragment from plasmid pMJ6/6

pMJ6/4.1 0.36 kb *Bam*HI/*Pst*I fragment from plasmid pMJ6/6

pMJ6/6.2.2 0.5 kb *Sal*I religand

pMJ6/6.2.3 3.3 kb *Sal*I fragment

pMJ6/6.2.4 1.2 kb *Sal*I fragment

pMJ6/6.2.5 1.0 kb *Sal*I fragment

pMJ6/6.2.6 0.7 kb *Sal*I fragment

pMJ6/6.2.7 0.14 kb *Sal*I fragment

pMJ6/6.2.8 0.13 kb *Sal*I fragment

pMJ6/8.1 1.1 kb *Cla*I/*Bam*HI fragment

pMJ6/10 1.5 kb *Pst*I/*Sal*I fragment

pAS6/3 2.8 kb *Bam*HI fragment from plasmid pAS6

pAS6/3.1 1.1 kb *Hinc*II fragment from plasmid pAS6/3

pAS6/3.2 1.2 kb *Sal*I fragment from plasmid pAS6/3

pAS6/3.3 1.45 kb *Pst*I fragment from plasmid pAS6/3

The following plasmids were constructed, and the sequences of the DNA inserts analysed, in order to determine the DNA sequence of the *Actinoplanes* sp 2.8 kb *Pst*I fragment (pMJ1).

pMJ1/1 0.6 kb *Sph*I/*Pst*I fragment from plasmid pMJI, religand following *Sph*I hydrolysis,

pMJ1/2 1.2 kb *Sal*I/*Pst*I fragment from plasmid pMJ1, religand following *Sal*I hydrolysis,

pMJ1/3 1.4 kb *Sst*I/*Pst*I fragment from plasmid pMJ1, religand following *Sst*I hydrolysis,

pMJ1/4.1 0.9 kb *Sal*I/*Sma*I fragment from plasmid pMJ1.

The method of Sanger et al. (1977), or a method derived from this, was employed for the DNA sequencing. Use was made of the Autoread sequencing kit (Pharmacia,

Freiburg, Germany) in combination with the Automated Laser Fluorescence (ALF) DNA sequencer (Pharmacia, Freiburg, Germnay). Suitable fluorescein-labelled pUC reverse-sequencing and sequencing primers were obtained commercially (Pharmacia, Freiburg, Germany). The sequence of the approx. 18.0 kb *Bgl*II/*Pst*I fragment is depicted in Fig. 3. Tab. 1 provides an overview of the properties of the *acb* genes and the products which they encode.

Tab. 2. Sequences of the primers for the PCR and the sequencing reaction.

Primers for the PCR:

Plasmid pAS5/17:

Primer designation	Sequence
acbD/E1	5' GGCGGCGATTCGGCCTGCGCGG 3'
acbD/E2	5' GCGGCGATGGCATGCCTGGCG 3'

Plasmid pAS5/18:

Primer designation	Sequence
acbD3	5' ACCAGGCCGAGGACGGCGCCC 3'
acbD4	5' AGCGGCATGTGCTTGACGGCG 3'

Plasmid pAS5/19:

Primer designation	Sequence
acbD5	5' ACCGGCTCGAACGGGCTGGCACC 3'
acbD6	5' CCCTCGACGGTGACGGTGGCG 3'

Primers for amplifying the *acbC* gene:

The sequence regions which were used to construct recognition sites for the restriction endonucleases *Nde*I and *Eco*RI are underlined.

Primer designation	Sequence
AS7	5'GGAAGCTCATATGAGTGGTGTCG3
AS8	5'CGAGACGGTACATATGCACGCGGATG3'
AS9'	5'CCGTCTCGCCCACCCGCATCACC3'
AS-C1'	5'AGGGAAGCTCATATGAGTGGTGTCGAG3'
AS-C2	5'GGTATCGCGCCAAGAATTCCTGGTGGACTG3

Primers for the sequencing reaction:

Primer designation	Sequence
universal primer	5'GTAAAACGACGGCCAGT3'
reverse primer	5'GAAACAGCTATGACCATG3'

The N-terminal sequence of the AcbE protein was analysed using a 473A gas-phase protein sequencer from Applied Biosystems (Forster City, CA, USA) and the standard Fastblott protein sequencing programme. The protein sequencer, the different programmes, the breakdown cycles and the PTH identification system, are all described in the sequencer's manual (User's manual; protein sequencing system model 473A (1989); Applied Biosystems, Forster City, CA 94404, USA).

The PTH amino acids were detected on-line using an Applied Biosystems RP18 column (220 mm × 2 mm, 5 µ material). The PTH amino acids were identified and quantified with the aid of a 50 pmol standard. The data were processed using an Applied Biosystems 610A sequencer data system.

All the chemicals used for the protein sequencer were supplied by Applied Biosystems.

Examples:

1. Culture of the *E.coli* strains, preparation of the plasmid DNA and isolation of DNA fragments

E.coli DH5α was incubated at 37°C in LB medium. Plasmid-harbouring bacteria were maintained under selection pressure (ampicillin, 100 µg/ml). The bacteria were cultured on an orbital shaker at 270 rpm. Samples which had been incubated for at least 16 h were designated overnight cultures (OC).

Cells from 1.5 ml of an OC which had been incubated under selection pressure were used for preparing plasmid DNA. The plasmids were isolated by the alkaline SDS lysis method [Birnboim, H.C., and J. Doly (1979)].

Restriction endonucleases were used exclusively, in accordance with the instructions of the producer (Gibco BRL, Eggenstein, Germany), for specifically hydrolysing vector DNA. 5 U of the relevant restriction endonuclease were used for restricting 10 µg of plasmid DNA, with the incubation being carried out at 37°C for 2 h. In order to ensure complete hydrolysis, the same quantity of restriction endonuclease was added once again and the mixture was incubated for at least a further 1 h.

The cleaved DNA was separated electrophoretically on horizontal agarose gels whose concentration varied from 0.5 to 1.2% depending on the size of the DNA fragments. For elution, the gel piece containing the DNA fragment was excised with a sterile scalpel and weighed. The DNA fragments were eluted from the agarose using the JETsorb kit (Genomed, Bad Oeynhausen, Germany) as instructed.

2. Culture of *Actinoplanes* sp. SE50/110, preparation and cleavage of the chromosomal DNA, and separation of the fragments by gel electrophoresis

Actinoplanes sp. SE50/110 was incubated at 30°C in TSB medium for 3 d on an orbital shaker. Preliminary culture (5 ml) was carried out at 240 rpm in culture tubes, while

the main culture (50 ml) was carried out at 100 rpm in 500 ml baffled flasks. After culturing, the cells were sedimented by centrifugation and washed twice in TE buffer.

1.5-2 mg of cells (fresh weight) were used to prepare the total DNA by the phenol/chloroform extraction method (Hopwood, D.A., et al. (1985)].

20 µg of chromosomal DNA were hydrolysed with 10 U of the relevant restriction enzyme (Gibco BRL, Eggenstein, Germany) at 37°C for 2 h in the appropriate buffer. In order to ensure complete hydrolysis, the same quantity of restriction endonuclease was added once again and the mixture was incubated for at least a further 1 h.

The cleaved DNA was separated electrophoretically on 0.6% horizontal agarose gels.

The DNA fragments were once again eluted using the JETsorb kit (see Example 1).

3. Preparation of acb gene probe II, acb gene probe III and acb gene probe IV

The fragments which were prepared from pAS2 (see DE 195 07214), pAS5/7.3 and pAS6/3 as described in Example 1 were labelled radioactively with the nick translation system supplied by Gibco BRL, Eggenstein, Germany, in accordance with the latter's instructions. 0.5 - 1.0 µg of DNA fragment was employed for this procedure, as was [α-^{32}P]dCTP (3000 Ci/mM; Amersham Buchler, Braunschweig). The mixture was then boiled for 10 minutes (denaturation) and immediately added to the hybridization solution (see Example 4).

4. DNA transfer to membranes and DNA hybridization (Southern hybridization and autoradiography)

The Southern transfer method [Southern, E.M. (1975)] was used to transfer DNA fragments from agarose gels onto membranes. The agarose gels which were obtained as described in Example 2 were tilted for 20 minutes in 0.25 M HCl. The gels were laid on 3 layers of absorbent Whatman 3 MM paper (Whatman, Maidstone, GB), and

a Hybond™-N⁺ membrane (Amersham Buchler, Braunschweig) was laid on top while excluding air bubbles. Several layers of absorbent paper were then laid on a membrane. A weight of approx. 1 kg was placed on top of the filter stack. The DNA was transferred by sucking through 0.4 M NaOH. After a transfer time of at least 12 h, the nylon filters were rinsed for 5 minutes with 2×SSC and then dried in air.

The nylon filters were then shaken in a water bath at 68°C for at least 2 h in 50-100 ml of prehybridization solution. During this period, the solution was changed at least twice. The hybridization was carried out for at least 12 h in a hybridization cabinet. 15 ml of hybridization solution, containing acb probe II (see Example 3), were employed.

The nylon filters were then washed for 15 minutes in each case with 6× postwash and 1× postwash. They were then covered with clingfilm while still moist. Autoradiography was carried out at -80°C for at least 16 h using Hyperfilm-MP (Amersham Buchler, Braunschweig) in a light-proof cassette provided with intensifying screens.

5. Isolation and cloning of the *Bgl*II, *Pst*I and *Sst*I fragments from *Actinoplanes* sp. total DNA

Actinoplanes sp. chromosomal DNA was completely hydrolysed with *Bgl*II, *Pst*I and *Sst*I and the hydrolysates were separated by agarose gel electrophoresis; the *Sst*I fragments of 9.0-12 kb in length, the *Bgl*II fragments of 11-13 kb in length and the *Pst*I fragments of 2.5-3.5 kb in length were then eluted from the agarose (see Example 1). The eluted *Sst*I fragments and *Pst*I fragments were ligated to vector plasmid pUC18, which had been prepared from *E.coli* DH5α and hydrolysed with *Sst*I and *Pst*I, respectively. The vector plasmids had been treated previously with alkaline phosphatase (Boehringer, Mannheim) in accordance with the manufacturer's instructions. The ligation was carried out in a volume of 20 µl, with the ratio of fragment to vector being 3:1 and with from 0.01-0.1 µg of DNA being present in the mixture. 1 U of T4 DNA ligase and the corresponding buffer (Gibco BRL, Eggenstein, Germany) were employed. The eluted *Bgl*II fragments were ligated into the vector

plasmid pBluescript II KS, which had been hydrolysed with *Bam*HI. The ligation was carried out as in the case of the *Sst*I and *Pst*I fragments.

Transformation-competent *E.coli* DH5α cells were transformed with complete ligation mixtures [as described by Hanahan, D. (1983)]. Ampicillin-resistant transformants were transferred to LB-Amp selection plates (100 µg/ml).

6. Identification of clones which harbour the 10.7 kb *Sst*I fragment, the 12 kb *Bgl* II fragment, the 2.8 kb *Pst*I fragment and the 6.3 kb *Sst*I fragment from the acarbose biosynthesis cluster

Ampicillin-resistant transformants were examined for the presence of the 10.7 kb *Sst*I fragment and the 12 kb *Bgl*II fragment which hybridizes with acb probe II. Ten of each of these clones were streaked out on a selection plate, incubated overnight and then washed from the plate with 3 ml of LB medium. The plasmid DNA was then isolated from 20 such pools of ten [using the method of Birnboim, H.C., and J. Doly (1979)]. In order to remove the cloned *Sst*I fragments from the polylinker, the 20 different plasmid preparations were hydrolysed with the restriction endonucleases *Eco*RI and *Hind*III, and *Sst*I and *Hind*III, respectively. The restriction mixtures were then separated electrophoretically on a 0.6% agarose gel and the DNA transferred by Southern transfer from the agarose gel to a nylon filter (see Example 4). Hybridization was once again carried out using acb probe II (see Example 4). One of the pools in each case reacted positively with acb probe II and was subdivided into the ten individual clones. Their plasmids were isolated likewise and subjected to the above-described procedure. The hybridizing plasmids were designated pAS5 and pAS6, respectively. They contained a 10.7 kb *Sst*I fragment (pAS5) and a 12 kb *Bgl*II fragment (pAS6), respectively.

The recombinant phage 10/3 was hydrolysed with *Pst*I, after which the DNA was [lacuna] on a horizontal agarose gel; the 2.8 kb *Pst*I fragment was then eluted from the matrix (see Example 1) and ligated into the pUC18 vector. The recombinant plasmid was designated pMJ1 and transformed into *E.coli* DH5α.

The recombinant phage 5/4 was hydrolysed with *Sst*I and the DNA was separated on a horizontal agarose gel; the 6.3 kb *Sst*I fragment was then eluted from the matrix (see Example 1) and ligated into the pUC18 vector. The recombinant plasmid was designated pMJ9 and transformed into *E.coli* DH5α.

7. Preparation of the GEM12 library and isolation of recombinant phages which harbour the acarbose biosynthesis genes, and preparation of the phage DNA.

Actinoplanes sp. chromosomal DNA was partially hydrolysed with *Sau*3AI. For this, 50 µg of *Actinoplanes* chromosomal DNA were incubated at 37°C for 30 min with 0.015 U of *Sau*3AI. The enzyme reaction was stopped by phenol extraction, chloroform and ethanol precipitation [as described by Sambrook et al. (1989)]. The subsequent treatment of the DNA fragments, and ligation to phage vector GEM12, were carried out in accordance with the instructions of the manufacturer (Promega, Heidelberg). The invitro packaging of the ligation mixture was carried out using the DNA-packaging kit supplied by Boehringer (Mannheim). The phages were multiplied in *E.coli* LE392 using the method described in Sambrook et al. (1989). The phages harbouring acarbose biosynthesis genes were identified by means of plaque hybridization (as described by Sambrook et al. 1989) using the acb III and acb IV gene probes. The phage DNA comprising the acarbose biosynthesis genes was prepared, as described by Sambrook et al. (1989), from phages which had been multiplied on *E.coli* LE392.

8. Polymerase chain reaction

PCR is used to amplify chosen DNA regions *in vitro* [Mullis, K.B., and F.A. Falloona (1987)]. For all the reactions, Taq DNA polymerase was employed, in accordance with the producer's (Gibco BRL, Eggenstein) instructions, in 25 reaction cycles. In order to suppress possible secondary structures in the case of GC-rich DNA, the mixtures contained 5% formamide. The volume was 100 µl, with 50 pmol of each primer and 200 µM dNTP's being employed. After an initial 5-minute denaturation of the DNA at 95°C, 2.5 U of the temperature-stable DNA polymerase were added to the mixtures in a hot start. Primer elongation took place at 72°C, and the DNA was denatured at the

beginning of each cycle at 95°C for 1 min. The reactions were carried out in a Biometra Thermocycler (Göttingen).

Tab. 3 Protocols for the PCR for amplifying DNA fragments from the acarbose cluster. The table lists the designations of the recombinant plasmids which contain the corresponding fragments.

Fragment size	Primer annealing	Primer extension	Recombinant plasmid
0.46 kb	72°C in 20 sec.	72°C in 20 sec.	pAS5/17
0.26 kb	68°C in 20 sec.	72°C in 20 sec.	pAS5/18
0.27 kb	68°C in 20 sec.	72°C in 20 sec.	pAS5/19

9. Subcloning of plasmid pAS5

Several subclones were prepared from plasmid pAS5 in order to elucidate the sequence of the double-stranded DNA.

pAS5/6 Plasmid pAS5 was hydrolysed with the restriction enzyme *Pst*I and the hydrolysate was fractionated by gel electrophoresis (0.7% agarose gel); the 5.4 kb *Pst*I fragment was eluted from the gel and cloned into pUC18 (hydrolysed with *Pst*I), and the recombinant plasmid was transformed into *E.coli* DH5α.

pAS5/3; pAS5/4; pAS5/13 and pAS5/16 Plasmid pAS5 was hydrolysed with the restriction enzyme *Bam*HI and the hydrolysate was fractionated by gel electrophoresis. The fragments were of the following sizes:

1.4 kb *Bam*HI fragment
1.2 kb *Bam*HI fragment
2.3 kb *Bam*HI fragment
0.5 kb *Bam*HI fragment
0.45 kb *Bam*HI fragment
7.5 kb *Bam*HI fragment (= 4.8 kb *Actinoplanes* DNA *Bam*HI/*Sst*I fragment ligated to pUC18).

The fragments which were envisaged for the subcloning, and which were of 1.4 kb and 0.5 kb in size, were eluted from the gel (see Example 1). The pUC18 vector was prepared for the cloning using the restriction enzyme *Bam*HI as described in Example 1. The ligations were carried out as described in Example 5. Ligation of the 0.5 kb fragment into the prepared pUC18 resulted in subclone pAS5/16. Subclone pAS5/3 resulted from ligating the 1.4 kb fragment to the prepared pUC18. Subclone pAS5/4 resulted from ligating the 1.2 kb fragment to the pUC18 vector. Subclone pAS5/13 resulted from religating the 7.5 kb *Bam*HI fragment.

pAS5/5; pAS5/7; pAS5/11 and pAS5/12 Plasmid pAS5 was hydrolysed with the restriction enzymes *Bam*HI and *Sst*I, *Pst*I and *Sst*I, *Bgl*II and *Pst*I, and also *Bgl*II and *Hind*III. The restriction mixtures were separated in a 1.2% agarose gel. The corresponding fragments were eluted from the agarose gel and cloned into pUC18 (hydrolysed with *Bam*HI and *Sst*I, *Pst*I and *Sst*I, *Bam*HI and *Pst*I or *Bam*HI and *Hind*III), with the recombinant plasmids being transformed into *E.coli* DH5α. Subclone pAS5/5 contains the 0.48 kb *Sst*I/*Bam*HI fragment, subclone pAS5/12 the 0.63 kb *Bgl*II/*Pst*I fragment and subclone pAS5/11 the 0.68 kb *Bgl*II/*Hind*III fragment.

pAS5/15.11 and pAS5/15.12 Plasmid pAS5/15 was hydrolysed with the restriction endonucleases *Nco*I and *Kpn*I. The resulting 0.9 kb *Nco*I/*Kpn*I and 1.1 kb *Nco*I/*Kpn*I fragments were eluted from a 1.2% agarose gel (see Example 1) and cloned into vector pUCBM21 (Boehringer, Mannheim) (hydrolysed with *Nco*I/*Kpn*I) with the recombinant plasmids being transformed into *E.coli* DH5α, resulting in the subclones pAS5/15.12 (0.9 kb fragment) and pAS5/15.11 (1.1 kb fragment).

10. Subcloning of plasmids pAS6 and pMJ6/6 and of phage 5/4

pMJ6/6: Plasmid pAS6 was hydrolysed with the restriction endonuclease *Sst*I (using the restriction cleavage site of the vector) and a 5.9 kb *Sst*I fragment was eluted from the agarose gel and ligated to plasmid pUC18. *E.coli* DH5α was transformed with the recombinant plasmid.

pMJ6/4.1 and pMJ6/4.2 Plasmid pMJ6/6 was hydrolysed with the restriction endonucleases *Bam*HI and *Pst*I, and a 0.36 kb *Bam*HI/*Pst*I fragment, and also an 0.5 kb *Bam*HI/*Pst*I fragment, were eluted from the agarose gel and ligated to plasmid pUC18. *E.coli* DH5α was transformed with the recombinant plasmids.

pMJ6/6.2.2, pMJ6/6.2.3, pMJ6/6.2.4, pMJ6/6.2.5, pMJ6/6.2.6, pMJ6/6.2.7 and pMJ6/6.2.8: Plasmid pMJ6/6 was hydrolysed with the restriction endonuclease *Sal*I, and a 3.3 kb fragment, a 1.2 kb fragment, a 1.0 kb fragment, a 0.7 kb fragment, a 0.14 kb fragment and a 0.13 kb fragment were eluted from the agarose gel and ligated to plasmid pUC18. *E.coli* DH5α was transformed with the recombinant plasmids. Plasmid pMJ6/6.2.2 was obtained by hydrolysis and subsequent religation.

pMJ6/8.1: Plasmid pMJ6/6 was hydrolysed with the restriction enzymes *Cla*I and *Bam*HI, and a 1.1 kb fragment was eluted from an agarose gel and ligated to plasmid pBluescript II KS. *E.coli* DH5α was transformed with the recombinant plasmid.

pMJ6/10: Plasmid pMJ6/6 was hydrolysed with the restriction enzymes *Pst*I and *Sal*I, and a 1.5 kb fragment was eluted from an agarose gel and ligated to plasmid pUC18. *E.coli* DH5α was transformed with the recombinant plasmid.

pAS6/3 Plasmid pAS6 was hydrolysed with the restriction endonuclease *Bam*HI, and a 2.8 kb *Bam*HI fragment was eluted from the agarose gel and ligated to plasmid pUC18; the recombinant plasmid was then transformed into *E.coli* DH5α.

pAS6/3.1: Plasmid pAS6/3 was hydrolysed with the restriction endonuclease *Hinc*II,

and a 1.1 kb fragment was ligated into pUC18, which had been hydrolysed with *Hinc*II, and the recombinant plasmid was cloned into *E.coli* DH5α.

pAS6/3.2 Plasmid pAS6/3 was hydrolysed with the restriction endonuclease *Sal*I, and a 1.2 kb fragment was ligated into pUC18, which had been hydrolysed with *Sal*I, and the recombinant plasmid was cloned into *E.coli* DH5α.

pAS6/3.3 Plasmid pAS6 was hydrolysed with the restriction endonuclease *Pst*I, and a 1.45 kb fragment was eluted from the gel and ligated to pUC18. *E.coli* DH5α was transformed with the recombinant plasmid.

11. Subcloning of plasmid pMJ1

pMJ1/1 Plasmid pMJ1 was hydrolysed with the restriction endonuclease *Sph*I, and a 3.3 kb *Sph*I fragment (0.6 kb *Sph*I/*Pst*I fragment ligated to pUC18) was eluted from the agarose gel. This fragment was religated and the recombinant plasmid was cloned into *E.coli* DH5α.

pMJ1/2 Plasmid pMJ1 was hydrolysed with the restriction endonuclease *Sal*I, and a 3.9 kb *Sal*I fragment (1.2 kb *Sal*I/*Pst*I fragment ligated to pUC18) was eluted from the agarose gel. This fragment was religated and the recombinant plasmid was cloned into *E.coli* DH5α.

pMJ1/3 Plasmid pMJ1 was hydrolysed with the restriction endonuclease *Sal*I, and a 4.1 kb *Sst*I/*Pst*I fragment (1.4 kb *Sst*I/*Pst*I fragment ligated to pUC18) was eluted from the agarose gel. This fragment was religated and the recombinant plasmid was cloned into *E.coli* DH5α.

pMJ1/4.1 Plasmid pMJ1 was hydrolysed with the restriction endonuclease *Sal*I, and a 0.9 kb *Sal*I/*Sma*I fragment was eluted from the agarose gel and ligated to plasmid pUC18. The recombinant plasmid was transformed into *E.coli* DH5α.

12. Preparation of subclones from pAS5/6

The pAS5/6 subclones were prepared using a double-stranded Nested Deletion Kit (Pharmacie, Freiburg, Germany). 10 µg of pAS5/6 DNA were prepared as described in Example 1 and hydrolysed with 10 U each of *Xho*I and *Sst*I. The subsequent incubation with exonuclease III was carried out in accordance with the producer's instructions for a total of 20 min. Aliquots corresponding to a quantity of approx. 2.5 µg of DNA were removed from the reaction mixture at intervals of 5 min. The aliquots were treated with S1 nuclease at 20°C for 30 min, in accordance with the producer's instructions, in order to prepare non-protruding DNA ends. These DNA molecules were then religated with T4 ligase and cloned into *E.coli* DH5α.

13. Sequencing the DNA of *Actinoplanes* sp. acarbose biosynthesis genes

The plasmids described in Examples 8 to 11 were sequenced. From 6 to 8 µg of plasmid DNA from a preparation (see Example 1) were employed in the sequencing reaction. The sequencing reaction was carried out using the Auto-read sequencing kit (Pharmacia, Freiburg, Germany). The standard protocol for sequencing dsDNA was used. In order to make it possible to use the A.L.F. (automated laser fluorescence (DNA) sequencer) to analyse the nucleotide sequence, the fluorescein-labelled universal and reversed sequencing primers were used as starter molecules for the sequencing reaction (see Tab. 2). For preparing the gel, 8 ml of Hydro Link Long Ranger (Serva, Heidelberg), 33.6 g of urea, 8 ml of 10× TBE buffer and H_2O to make up to 80 ml were mixed, sterilized by filtration and degassed for 1 minute. The polymerization was initiated by adding 350 µl of 10% (w/v) ammonium persulphate and 40 µl of N,N,N',N'-tetramethylethylenediamine. The solution was poured into a gel mould (50×50×0.05 cm). Electrophoresis was carried out at 38 W and a constant temperature of 45°C. 1× TBE buffer was used as the running buffer. A linked-in computer (Compaq 386/20e) was used to process the measured fluorescence into a DNA sequence, with the computer also serving to control the electrophoresis unit (A.L.F. Manager 2.5 program; Pharmacia, Freiburg).

14. Transformation of *S. lividans*

S. lividans TK23 and 1326 were protoplasted and transformed using the method of Babcock and Kendrick (1988), with the cells being grown in TSB-PEG 8000.

15. Overexpression of AcbC

15.1 Overexpression of AcbC in *E.coli*

The DNA sequence of the *acbC* gene shows two possible translation start points for AcbC. Although start point 1 represents the probable start of AcbC due to a more significant ribosome binding site, both possible AcbC proteins were overexpressed. For this, plasmids pET11a and pET16b (Novagen, Heidelberg) were used for expressing in *E.coli*. In order to ensure an optimal translation start for the expression, the ATG start codon of the pET vectors, which is at a suitable distance from an *E.coli*-like RBS, should be used. In order to do this, it is necessary to construct an *Nde*I recognition sequence at the start codon of *acbC*. The oligonucleotides AS7 (sequence position 6617) and AS8 (sequence position 6638) were employed for synthesizing an *Nde*I recognition site at the two possible start codons. The oligonucleotide AS9 binds to the DNA 66 bp downstream of a *Bam*HI recognition sequence at sequence position 6877. Using the PCR method (see Example 8), two DNA fragments were amplified which were used for expressing the two possible AcbC proteins. The primers were annealed at 45°C for 40 sec, and primer elongation took place in 30 sec. The two amplified DNA fragments were hydrolysed with the restriction endonucleases *Nde*I and *Bam*HI and correspondingly ligated into vectors pET11a and pET16b. The 2.2 kb *Bam*HI fragment was isolated from recombinant plasmid pAS2 [EP A 0 730 029/DE 19507214] and fused to the cloned PCR fragments by way of the *Bam*HI recognition site. After the orientation of the 2.2 kb *Bam*HI fragment had been checked, the complete *acbC* gene was present in the expression vectors. The expression vectors were given the designations pAS8/1 - pAS8/4 (Fig. 4). In addition, the complete *acbB* reading frame (in the opposite orientation) and the beginning of the *acbA* gene are present on the cloned DNA in the expression vectors. For each of the

vectors, it was posisble to identify an additional protein which was expressed in IPTG-induced *E.coli* BL21pLys cultures. The sizes of the overexpressed AcbC proteins are given in Tab. 4. However, all the proteins were synthesized in the form of insoluble inclusion bodies.

Tab. 4: The structures of the AcbC expression vectors for expression in *E.coli*

Recombinant plasmid	Start point	Vector plasmid	Recombinant protein
pAS8/1	1	pET11a	42 kDa
pAS8/3	2	pET11a	41 kDa
pAS8/2	1	pET16b	44.5 kDa
pAS8/4	2	pET16b	43.5 kDa

15.2 Overexpression of AcbC in *S. lividans* 1326

The AcbC protein was expressed in *S. lividans* 1326 using the plasmid vector pIJ6021 [Takano, E., et al. (1995)]. A fragment encompassing only the coding region of the *acbC* gene was amplified from chromosomal DNA using the PCR method [Mullis and Falloona, (1987)]. The oligonucleotide AS-C1 and AS-C2 were employed for the PCR, with the AS-C1 primer (sequence position 6089) being used to construct an *Nde*I recognition sequence at start codon 2 of the *acbC* gene. Oligonucleotide AS-C2 binds to sequence position 7882 and was used to construct an *Eco*RI recognition sequence. The primers were annealed in 20 sec at 50°C, and the primers were extended in 40 sec. The resulting *acbC* DNA fragment was first of all blunt-end cloned into the pUC18 vector and the DNA sequence was checked for correctness following the PCR. This recombinant plasmid, containing the cloned *acbC* gene, was given the designation pAS8/5.1. Plasmid pAS8/5.1 was hydrolysed with the restriction endonucleases *Nde*I

and *Eco*RI and the DNA was separated on an agarose gel and then eluted from the matrix. The *acbC* fragment which had been prepared in this way was ligated into Vector pIJ6021. The recombinant expression plasmid was given the designation pAS8/7.2 (Fig. 5). Protoplasts of *S. lividans* 1326 were transformed with plasmid pAS8/7.2. The resulting clone was used to overexpress the AcbC protein in soluble form in thiostrepton-induced cultures (Fig. 6).

16. Overexpression of AcbE in *S. lividans* TK23

The *acbE* gene was isolated from plasmid pAS5/6.9-6 by hydrolysing the plasmid with restriction endonucleases *Eco*RI and *Hind*III. After the DNA had been separated on an agarose gel, and the 3.8 kb *Eco*RI/*Hind*III fragment had been eluted from the matrix, this *acbE* fragment was appropriately ligated into vector pUWL219 [J. Wehmeier, U.F. (1995)]. The intention was to use a possible promoter sequence on the 200 bp upstream region in these vectors for subsequently expressing AcbE in *S. lividans* (see Tab. 5). The recombinant plasmid was given the designation pAS11 (Fig. 7).

Tab. 5 The intercistronic region between genes *acbE* and *acbD*. Inverted repeat (IR) and direct repeat (DR) sequences which could be involved in regulation are underlined.

Protoplasts of *S. lividans* TK23 were transformed with plasmid pAS11. Both in the case of the *S. lividans* TK23/pAS11 samples and in the case of the *Actinoplanes* sp.

samples, it was possible to detect an extracellular protein of 110 kDa in size in the supernatants from MD 50 cultures (Fig. 8). This size corresponds to the molecular weight of the protein derived from *acbE*. The identities of these proteins were demonstrated by appropriate enzyme tests (see Example 19.2) and by sequencing the N-terminal amino acids (see Example 18). It was not possible to detect any corresponding protein in the supernatant from the control *S. lividans* TK23/pUWL219 culture in MD 50 medium. For this reason it is probable that the possible promoter sequence (Tab. 5) upstream of the *acbE* gene is responsible for expressing AcbE in the *S. lividans*/pAS11 cultures in MD 50 medium.

17. Gel-electrophoretic preparation of proteins

Proteins were separated in denaturing SDS polyacrylamide gels, and were stained with Coomassie dye, in accordance with the method of Lugtenberg (1975). 8% or 11% gels were used depending on the sample.

Gel composition (11% gel)

	Resolving gel	Stacking gel
Solution A*	8.0 ml	
Solution B*		0.64 ml
APS (2 mg/ml)	0.8 ml	0.15 ml
10% (w/w) SDS	0.64 ml	0.064 ml
0.75M tris/HCl, pH 8.8	16 ml	
0.25M tris/HCl, pH 6.8		3.2 ml
Distilled water	6.56 ml	
TEMED	25 µl	10 µl

* see buffers and solutions

The electrophoresis was carried out using either a SERVA blue vertical 100/C apparatus (gel dimensions, 80 × 100 × 0.75 mm) or using a Renner twin vertical apparatus (gel dimensions, 180 × 170 × 1 mm).

The protein concentrations of the samples to be analysed were determined by means of the BioRad, Munich, protein assay, with BSA being used to construct a calibration curve. The VIIL Dalton marker (14.2 kDa - 66 kDa) and the high molecular weight standard (29 kDa - 210 kDa) supplied by Sigma (Deisenhofen) were used as standards for the molecular weights of the separated proteins.

18. Determination of the N-terminal amino acid sequence

The N-terminal amino acid sequences of the AcbE protein derived from *Actinoplanes* sp. and the *S. lividans* TK23/pAS11 clone were determined for comparison. For this, 50 ml cultures were incubated in MD 50 medium for 3 days. The cells were removed by centrifugation and the culture supernatants were dialysed at 4°C for 12 h against buffer (5 mM tris/HCl, pH 7.5, 1 mM $CaCl_2$). The supernatants were subsequently freeze-dried for 48 h and the freeze-dried residues then taken up in 1.5 ml of sample buffer. The culture supernatants which had been prepared in this way were separated by SDS-PAGE using the Renner twin vertical apparatus (gel dimensions,

180 × 170 × 3 mm). A gradient gel (5% → 10%) was employed in order to ensure the best possible separation of the AcbE protein from other extracellular proteins. The proteins were transferred from the SDS polyacrylamide gel to a polyvinyl fluoride (PVDF) membrane (Amersham Buchler, Braunschweig) in a semi-dry electrophoretic procedure using a Fast Blot B33 apparatus (Biometra, Göttingen) in accordance with the manufacturer's instructions. The transfer was carried out at 250 mA for 45 min. Running buffer diluted 1:2 (see Buffers and Solutions) was used as the transfer buffer. The membranes were stained for 30 min and then destained with destaining solution (see Buffers and Solutions). In order to determine the N-terminal amino acid sequence, the blot specimen was washed twice with on each occasion 100 µl of 50% methanol prior to the sequencing in order to remove excess salts. After drying, the sequence analysis was carried out using a blot cartridge and a filter which had been pretreated with polybrene. The fast blot cycle was used for the sequence analysis. The result is shown in Tab. 6.

Tab. 6: The results of sequencing the N-terminal amino acid sequence of the AcbE protein from *Actinoplanes* sp. and the *S.lividans* TK23/pAS11 clone

Organism	**N-terminal amino acid sequence determined**
Actinoplanes sp.	Sequence 1: ESPPDRPSHAEQLYL Sequence 2: SPPDRPSHAEQLYL
S.lividans TK 23/pAS11	Sequence 1: ESPPDRPSHAEQLYL Sequence 2: SPPDRPSHAEQLYL

19. Determination of enzyme activities

19.1. Determination of valiolone synthase activity

For overexpressing AcbC, 10 ml of YEME medium (50 µg/ml Km) were inoculated with a suspension of *S. lividans* 1326/pAS8.7.2 spores. After culturing for from 1 to 2 days, the cultures were in the early logarithmic phase of growth. At this time, the cultures were induced with 7.5 µg/ml thiostrepton. The cultures were then harvested at 20 h after the induction. The sedimented cells were taken up in 1.5 ml of cold disruption buffer (see Buffers and Solutions) and carefully disrupted by means of ultrasonication. The cell debris were removed by centrifuging at 15,000 g for 30 minutes at 4°C. The AcbC extract required for the enzyme test was prepared by dialysing (12 h) at 4°C against 2.5 litres of disruption buffer. It was possible to store this extract at -20°C for two months without any noticeable loss of activity. The protein content of the extract was determined using the Biorad, Munich, protein assay and 15 µg were analysed in an SDS-PAGE run (Fig. 6). The enzyme test was carried out, at RT for 2 h, in a 20 mM P buffer (pH 7.5) containing 40 µM $CoCl_2$. 20 µg of total protein from the AcbC extract and 8 mM sedoheptulose 7-phosphate were employed in the enzyme test. In addition, the reaction mixture contained 2 mM NaF in order to inhibit unspecific phosphatases in the extract. The reaction volume was 100 µl. The results of the test were determined by analysing 25 µl from a reaction mixture by TLC on silica gel films using butanol/ethanol/H_2O (9:7:4) as the mobile phase. The organic compounds were visualized by spraying the TLC films with cerium reagent (see Buffers and Solutions) and then incubating the films at 90°C for 15 minutes in a drying oven. A mixture of valienone and valiolone (Prof. H.G. Floss, Seattle) was used as the reference substance.

The AcbC protein expressed in *S. lividans* specifically converted sedoheptulose 7-phosphate (Fig. 9). However, the reaction product exhibited a migratory behaviour in the TLC which differed to a minor extent from that of the valienone/valiolone standard. The possibility that the reaction buffer had decreased the distance migrated by the reaction product on the silica gel film could not be excluded (Fig. 9, Track 5).

19.2. Determination of α-amylase activity

The cultures of *S. lividans* TK23/pAS11 were grown in TSB medium and MD 50 medium in the presence of 25 µg/ml thiostrepton. The cultures were harvested after having been incubated for 3-4 days. The cells were removed by centrifuging (3500 g) for 10 min at 4°C. The supernatants were dialysed at 4°C for 12 h against buffer (25 mM tris/HCl, pH 7.5, 1 mM $CaCl_2$). 500 µl volumes from the supernatants which had been prepared in this way were dried in vacuo and the dried residues were taken up in sample buffer (see buffers and solutions); the proteins in the supernatants were separated in an SDS-PAGE run (Fig. 8). Supernatants from *Actinoplanes* sp. cultures which had been grown under identical conditions were used as references. The α-amylase activity was determined by measuring the decrease in the turbidity of a 1% starch suspension. In order to carry out a measurement, 100 µl of dialysed culture supernatant were mixed with 900 µl of starch suspension and the decrease in the extinction at 300 nm with time was recorded [Virolle, M.J., et al. (1990)]. Corresponding investigations using a *Bacillus* sp. (Sigma, Deisenhofen) α-amylase (Sigma, Deisenhofen) were carried out for comparison. The results are presented in (Fig. 10). In the test, it was not possible to inhibit the AcbE activity in *Actinoplanes* sp. MD 50 cultures and in *S. lividans* TK23/pAS11 MD 50 cultures with 1 mM acarbose. On the other hand, the background activity in *S. lividans* TK23/pUWL219 MD50 cultures was inhibited by 0.1 mM acarbose. The *Bacillus* sp. α-amylase was also inhibited by 0.1 mM acarbose (Fig. 10).

Buffers and Solutions:

Media for growing bacteria

LB medium:

Tryptone	10 g
NaCl	10 g
Yeast extract	5 g
H_2O	to 1000 ml

The pH was adjusted to 7.5 with 4M NaOH

MD 50 medium

Solution I

MD50 starch hydrolysate	70 g
$(NH_4)_2SO_4$	5 g
Yeast extract	2 g
H_2O to 400 ml	

Solution II

K_2HPO_4	1 g
KH_2PO_4	1 g
Trisodium citrate	5 g
H_2O to 400 ml	
pH adjusted to 7.0 with 1M NaOH	

Solution III

$MgCl_2 \cdot 6H_2O$	1 g
$FeCl_3 \cdot 6H_2O$	0.25 g
$CaCl_2 \cdot 2H_2O$	2 g
H_2O to 200 ml	

After mixing, the solutions are sterilized by filtration.

TSB medium:

Tryptone-soya broth (Oxoid)	30 g
H_2O	to 1000 ml

TSB PEG 8000 [see Babcock et al. (1988)]:

Tryptone soya broth (Oxoid)	30 g/l
PEG 8000	50 g/l
after autoclaving:	
Glycine (20%)	25 ml
$MgCl_2$ (2.5M)	2 ml

YEME [Hopwood, D.A., et al. (1985)]

Yeast extract	3 g/l

Peptone	5 g/l
Malt extract	3 g/l
Glucose	10 g/l
Sucrose	340 g/l
after autoclaving	
$MgCl_2$ (2.5M)	2 ml

Standard preparation of plasmid DNA [modified from Birnboim and Doly (1979)]

Mix I	50 mM	glucose
	50 mM	tris/HCl (pH 8.0)
	10 mM	EDTA (pH 8.0)
	5	mg/ml lysozyme
Mix II	200	mM NaOH
	1% (w/v)	SDS (Sodium dodecyl sulphate)
Mix III	3M	potassium acetate
	1.8M	formate

TE buffer (pH 8.0)

Tris/HCl	10 mM
Na_2 EDTA	1 mM

DNA-DNA/hybridization

20× SSC

3M	NaCl
0.3M	Na citrate

Prehybridization solution:

6× SSC

0.01M	sodium phosphate buffer, pH 6.8
1 mM	EDTA
0.5%	SDS
0.1%	skimmed milk powder

Hybridization solution:

After the labelling reaction, the acb probe is added to 15 ml of prehybridization solution.

6× Postwash

6×	SSC
0.5%	SDS

DNA sequencing:

TBE buffer (pH 8.0)

1M	Tris base
0.83M	Boric acid
10 mM	EDTA

Denaturing polyacrylamide gel electrophoresis of proteins

5× sample buffer

Glycerol	25 ml
SDS	5 g
BPB	2.5 mg
2-Mercaptoethanol	12.5 ml

0.625M Tris/HCl (pH 6.8) to 50 ml

Electrode buffer

Tris/HCl (pH 8.3)	25 mM
Glycine	190 mM
SDS (w/v)	0.1%

pH adjusted before adding SDS

Solution A

Acrylamide	44 g
N,N-methylene-bisacrylamide	0.8 g

H_2O to 100 ml

Solution B

Acrylamide	30 g
N,N-methylene-bisacrylamide	0.8 g
H_2O to 100 ml	

Dye solution

SERVA blue R-250 (w/v)	0.15%
Methanol (v/v)	50%
Acetic acid (v/v)	10%

Destaining solution

Methanol (v/v)	25%
Acetic acid (v/v)	10%

AcbC disruption buffer

K_2HPO_4/KH_2PO_4 (pH 7.5)	20 mM
NAD	0.2 mM
DTT	0.5 mM

α-Amylase test phosphate buffer

K_2HPO_4/KH_2PO_4 (pH 6.8)	50 mM
KCl	50 mM

Cerium reagent

Molybdophosphoric acid	1.25 g
Cerium(IV) sulphate	0.5 g
H_2SO_4	3 ml
H_2O to 50 ml	

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Figure legends

Fig. 1 Restriction map of the approx. 18 kb sequenced segment from the genome of *Actinoplanes* sp. SE50/110 (cf. Fig. 2). The thick black line indicates the region claimed in the original patent, which region partially overlaps the *acbCBA* genes (order as indicated from left to right).

Fig. 2 Gene map of the acarbose biosynthesis clusters.

Fig. 3 DNA sequences from the acarbose biosynthesis cluster.

Fig. 4 The recombinant plasmids which were constructed from plasmids pET11a and pET16b for expressing AcbC in *E.coli*.

Fig. 5 The recombinant plasmid pAS8/7.2 which was constructed from plasmid pIJ6021 for expressing AcbC in *S. lividans* 1326.

Fig. 6 Gel-electrophoretic separation of cell lysates (see Example 15.2). The expression of AcbC (42 kDa) in the thiostrepton-induced *S. lividans* 1326/pAS8/7 culture is shown in track 3.

Fig. 7 The recombinant plasmid pAS11 which was constructed from plasmid pUWL219 - for expressing AcBE in *S. lividans TK 23*.

Fig. 8 Gel-electrophoretic separation of proteins from culture supernatants (see Example 16). The expression of AcbE (110 kDa) can be seen in track 2, track 5 and track 6.

Fig. 9 Detection of AcbC enzyme activity by means of thin layer chromatography on silica gel films (see Example 19.1).

1) Extract from *Actinoplanes* sp.
2) Extract from *S. lividans* 1326/pJ6021

3) Extract from *S. lividans* 1326/pAS8/7.2 (extract stored at -20°C for 2 months)
4) Extract from *S. lividans* 1326/pAS8/7.2 (boiled)
5) Extract from *S. lividans* 1326/pAS8/7.2 (valienone instead of sedoheptulose 7-phosphate as the substrate)
6) Valiolone/valienone standard
7) Sedoheptulose
8) Sedoheptulose 7-phosphate
9) Extract from *S. lividans* 1326/pAS8/7.2 (extract freshly prepared)

Fig. 10 Determination of the α-amylase activity in culture supernatants. The bacteria are grown in MD50 medium. No activity was measured with boiled culture supernatants. The duration of the test was 6 min. For comparison, 2.8 mU of purchased α-amylase were used in each of samples 9-11.

Patent claims

1. Acarbose biosynthesis gene cluster comprising DNA from Fig. 3.

2. Acarbose genes comprising DNA from Fig. 3.