Genomic Analysis of Secondary Metabolite Producing Actinomycetes: AcbM is a 2-*epi*-5-*epi*-valiolone 7-kinase

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CONTENTS

ABBREVIATIONS

A	adenine
aa	amino acid
ALF	automatic laser fluorescence DNA sequencer
App.	appendix
APS	ammonium persulfate
ATCC	American Type Culture Collection, Maryland
ATP	adenosine 5'-triphosphate
AUD	amplifiable unit of DNA
BBA	Biologische Bundesanstalt für Land- und Forstwirtschaft, Dresden
bp	base pair
BSA	bovine serum albumin
C	cytosine
ca.	*circa*
cf.	confers to
Ci	curie
CIAP	calf intestine alkaline phosphatase
Cml	chloramphenicol
COSY	correlation spectroscopy
DEPT	distortionless enhancement by polarization transfer
DMF	dimethylformamide
DMSO	dimethylsulfoxide
(r)DNA	(ribosomal) deoxyribonucleic acid
dNTP	deoxynucleoside-5´-triphosphate
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig
dTDP	2'-deoxythymidine diphosphate
EDTA	ethylene diaminetetraacetic acid
e.g.	example given, for instance
ETH	Eidgenössische Technische Hochschule, Zürich
FAD	falvin adenine dinucleotide
Fig.	figure
g	gram
G	guanine
h	hour
IC-MS	ion chromatography coupled with mass spectrometry
IMET	Institut für medizinische experimentelle Technologie, Jena
IPTG	isopropylgalactoside
kb	kilobase
kDa	kilodalton
min	minute
Mr	relative molecular weight
NAD(P)	nicotinamide adenine dinucleotide (phosphate)

NAD(P)H	nicotinamide adenine dinucleotide (phosphate), reduced form
NMR	nuclear magnetic resonance
nt	nucleotide
NRRL	Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinios
OD_X	optical density at x nm and 1 cm depth
orf	open reading frame
ori	origin of replication
p.a.	pro analysis
PAGE	polyacrylamide gelelectrophoresis
PAA	polyacrylamide
p.c.	personal communication
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed field gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinyl difluoride
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphisms
rpm	rounds per minute
RT	room temperature
S	Svedberg unit
S.	*Streptomyces*
SDS	sodiumdodecylsulfate
Sect.	section
Sm	streptomycin
Spc	spectionmycin
T	thymine
Tab(s).	table(s)
TEMED	N, N, N´, N´-tetramethylethylendiamine
TES	trishydroxymethylaminoethansulfonic acid
Thio	thiostrepton
TIR	terminal inverted repeat
TLC	thin-layer chromatography
TPP	thiaminpyrophosphate
Tris	trishydroxymethylaminomethane
U	enzyme units
UV	ultraviolet
V	volt
X-Gal	5´-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

SUMMARY

This dissertation falls into two only distantly related parts - "gene sponge" and acarbose biosynthesis. The following aspects and results have been worked on and achieved.

I. Gene sponge:

The basic steps for the use of deletion strains of a streptomycete as so called "gene sponges" for picking up large-scale DNA from other microorganisms in soil have been done. (1) A natural transfer of a non-conjugative plasmid pIJ702 from *S. lividans* 66 TK23 to recipient strain Jni13C1 was demonstrated in sterile soil; (2) Some soil isolates with recipient phenotype were analyzed by PCR, RAPD, comparison of 16S rRNA-RFLPs and PFGE experiments on the genomic DNA; (3) Three conjugative recombinants from matings between non-deletion (donor) and deletion strains of *S. lividans* 66 resulted from transfer events, in which large DNA fragments (ca. 500 kb) from the left chromosomal arm were integrated into the recipient strains. Two of these, M3-1 and M1-107, had linear chromosomes with duplicated end regions including a chloramphenicol resistance gene at both ends of the chromosome.

II. Acarbose biosynthesis:

(1) The genome size of *Actinoplanes* sp. SE50/110 was estimated to be of about 6.5 Mb and the complete acarbose gene cluster (*acb*) was localized on a 45 kb *Xba*I/*Ase*I chromosomal fragment as a single copy; (2) The proteins, AcbK, M, L, N, O and U were heterologously overproduced in *E. coli* and/or *S. lividans* in soluble forms mostly as their His-tag versions; (3) The activity of AcbK was confirmed and used to demonstrate the heterologous production of acarbose-related compounds in a *S. lividans* strain containing the whole *acb*-cluster; (4) 1-*epi*-valienol was the only cyclitol phosphorylated by crude extracts of *Actinoplanes* sp.; but no Acb-proteins could be identified catalyzing this reaction; (5) The cyclitol precursor 2-*epi*-5-*epi*-valiolone was enzymatically and preparatively synthesized by use of transketolase and AcbC; (6) AcbM was characterized as a 2-*epi*-5-*epi*-valiolone 7-kinase and its product was characterized by IC-MS and NMR analysis; (7) 2-*epi*-5-*epi*-valiolone-7-phosphate was identified as the substrate for the isomerase AcbO, the product of which was likely to be 5-*epi*-valiolone-7-phosphate; (8) A new pathway for the biosynthesis of acarbose is presented.

ZUSAMMENFASSUNG

Diese Dissertation behandelte zwei nur entfernt miteinander verwandte Teile – Genschwamm und Biosynthese der Acarbose. Die folgenden Aspekte und Ergebnisse wurden ausgearbeitet.

I. Genschwamm:

Die grundlegenden Schritte für die Verwendung von Deletionsstämmen eines Streptomyceten als sogenannte Genschwämme, die der DNA-Aufnahme durch andere Mikroorganismen im Boden dienen, wurden durchgeführt. (1) Ein natürlicher Transfer eines nicht konjugativen Plamides, pIJ702, von *S. lividans* 66 TK23 auf den Stamm Jni13C1 konnte in sterilem Boden demonstriert werden; (2) Einige Isolate aus dem Boden mit Rezipientenphänotyp wurden durch PCR, RAPD und den Vergleich der genomischen DNA mit 16S rRNA-RFLPs und PFGE analysiert; (3) Drei Rekombinanten aus Konjugationsexperimenten mit Nicht-Deletionsstämmen und Deletionsstämmen von *S. lividans* resultieren aus Übertragungen, bei denen große DNA Fragmente (ca. 500 kb) aus dem linken Chromosomenarm in den Empfängerstamm integriert wurden. Zwei solcher Stämme, M3-1 und M1-107, hatten lineare Chromosomen mit duplizierten Endbereichen einschließlich eines Chloramphenicol Resistenzgenes an beiden Chromosomenden.

II. Biosynthese der Acarbose

(1) Die Größe des Genomes von *Actinoplanes* sp. SE50/110 wurde auf ca. 6.5 Mb geschätzt. Das komplette Gencluster der Acarbose (*acb*) befindet sich als einfache Kopie auf einen ca. 45 kb *Xba*I/*Ase*I Fragment; (2) Die Proteine, AcbK, M, L, N, O und U wurden heterolog in *E. coli* und/oder *S. lividans* in löslicher Form, meistens als His-tag Versionen, überproduziert; (3) Die Aktivität des AcbK Proteins wurde bestätigt und genutzt um die heterologe Produktion der Acarbose-verwandter Substanzen in einem *S. lividans* Stamm, der das komplette *acb*-Cluster enthielt, zu demonstratieren; (4) Das 1-*epi*-Valienol wurde als einziges der getesten Cyclitole durch Rohextrakte aus *Actinoplanes* sp. phosphoryliert; aber kein Acb-Protein wurde identifiziert, das diese Reaktion katalysiert; (5) Das 2-*epi*-5-*epi*-Valiolon wurde durch Transketolase und AcbC enzymatisch und präparativ synthesiert; (6) AcbM wurde als eine 2-*epi*-5-*epi*-Valiolon 7-Kinase charakterisiert und ihr Produkt wurde durch IC-MS und NMR analysiert; (7) 2-*epi*-5-*epi*-Valiolon-7-Phosphat wurde als das Substrat der Isomerase AcbO identifiziert, deren warscheinliches Produkt 5-*epi*-Valiolon-7-Phosphat ist; (8) Ein neuer Biosyntheseweg der Acarbose wird vorgestellt.

1: INTRODUCTION I - Gene Sponge

1.1: Taxonomy, ecology and genetic features of the genus *Streptomyces*

Members of the genus *Streptomyces* are filamentous gram-positive soil bacteria (Wakesman and Lechevalier, 1962) with a high potential in biotechnology (Piepersberg, 1993). The genus *Streptomyces* is defined by both chemotaxonomic and phenotypic characters (Miyadoh, 1990). The major taxonomical emphasis lies now on 16S rRNA homologies, in addition to cell wall analysis and fatty acid and lipid patterns (Williams *et al.*, 1989; Wellington *et al.*, 1992; Mehling *et al.*, 1995b). One of the quickest methods for preliminary identification to genus level was the presence of the LL isomer of diaminopimelic acid (LL-DAP) as the diamino acid in the peptidoglycan (Kieser *et al.*, 2000).

Streptomycetes are ubiquitous in nature. The genus *Streptomyces* has been isolated from any type of natural habitat including soils, sediments of lakes, rivers, estuaries and marine environments (Piepersberg, 1993). *Streptomyces* undergo complex morphological differentiation including growth of substrate mycelia in the initial phase, followed by development of aerial mycelium and its subsequent conversion to spores (Chater, 1993; Piepersberg, 1993). Streptomycete spores are not particularly heat resistant although they tolerate mildly elevated temperatures (Ensign, 1978). Spores are resistant to dessication and are metabolically almost dormant (Dworkin *et al.*, 1985). The cytoplasmic constituents do not differ greatly from those of mycelium indicating that spores may essentially be a dispersal phase, allowing rapid germination when nutrients become available (Ensign, 1978). The mycelial stage is sensitive to drought, whereas the spores impart resistance to low nutrient and water availability (Karagouni *et al.*, 1993), enabling the survival of streptomycetes in soil for long time (Mayfield *et al.*, 1972; Ensign, 1978). For instance, viable *Streptomyces* cultures were recovered from 70 year old soil samples (Morita, 1985). Members of the genus *Streptomyces* are well known as producers of many secondary metabolites possessing some biological activities (cf. **Sect.** 1.2). Actinomycetes make two-thirds of the known antibiotics that are produced by microorganisms and among them nearly 80% are made by members of the genus *Streptomyces* (Piepersberg, 1993, Kieser *et al.*, 2000).

A typical property of the genomic DNA of streptomycetes is the high G+C content of 72 to 75 mole % (Enquist and Bradley, 1971; Piepersberg, 1993) which results in a highly biased codon usage (Wright and Bibb, 1992). The G+C content is of intermediate concentration (ca. 70%) in

the first codon position, lowest in the second (ca. 50%) and highest (ca. 90% and above) in the third codon position. These features can be perfectly used in gene identification (Bibb *et al.*, 1984). *Streptomyces* are one of the few examples of bacteria with a proven linear chromosome (Chen, 1996; Kolsto, 1997). The linearity of the chromosome of *Streptomyces* sp. was first demonstrated in *Streptomyces lividans* (Lin *et al.*, 1993), and then generally confirmed for other species. *Streptomyces* chromosomes have a size of about 8 Mb, as revealed by pulsed field gel electrophoresis (Lezhava *et al.*, 1995; Leblond *et al.*, 1996; Pandza *et al.*, 1997). Recently, the complete sequence of the *Streptomyces coelicolor* A3(2) genome revealed that the chromosome is 8,667,507 bp long with a G+C content of 72.1% and is predicted to contain 7825 protein encoding genes (http://www.sanger.ac.uk/Projects/S_coelicolor/). The linear ends of the chromosomes of *Streptomyces* sp. consist of terminal inverted repeats (TIRs) of various sizes (24-500 kb; Leblond *et al.*, 1993; Lin *et al.*, 1993; Redenbach *et al.*, 1993; Lezhava *et al.*, 1995; Pandza *et al.*, 1997). They are equipped with proteins covalently bound to both free 5´ ends (Lin *et al.*, 1993). These proteins probably act as primers for the synthesis of the last Okazaki fragment of the lagging strand when bidirectional DNA replication reaches the free ends, after initiation at a typical *oriC* (Musialowski *et al.*, 1994) located centrally in the chromosome (Chang and Cohen, 1994; Chen, 1996; Redenbach *et al.*, 1996). The linear chromosome of *Streptomyces* sp. represents one of the most spectacular examples of genetic instability among prokaryotes (Volff and Altenbuchner, 1998). Thus, the *Streptomyces* sp. chromosomes undergo very large deletions spontaneously at rates higher than 0.1% of spores, removing up to 25% of the genome (Volff and Altenbuchner, 2000). This phenomenon of genetic instability seems to be ubiquitous in the genus *Streptomyces* (Leblond and Decaris, 1994; Dharmalingam and Cullum, 1996; Volff and Altenbuchner, 1998). Frequently, the telomeres of the chromosomes are included in the deletions. Loss of both telomeres leads to circulation of the chromosome (Redenbach *et al.*, 1993). Deletions are frequently accompanied by high-copy-number tandem amplifications of specific sequences called amplifiable units of DNA (AUDs) and probably by large rearrangements of the chromosome (Redenbach *et al.*, 1993; Rauland *et al.*, 1995; Volff *et al.*, 1996). The wild type chromosome can also be circularized artificially by targeted recombination. Nevertheless, spontaneously or artificially circularized chromosomes are at least as unstable as the corresponding linear ones (Volff *et al.*, 1997a; Lin and Chen, 1997; Fischer *et al.*, 1997). Genetic instability in *Streptomyces* sp. affects different phenotypic properties,

including morphological differentiation, production of secondary metabolites, antibiotic resistance, secretion of extracellular enzymes and sometimes genes for primary metabolism, e.g., in *S. coelicolor* A3(2), particularly one or more steps in the arginine biosynthesis pathway (Volff and Altenbuchner, 1998).

1.2: Assessing the microbial and genetic diversity in soil

After the oceans, soil is the most useful and valuable habitat on earth, which has been used for planting crops, for mining for minerals, for building on and for discovering medicinal chemicals. Soil is a highly complex environment dominated by the soil solid phase, which is relatively recalcitrant to mixing in contrast to watery ecosystems (van Elsas and Smalla, 1997). The soil microbiota, including bacteria, fungi and protozoa, is localized in close association with soil particles, mainly clay-organic matter complexes (Foster, 1988).

Over the past 60 years, products derived from microbial secondary metabolites have been used to meet medical, industrial and agricultural needs, e.g., antibiotics, anticancer drugs, antifungal compounds, immunosuppressive agents, enzyme inhibitors, anti-parasitic agents, herbicides, insecticides and growth promoters (Omura, 1992). Most microbial secondary metabolites in use today come from soil-dwelling microorganisms, the most prolific of which have been the actinomycetes. Pharmaceutical chemists and microbiologists have been culturing the diverse microbes of the soil and screening them for useful products. But of late, the yield of new natural products from the soil microflora has been poor, in part because culturing recovers the same organisms again and again. In actinomycetes, for example, the rediscovery rate for antibiotics is 99% (Zaehner and Fiedler, 1995). However, most microorganisms are not recovered by cultivation (DeLong, 1996). Research over the past 20 years has shown that the extent of microbial diversity in soil is largely unknown (Rondon *et al.*, 1999b). Current attempts to describe and understand microbial diversity are aimed at overcoming the culturing bias in an effort to further characterize those "unculturable" microorganisms, using molecular methods and phylogenetic analysis based on DNA sequence information (**Fig.** 1.1, Theron and Cloete, 2000). Many methods for investigating microbial diversity are based on 16S-rRNA-gene sequence analysis. Commonly, 16S-rRNA-gene sequences are amplified from DNA isolated from environmental sources and analyzed by several methods, including cloning and sequencing, amplified-ribosomal-DNA-restriction analysis (ARDRA), denaturing gradient gel electrophoresis

(DGGE), temperature gradient gel electrophoresis (TGGE), dot blots, single-strand conformational polymorphisms (SSCP), and terminal-restriction-fragment-length polymorphism (T-RFLP) analysis (**Tab.** 1.1). The results based on these methods have revealed that soil represents one of the most diverse habitats for microorganisms with great genetic diversity. Many lines of evidence show that fewer than 0.1% of the microorganisms in soil are readily cultured using current techniques (Torsvik *et al.*, 1996). And, most impressively, the other 99.9% of the soil microflora are emerging as a world of stunning, novel genetic diversity. New groups of bacteria have been identified in soil that appear to diverge so deeply from the cultured bacteria that they could represent new phyla, or even new kingdoms of life (Borneman *et al.*, 1996; Kuske *et al.*, 1997). Tapping into this source of genetic diversity in soil should be a great, future joint adventure for biologists and chemists (Rondon *et al.*, 1999b).

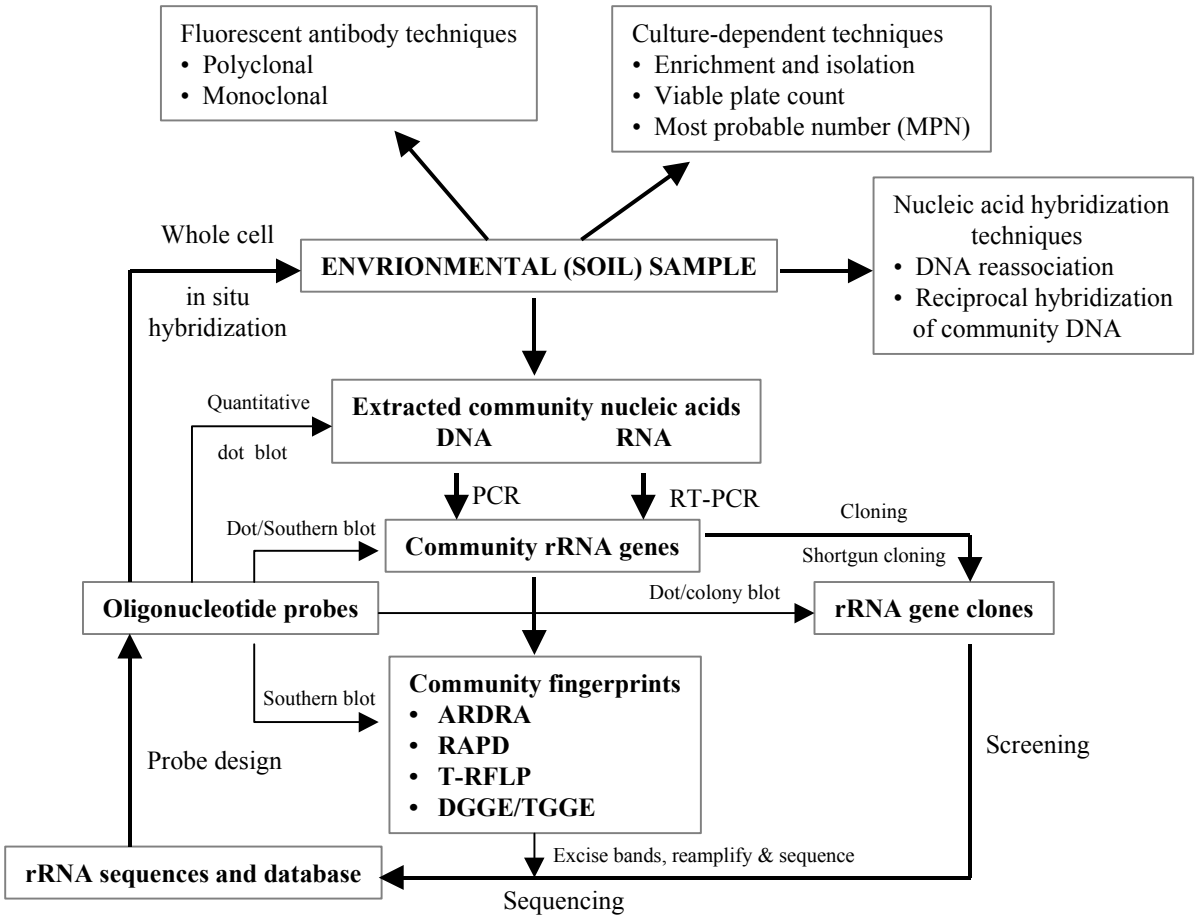


Fig. 1.1: Commonly used molecular approaches in microbial ecology (After Theron and Cloete, 2000). Abbreviations see Tab. 1.1.

Table 1.1: Methods for analysis of microbial diversity

Method	Comments	References
Culturing	Not representative	
16S rRNA gene sequence analysis	Cloning required; provides identification of members of community	Hugenholtz and Pace (1996)
In situ hybridization	Labor intensive, can be used to identify metabolically active microorganisms	Amann and Kühl (1998)
Substrate utilization	Measures metabolic diversity	Garland (1996); Smalla (1998)
DNA–DNA reassociation kinetics	Provides a global view of genetic complexity of sample	Torsvik *et al.* (1990)
ARDRA	More useful for simple communities, useful for comparative analysis	Heyndrickx *et al.* (1996)
PCR amplification or expression cloning	Functional diversity targeted	Dalboge (1997)
BAC libraries	Permanent archive of genetic information from sampled environment; phylogenetic and functional diversity	Handelsman *et al.* (1998)
Flow cytometry	Enumeration of microorganisms	Wallner *et al.* (1997)
RNA dot or slot blot	Representation of metabolically active members of a community	Mobarry et al. (1996)
SSCP	Comparative analysis	Schwieger and Tebbe (1998)
%GC content	Global view of community diversity	Nüsslein and Tiedje (1998)
T-RFLP	Comparative analysis	Marsh (1999)
DGGE or TGGE	Used to monitor enrichment, comparative analysis	Muyzer (1999)

Abbreviations: ARDRA, amplified ribosomal DNA restriction analysis; BAC, bacterial artificial chromosomes; DGGE, denaturing-gradient gel electrophoresis; SSCP, single-strand conformational polymorphisms; TGGE, temperature-gradient gel electrophoresis; T-RFLP, terminal-restriction-fragment-length polymorphisms. (after Rondon *et al.*, 1999b)

1.3: Natural gene transfer in soil

The large-scale transfer of genetic information between distantly or even unrelated organisms during evolution had been inferred from whole genome comparisons (Dröge *et al.*, 1998). Soil represents one of the most diverse habitats of microorganisms, in which share a vast pool of genetic material. Hence, more and more studies are emphasized on the natural gene transfers in soil. Genetic interactions between microorganisms in natural soil environments are accomplished by one of the three basically distinctive transfer mechanisms, transduction, transformation or conjugation (Lorenz and Wackernagel, 1994; Nielsen *et al.*, 1998). Each of these transfer mechanisms has its own characteristics (**Tab.** 1.2). In bacterial transduction, genetic information is transferred from donor to recipient by bacteriophages (Keppel *et al.*, 1988). Phages often display a very narrow host range of infection. Gene transfer by transduction requires a metabolically active donor cell. The recipient can be spatially and temporarily separated form the donor because the genetic information in the transducing particle can persist. Phages are often resistant to many physical and chemical agents and can survive in soil particularly when absorbed on clay minerals (Lorenz and Wackernagel, 1994). Conjugation requires a physical

contact from cell to cell sufficiently stable to allow the transfer of plasmids or transposons from donor to recipient (Clewell, 1993). Conjugation is known to occur frequently between bacteria and the transferable genes usually reside on plasmids. Moreover, chromosomal genes can be transferred if mobilized by a plasmid (Chater *et al.*, 1997). Retro-transfer, the conjugative back-transfer of chromosomal or plasmid genes from infected bacteria to the donor bacteria, has been suggested to mediate chromosomal gene exchange or even of full-size chromosomes (Mergeay *et al.*, 1987). Hence, conjugation is regarded to be an important factor for gene flux among soil bacteria (Dröge *et al.*, 1998). Natural genetic transformation of bacteria encompasses the active uptake by a cell of free or "naked" DNA (plasmid and chromosomal) and the inheritable incorporation of its genetic information (Lorenz and Wackernagel, 1994). Over 43 bacterial species of 24 genera have been identified as naturally competent for transformation (Lorenz and Wackernagel, 1994; Palmen and Hellingwerf, 1997). Evidence indicates that extracellular high-molecular-weight DNAs are present in considerable quantities in soil, e.g. those released from decaying cells (Lorenz and Wackernagel, 1994). The fate of such DNA and the process of natural transformation are schematically represented in **Fig.** 1.2.

Tab. 1.2: Some characteristics of the recognized mechanisms of horizontal gene transfer in bacteria

Characteristics	**Mechanism of gene transfer**		
	Transduction	Conjugation	Transformation
Donor organism	Bacterium infected with a bacteriophage	Bacterium harboring a plasmid or a conjugative transposon	Any organism with double-stranded DNA
Requirements to the recipient bacterium [a]	Attachment sites for binding of bacteriophage (phage receptors)	Able to bind pili from donor bacterium	Competence for uptake of DNA
Vector	Bacteriophage	Plasmid or chromosome with an inserted plasmid or a conjugative transposon	Free DNA
Stability of vector DNA in soil	Long-term stability expected due to protection of DNA in the protein envelopes of bacteriophages	Stability depends on the survival and activity of the donor bacterium	Stability of free DNA is poor due to nucleases, chemical modifications, shearing or binding to solids
Host range dependence	Presence of phage attachment sites	Binding of pili and plasmid incompatibility [b]	Ability of the DNA to integrate into the genome or recircularize into plasmids
Size range of transferred DNA	<100 kb (45 kb)	100 bp to 10^6 kb [c] (size of bacterial plasmids or even full-length of chromosomes)	0 - 25 kb, experimentally. In nature maybe much longer
References	Herron and Wellington (1994); Herron (1995)	Troxler *et al.* (1997); Vionis *et al.* (1998); Ravel *et al.* (2000)	Stotzky *et al.* (1996); Nielsen *et al.* (1997); Sikorski *et al.* (1998)

[a] DNA sequences must be available for several genes, including the gene of interest, in both evolutionarily close and distant organisms.
[b] A low sensitivity is expected due to differences in cultivation requirements for soil bacteria, the level of acquired resistance to the selecting agent, efficiency of plating, and interference from the natural background of resistant bacteria to the selective agent, e.g. antibiotic resistance (Huddleston *et al.*, 1997; Smalla *et al.*, 1997; Nwosu, 2001).
[c] Potential ecological interactions and factors that are important for the transfer process are not evaluated unless natural conditions are mimicked by the use of soil microcosms (Hill and Top, 1998). (After Nielsen *et al.*, 1998)

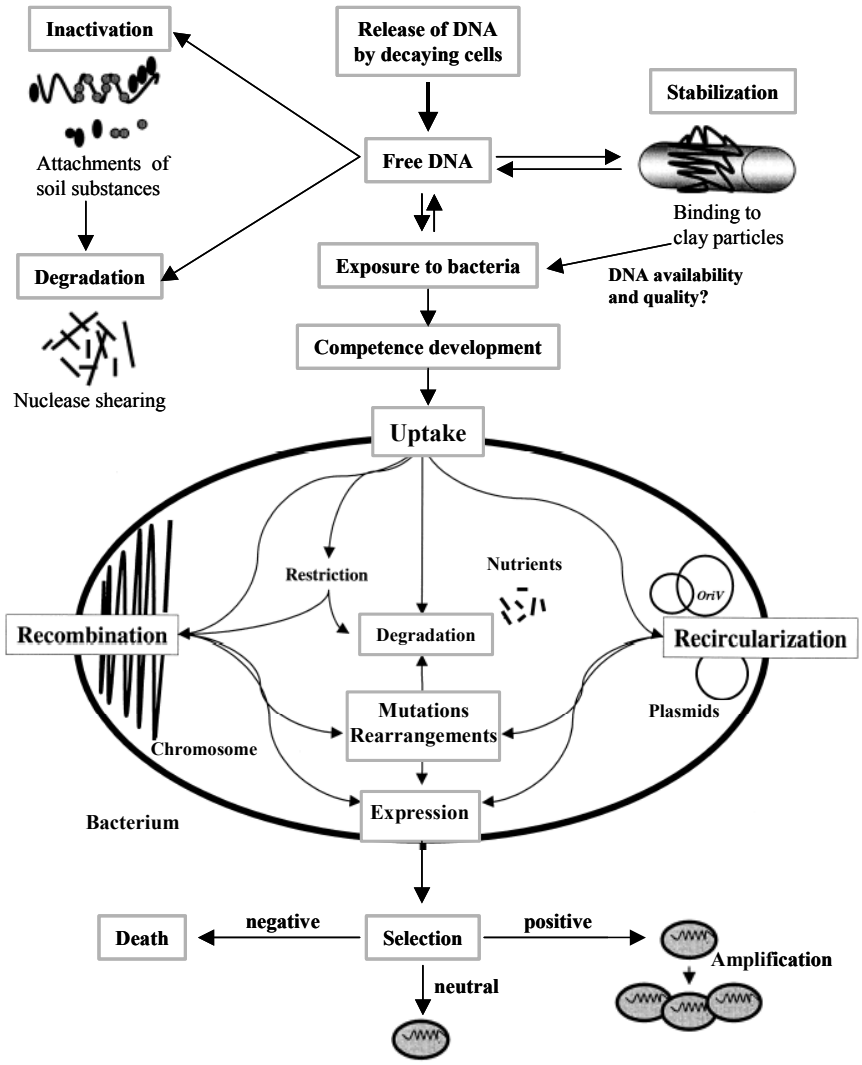


Fig. 1.2: A proposed fate of DNA released into soil from decaying cells. (After Nielsen, 1998)

Sterile and non-sterile soil model systems (microcosms) have been employed and revealed to be a valuable tool to detect gene transfer in soil (Lorenz *et al.*, 1988; Bleakley and Crawford, 1989; Wellington *et al.*, 1990). Over the last few years streptomycetes have provided a useful model for studying microbial interactions and activities in soil, because of their ubiquitous presence in many different soil types (Vionis *et al.*, 1998). Plasmid transfer between streptomycetes in soil has been studied (Bleakey and Crawford, 1989; Wellington *et al.*, 1992). The covalently closed circular plasmid pIJ673 was transferred intra- or interspecifically in sterile and nonsterile soil (Wellington *et al.*, 1990). The interspecific transfer of two giant linear plasmids pRJ3L (322 kb) and pRJ28 (330 kb) has also been demonstrated between *Streptomyces* sp. in sterile amended soil

microcosms (Ravel *et al.,* 2000). The phylogenetic relationships of a collection of streptomycete soil isolates and type strains were resolved by sequence analysis of 16S rRNAs and *trpB*, a housekeeping gene involved in tryptophan biosynthesis. The analysis confirmed that two *S. coelicolor*-like isolates were recipients in a gene transfer event, demonstrated by phylogenetic incongruencies between *trpB* and *strB1* trees. One strain had acquired the entire streptomycin biosynthetic cluster, whilst the other contained only *strRAB1*, originated from *S. griseus* (Huddleston *et al.*, 1997; Wiener *et al.*, 1998; Egan *et al.*, 1998; Egan *et al.*, 2001).

Many factors, such as soil type, volume and moisture, donor/recipient ratios, temperature, pH, antibiotics and nutrient status, have been investigated for effects on the gene transfer events occurring in soil systems (Cresswell and Wellington, 1992; Hill and Top, 1998). A recent report gives laboratory evidence for lightning-mediated gene transfer in soil, indicating that thunder-storms and lightning discharges could lead to the "natural" transformation of soil bacteria (Demaneche *et al.*, 2001).

1.4: Approaches to make use of the genetic diversity in soil

It is believed that production of so-called secondary metabolites in general facilitates the survival of the producing organisms in their biotope like soil and that the end products of secondary metabolism are used as communication systems/substances in a broader sense (Piepersberg, 1993; 1994). All available data support the idea that secondary metabolic traits are inherited as part of a highly variable and mobile gene pool, transferable between widely distant taxonomic branches (**Sect.** 1.3). Accessing the chemistry of microbial diversity presents an enticing but difficult challenge, in part because most of the novel structures are likely to be in organisms present in low abundance in the soil. Developing methods to culture the enormous diversity of soil microflora will be slow and tedious. A more direct, global and rapid method is needed to access the genetic riches of the soil and other microfloras.

1.4.1: The cloning approach

An effort to access the chemical diversity of soil life is by cloning and expressing the metagenome of the soil microflora. The strategy is to isolate metagenomic DNA directly from soil, clone it into a readily culturable organism, e.g. *E. coli*, and screen the clones for biological activity (**Fig.** 1.3).

A route to access the genomes of uncultured organisms is expression-cloning. The directly isolated soil microflora DNA is digested and cloned into a high-copy-number expression vector (**Fig.** 1.3) and the resulting clones are screened for the production of industrial and biotechnological enzymes (Short, 1997). An adaptation of this approach involves PCR-based amplification of selected sequences form soil DNA. Novel polyketide-synthase-gene fragments can be isolated by this method (Dalboge, 1997), providing new sources of molecular diversity for combinatorial biosynthesis and module switching (Hutchinson, 1997).

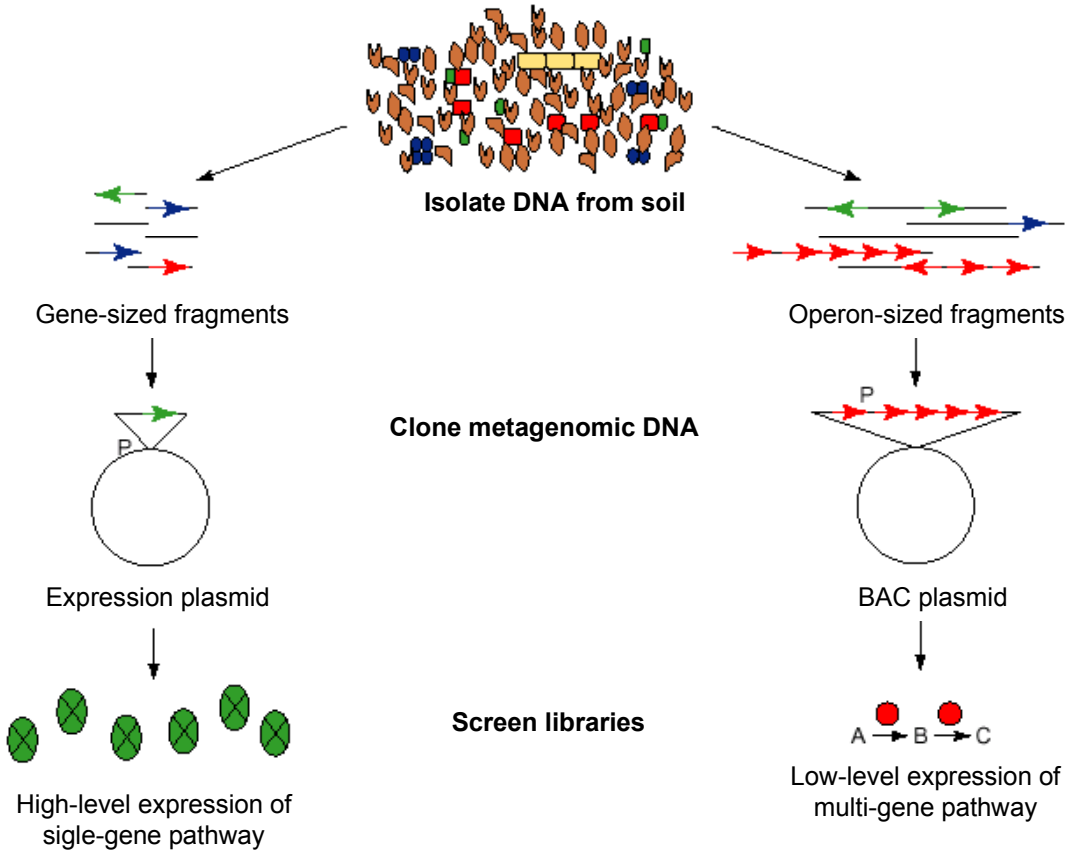


Fig. 1.3: The approach of the expression-cloning and BAC-cloning for capturing the soil metagneome. Genes are represented by horizontal arrows; P = promoters. (After Rondon *et al.*, 1999b).

Another route is to clone soil metagenomic DNA in large pieces into a BAC (bacterial artificial chromosome) vector which is able to maintain large fragments of foreign DNA stably in the *E. coli* host (Rondon *et al.*, 1999a). The genes for natural product biosynthetic pathways are usually clustered in prokaryotes (Distler *et al.*, 1987; Martin *et al.*, 1989; Skaugen *et al.*, 1997; Lomovskaya *et al.*, 1998), therefore, it is feasible to clone an entire pathway in one BAC vector on a

contiguous piece of DNA, which provides a method for capturing, expressing and detecting natural products produced from a BAC library made from soil metagenomic DNA (**Fig.** 1.3). Two such libraries have been constructed. Initial screening has shown that the phenotypes expressed by the clones from these two libraries included antibacterial, lipase, amylase, nuclease and hemolytic activities (Rondon *et al.*, 2000).

Besides the difficulty to reach insert sizes in the range (25 to 100 kb) from soil-extracted DNA, there are several pitfalls and difficulties which could further depress actual chances to find new substances via that way: (i) the expressibility of the whole gene cluster in the host (e.g. *E. coli*); (ii) the stability of plasmid containing gene cluster; (iii) the regulation and metabolic adaptation of substrate fluxes; (iv) resistance development or toxicity of the end product in the host; (v) active secretion of the end product from the host; (vi) sensitive screening test for new substances (chemical/biological).

1.4.2: The *in vivo* "gene sponge" approach

An alternative approach to isolate a complex metabolic trait encoded by a gene cluster form soil samples (and other materials) is *in situ* gene transfer into a seeded recipient ("gene sponge", Piepersberg, 1996, patent). Owing to the need for the seeded recipient (e.g. *Streptomyces*) to adapt its life style and to cope with the environment, it is feasible to think that it is a rapid process to acquire adaptive phenotypes via new genetic material. This could be obtained from the variable gene pool by natural gene transfer when a given cell line/biovar is transplaced into a different biotope (e.g. soil), bringing about a high selective pressure for adaptation. The need for an adaptation of the communication metabolism might even be more pressing, if the secondary gene pool of the seeded recipient has been emptied before of at least some of the extracellular-prone metabolism, such as antibiotic production and release.

In principle, every bacterial (or lower eucaryote) which is well fermentable and is known to be an active recipient in gene transfers could be used to serve as "gene sponge" (soaking up foreign genetic material). Even better would be strains with: loss of all or parts (as much as possible) of the variable gene pool, e.g. large deletions at the chromosome ends of *Streptomyces* sp., positive-ly marked strains which are selectable by genetic tags inserted into the stable genetic regions, e.g. antibiotic resistance genes; loss of as much as possible of barriers of gene establishment, especially production of restriction enzymes and other host exclusion mechanisms, etc..

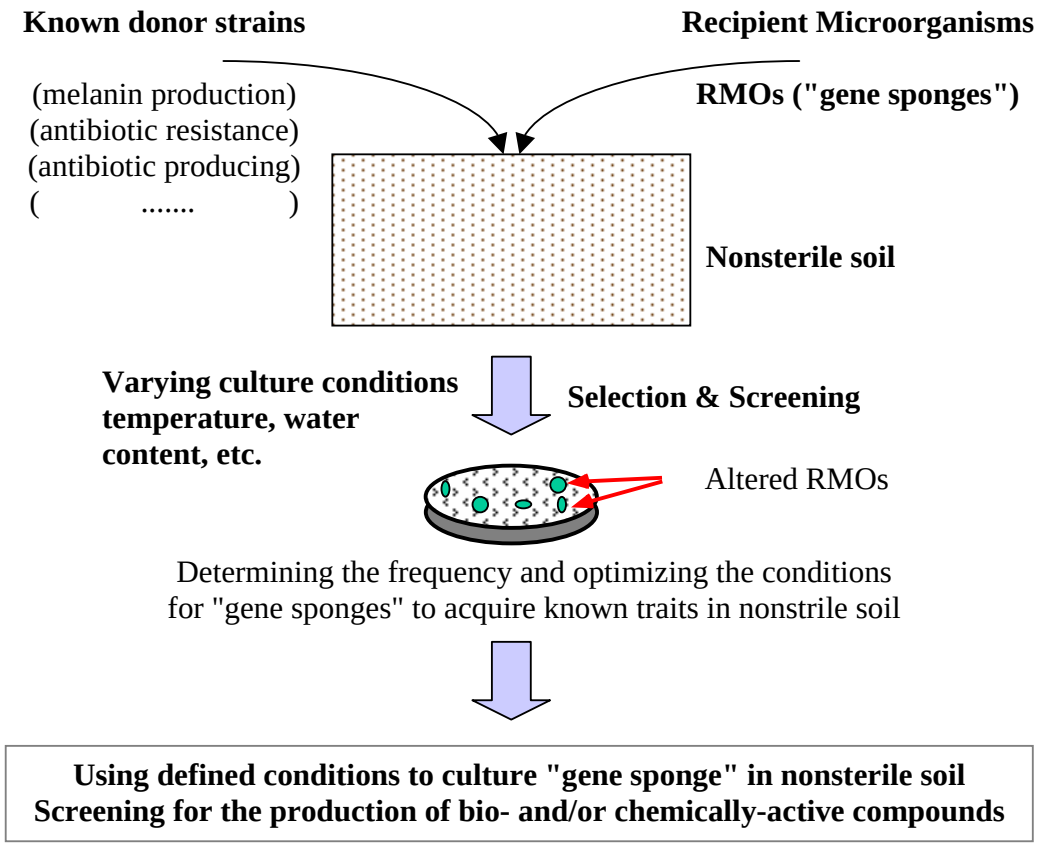


Fig. 1.4: The procedure of *in vivo* gene sponge approach for accessing soil genetic diversity.

A basic procedure of this approach is presented in **Fig.** 1.4. The putative "gene sponge" is first seeded into nonsterile soil together with some strains with known traits such as pigment production (e.g. melanin), antibiotic resistance (e.g. *cmlR*), antibiotic production (e.g. streptomycin, lincomycin), to optimize the conditions (e.g. culturing temperature, soil moisture, soil types, and presence of other physical, chemical or biological pressure of selection) for the potential gene transfer. The "gene sponge" will then be cultured in the nonsterile soil using the defined conditions with a higher gene transfer frequency. The reisolates will be screened for the production of new useful products.

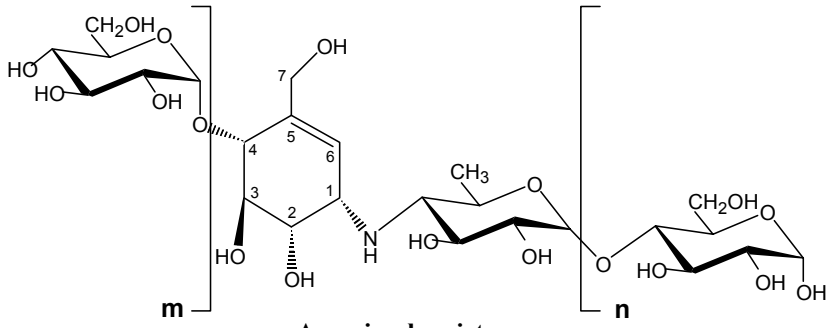
1: INTRODUCTION II – Acarbose Biosynthesis

1.5: The genus *Actinoplanes*

The actinomycete *Actinoplanes* sp. forms a genus of aerobic, sporogenous, Gram-positive bacteria belonging to the large order *Actinomycetales* and is within the family *Actinoplanaceae*. These are commonly found as branching mycelia in soil and aquatic habitats (Parenti and Coronelli, 1979; Vobis, 1989). *Actinoplanes* is characterized by the formation of spores within sporangia. The spores of *Actinoplanes* are spherical or subspherical; they are released shortly after the mature sporangia are immersed in water and swim actively from the moment of their release or some time thereafter (Palleroni, 1976; Parenti and Coronelli, 1979). An aerial mycelium is usually not present in most isolates of *Actinoplanes* sp. and the large majority of *Actinoplanes* strains exhibit an orange or yellow color when grown on a variety of solid or liquid media (Parenti and Coronelli, 1979). The composition of the cell wall of *Actinoplanes* contains mostly generic specific meso-2,6-diaminopimelic acid (60 – 70 %), LL-2,6-diaminopimelic acid (10 – 15 %) and/or hydroxy-aminopimelic acid (Lechevalier *et al.*, 1970; Parenti and Coronelli, 1979). The GC content of the genomic DNA of *Actinoplanes* is 70 - 73 mol % which is typical for actinomycetes (Farina and Bradley, 1970). The phylogenetic structure of the genus *Actinoplanes* has been recently determined by comparative 16S rDNA sequence analysis of the type strains of all validly described *Actinoplanes* species (Tamura and Hatano, 2001). Sequence analysis of the 16S rDNA of *Actinoplanes* sp. SE50/110, the strain used in this work, showed it has the highest degree of sequence similarity to those from the genus *Micromonospora* and *Frankia* (Mehling *et al.*, 1995). *Actinoplanes* strains represent the richest group of rare actinomycetes which produce microbial products and more than 120 antibiotics have been reported from *Actinoplanes* strains (Lazzarini *et al.*, 2000). Amino acid derivatives, such as peptides and depsipeptides, are prevalent among these compounds. Some are of clinical relevance such as teicoplanin (Bardone *et al.*, 1978) and ramoplanin (Ciabatti & Cavalleri, 1989). Other interesting antibiotics discovered by exploiting the chemical diversity of *Actinoplanes* strains are purpuromycin (Coronelli *et al.*, 1974; Rambelli *et al.*, 1989), lipiarmycin (Parenti *et al.*, 1975; Cavalleri *et al.*, 1988), actagardine (Parenti *et al.*, 1976; Malabarba *et al.*, 1985) and friulimicins (Vertesy *et al.*, 2000). In the fermentation broth of *Actinoplanes* sp. SE50/110, a heat- and acid-stable α-glucosidase inhibitor acarbose was found for the first time (Schmidt *et al.*, 1977).

1.6: Structure and pharmaceutical applications of acarbose

The α-glucosidase inhibitor acarbose, produced by strains of the genera *Actinoplanes* and *Streptomyces*, is a member of an unusual group of bacterial (mainly actinomycetes) secondary metabolites, all of which inhibit various α-glucosidases, especially in the intestine (Truscheit *et al.*, 1981; Müller, 1989). Acarbose is a part of the amylostatin complex (**Fig.** 1.5) and is produced industrially using the developed strain of *Actonplanes* sp. SE50 (former designation *A. utahensis*). The pseduooligosaccharide acarbose consists of an unsaturated cyclitol (valeinol), a 4-amino-4,6-dideoxyglucose and maltose (**Fig.** 1.5). The valienol and the 4-amino-4,6-dideoxyglucose are linked via an *N*-glycosidic bond to form the acarviosyl moiety which is primarily responsible for the inhibitory effect on α-glucosidases (Müller *et al.*, 1980; Junge *et al.*, 1980). Besides acarbose the organism *Actinoplanes* sp. SE50 also produces an extensive series of acarviosyl {4-*N*-4,6-didesoxy-4-([4,5,6-trihydroxy-3-hydroxymethyl-2-cyclohexen-1-yl]amino)-α-D-glucopyranose} containing pseudooligosaccharides. These compounds differ in the number of glucose units connected among each other by α-1,4 glycosidic bonds which are attached to the acarviosyl core at the reducing and non-reducing end (Müller *et al.*, 1980; Hemker *et al.*, 2001). In addition, some compounds show variations in the type of the terminal glycosidic bond or in the nature of the terminal sugar moiety (Hemker *et al.*, 2001; **Tab.** 1.3). The different homologues are formed dependent on the sugar source in the culture broth (Schmidt *et al.*, 1977). If glucose or maltose are supplied as the sole carbon source inhibitors with a small number of glucose units are produced, preferentially acarbose, while addition of starch leads to compounds with a higher number of glucose units. The number of glucose units determines the inhibitory specificity against different α-glycosidases. Acarbose and homologues with low molecular weight (component 2) have strong inhibition of disaccharidases whereas those with high molecular weight have strong inhibition of amylases (Müller *et al.*, 1980). The pseudooligosaccharides exerted an inhibitory effect on glucoamylase, sucrase, and maltase, but trehalases, lactases, β-amylases, β-glucosidases, β-galactosidases, pullulanases and isoamylases from both microbes and plants were not affected. Acarbose inhibited the cyclotdextrin-glycosyltransferase (CGTase) and α-amylase from *Bacillus* sp. or *Aspergillus* sp. (Truscheidt *et al.*, 1981; Müller, 1986; Strokopytov *et al.*, 1995).



Acarviosyl moiety

Name	m	n
Component 2	0	1
Component 3 (acarbose)	0	2
Component 4-isomer *	0	1
Component 5-isomer *	0	1
Component 6-isomer *	0	1

Fig. 1.5: Chemical structures of the acarbose and other members of amylostatin family of α-glucosidases inhibitors from *Actinoplanes* sp. SE50/110. For components marked with an asterisk, the main ingredient of the isomer mixture with m + n is 3 (4 or 5). (after Hemker *et al.*, 2001).

Tab. 1.3: Names and compositions of acarviosyl-containing compounds [a]

Name	Composition
Acarbose (component 3)	Acarviosyl-1-4-Glc-1-4-Glc
Component A	Acarviosyl-1-4-Glc-1-4-**Fru**
Component B	Acarviosyl-1-4-Glc-1-4-**Val**
Component C	Acarviosyl-1-4-Glc-1-**1-Glc**
Component D	Acarviosyl-1-4-Glc-1-4-**Man**
Component 4a	Acarviosyl-1-4-Glc-1-4-Glc-1-4-**Fru**
Component 4b	Acarviosyl-1-4-Glc-1-4-Glc-1-4-Glc
Component 4c	Acarviosyl-1-4-Glc-1-4-Glc-1-**1-Glc**
Pseudo-acarbose	Acarviosyl-1-4-(**6-desoxy**)Glc-1-4-Glc

[a] : Structural differences compared to acarbose or component 4b, respectively, are indicated in boldface. Glc, glucose; Fru, fructose; Man, mannose; Val, 1-*ep*i-2-*ep*i-valienol. (after Hemker *et al.*, 2001)

The acarviosyl moiety of the pseudooligosaccharides is essential for the inhibitory effect on α-glucosidases. The *N*-glycosidic bond between the valienol moiety and the 4,6-didesoxyglucose, in contrast to the α-1,4-glycosidic bond, was not hydrolyzed by the active center of α-glucosidases (Heiker *et al.*, 1981). The inhibitory effect on α-glucosidases was based on a competitive mechanism, revealed by the X-ray structure analysis of a crystal of a sucrase-isomaltase complex, isolated from the small intestine of rats (Sigrist *et al.*, 1975; Hanzoet *et al.*, 1981; Samulitis *et al.*, 1987). Kinetic analysis also revealed a fully competitive type of inhibition,

the inhibitor acarbose had a 15,000-fold higher affinity to the enzyme sucrase than its natural substrate sucrose (Caspary and Graf, 1979). The molecular mechanism of the inhibitory effect was studied by the crystallographic analysis of pig pancreatic α-amylase isoenzyme II (PPAII), and the cyclodextrin-glycosyltransferase (CGTase) from *Bacillus circulans* (Strokopytov *et al.*, 1995) in complexes with acarbose in the active centers.

Several other aminoglycosidic α-glycosidase inhibitors with acarviosyl moieties have been found in the culture broth of various actinomycetes, including amylostatin (Mauro and Ohyama, 1975), adiposins (Namiki *et al.*, 1982a, 1982b), oligostatins (Omoto *et al.*, 1981), and trestatins (Yokose *et al.*, 1983). Another member group of this class of compounds are the trehalase and chitinase inhibitors validamycins and validoxylamines produced by *S. hygroscopicus* var. *limoneus* (Horii and Kameda, 1972). They all contain 1 or 2 units of a valiolol-derived C7-cyclitols.

Since 1990 the α-glucosidase inhibitor acarbose is used as an oral antidiabetic agent (Glucobay, Bayer) in the therapy of non-insulin-dependent type II diabetes mellitus (Bischoff *et al.*, 1994). This drug enables the diabetes patients to better utilize starch- or sucrose-containing diets by slowing down the intestinal release of α-D-glucose. Acarbose is considered non-toxic because there is negligible systemic absorption of the intact drug and only 20% absorption of the non-glucose moieties (Welborn, 1998).

1.7: The *acb*-gene cluster for acarbose production in *Actinoplanes* sp. SE50/110

The studies on the genetics of amylostatin (acarbose) production were started by isolation and analyzing DNA-fragments around the dTDP-D-glucose 4,6-dehydratase-encoding genes in the two organisms *Actinoplanes* sp. SE50 (Crueger *et al.*, 1995; Stratmann, 1997) and *Streptomyces glaucescens* GLA.0 (Decker, 1996). The 6-deoxyhexoses are frequent building units or side chains in many actinomycete secondary metabolites and are mostly synthesized via a dTDP-hexose pathway (Stockmann and Piepersberg, 1992; Piepersberg and Distler, 1997). Therefore, the highly conserved gene sequence of the dTDP-D-glucose 4,6-dehydratase (*strE*) from *S. griseus* N2-3-11 was used as a probe to screen for related genes in the acarbose producer strain *Actinoplanes* sp. SE50/110. Using this approach, a 17 kb fragment of the *acb*-gene cluster including the genes *acbQKMLNOCBAEDGF* was identified, isolated and analyzed (Stratmann, 1997; Stratmann *et al.*, 1999; M. Jarling, p.c.; **Fig.** 1.6). The same approach was also applied to

identify another *acb*-gene cluster from *S. glaucescens* GLA.0 (Decker, 1996). A λGEM12 phage gene bank (Jarling, p.c.) and a cosmid bank (Thomas, 2001) were constructed to screen for the whole *acb*-gene cluster using various known *acb*-genes as probes in *Actinoplanes* sp. SE50/110. By this way, the full-length *acb*-gene cluster putatively responsible for acarbose production in *Actinoplanes* sp. SE50/110 was identified, cloned and sequenced (Stratmann, 1997; Stratmann *et al.*, 1999; Diaz-Guardamino, 2000; Thomas, 2001; M. Jarling, p.c.; U. Wehmeier, p.c.). It covers about 35 kb of chromosomal DNA and encompasses 25 ORFs (**Fig.** 1.6; **Tab.** 1.4). The 25 *acb* genes are organized in at least 8 transcription units, *acbZ*, *acbWXY*, *acbVUSRPIJQKMLNOC*, *acbB*, *acbA*, *acbE*, *acbD* and *acbHGF*, 3 of which are operons (**Fig.** 1.6).

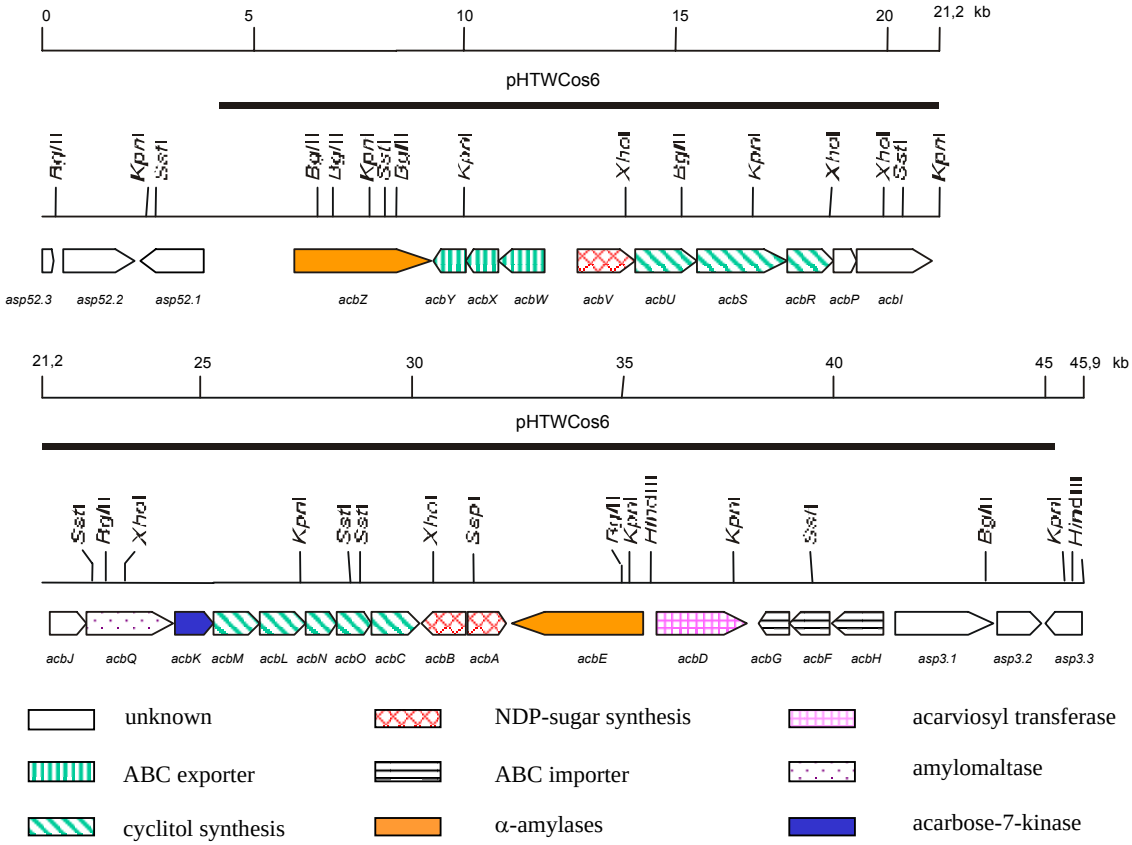


Fig. 1.6: Organization of the complete *acb*-gene cluster for acarbose biosynthesis in *Actinoplanes* sp. SE50/110. Genes of the *acb*-cluster are denoted from *acbA* to *acbZ* and indicated by rectangle arrows orientated in the direction of transcription. Genes denoted with *asp* are probably not responsible for acarbose production. The functions of the deduced Acb-proteins are symbolized by different colors with different filling patterns. The numbers in the figure represent the length of the segment in kb. The chromosomal region covered by the recombinant cosmid pHTWCos6 (Thomas, 2001) is indicated with the line in bold. The main restriction sites are also given.

Tab. 1.4: The *acb*-genes and the postulated functions of their products from the *acb*-gene cluster of *Actinoplanes* sp. SE50/110.

Genes	Postulated function of the gene product	Reference[a]
asp52.3	unknown, regulator?	1
asp52.2	unknown, signal transduction?	1
asp52.1	arabinofuranosidase	1
acbZ	α-amylase	1
acbY	membrane component of ABC-exporter AcbWXY	1
acbX	membrane component of ABC-exporter AcbWXY	1, 2
acbW	ATP-binding protein of ABC-exporter AcbWXY	1, 2
acbV	NDP-sugar aminotransferase	1, 2
acbU	cyclitol kinase?	1, 2
acbS	NDP-cyclitol transferase	1
acbR	NDP-cyclitol synthase	1
acbP	NUDIX hydrolase?	1
acbI	unknown	1, 7
acbJ	unknown	7
acbQ	amylomaltase-like function using acarbose as substrate instead of maltodextrins.	3, 7
acbK	acarbose 7-kinase	3, 6
acbM	unknown	7
acbL	unknown (oxidoreductase?)	7
acbN	unknown (oxidoreductase?)	7
acbO	unknown	7
acbC	2-*epi*-5-*epi*-valiolone synthase	4
acbB	dTDP-glucose 4,6-dehydratase	3
acbA	dTDP-glucose synthase	3
acbE	α-amylase	3
acbD	acarviosyltransferase	3, 5
acbG	membrane protein of ABC-importer AcbHGF	3
acbF	membrane protein of ABC-importer AcbHGF	3
acbH	binding protein of ABC-importer AcbHGF	3
asp3.1	galactocerebrosidase	7
asp3.2	xylanase	7
asp3.3	unknown	7

[a] 1: Thomas, 2001; 2: Diaz-Guardamino, 2000; 3: Stratmann, 1997; 4: Stratmann *et al.*, 1999; 5: Hemker *et al.*, 2001; 6: Goeke *et al.*, 1996, Drepper and Pape, 1996; 7: M. Jarling, personal communication.

Evidences for the functionality of this *acb*-cluster to represent the real production genes for acarbose stem from (1) the enzymology of individual Acb-proteins (**Tab.** 1.4) and (2) the expression of the cosmid pHTWCos6 which contains the full-length *acb*-cluster of *Actinoplanes* sp. SE50/110 in *S. lividans* 66 strains resulting in heterologous production of acarbose or an acarbose-like compound (Thomas, 2001).

Some genes of the *acb*-gene cluster have been characterized experimentally. The *acbC* gene which encodes an AroB-like protein (dehydroquinate synthase, DHQS) was expressed heterologously in the strain of *S. lividan*s 66, and employing the same reaction conditions as used in *in vitro* studies on DHQS proteins. Its product was shown to be a C7-cyclitol synthase using *sed*o-heptulose-7-phosphate as a substrate for the production of 2-*epi*-5-*epi*-valiolone (Stratmann *et al.*, 1999). The *acbD* gene encodes an acarviosyl transferase (ATase), which catalyzes the transfer of the acarviosyl moiety of acarbose to maltooligosaccharides (Hemker *et al.*, 2001). The gene *acbE* was shown to encode an acarbose-insensitive α-amylase using regular starch and amylose as substrates and was inducible by the addition of maltotriose to the medium (Stratmann, 1997). The activity of the *acbK* gene product (acarbose-7-kinase) was first detected in the cell-free extracts of *Actinoplanes* sp., which modifies acarbose by phosphorylation at its C7-position (Goeke *et al.*, 1996; Drepper and Pape, 1996). Experimental evidences showed that the *acbV* gene encodes a GabT like aminotransferase which uses dTDP-4-keto-6-deoxy-D-glucose and glutamate as substrates for the synthesis of dTDP-4-amino-4,6-dideoxy-D-hexose (Diaz-Guardamino, 2000).

The other *acb*-genes have not been experimentally investigated. Their functions are postulated on the basis of the comparisons of the deduced protein sequences with known proteins (**Tab.** 1.4). The two genes, *acbA* and *acbB*, encoding the dTDP-glucose synthase and the dTDP-glucose 4,6-dehydratase, respectively, are evidently involved in the formation of the dideoxyglucose moiety of acarbose. The genes *acbL* and *acbN* encode proteins related to two different dinucleotide-utilizing oxidoreductases. The *acbQ* gene encodes a protein with significant similarity throughout the full-length polypeptide chain with that of the amylomaltase MalQ of *E. coli* (Boos and Shuman, 1998) and therefore might be responsible for the addition/removing of the maltose unit to/from the dideoxyglucose moiety. The *acbZ* gene encodes another α-amylase, the functionality of which should be very similar to that of AcbE, due to its highly sequence identity to that protein. In the *acb*-gene cluster, the gene *acbR* which encodes a GlgC (ADP-glucose synthase)

like protein and the gene *acbS* which encodes a protein sharing motifs with glycosyltransferase are also identified, both of them are functionally grouped to the cyclitol pathway. The operons of an ABC sugar importer encoded by *acbHGF* and the ABC exporter encoded by *acbWXY* are also identified in the cluster, which might encode the export system for release of acarbose. The genes *acbM*, *acbO* and *acbPIJ* show no significant similarities to the known proteins. They are considered to be probably involved in the biosynthesis of the cyclitol moiety of acarbose.

Only five genes *acbABCDE*(*Sgl*) of the partially analyzed *acb*-gene cluster (6.8 kb) had been identified in *S. glaugescens* GLA.0. The deletions of the genes for the dTDP-glucose synthase (*acbCSgl*), the dTDP-glucose 4,6-dehydratase (*acbDSgl*) and the aminotransferase (*acbBSgl*) resulted in the loss of acarbose production in *S. glaucescens* GAL.0, therefore these genes were assigned to be responsible for the biosynthesis of acarbose in this strain. But the organization of the *acb*-gene cluster in *S. glaugescens* GLA.0 is totally different from that in *Actinoplanes* sp. SE50/110, and three of the ORFs, *acbCDE*(*Sgl*) had either little sequence identity to their common parts in *Actinoplanes* sp. SE50/110 (*acbAB, acbV*) or are absent from the latter cluster.

1.8: The acarbose metabolism in *Actinoplanes* sp. SE50/110

The acarbose metabolism in *Actinoplanes* sp. SE50/110 is only partially understood and the knowledge is based on feeding experiments and some enzymological investigations.

The biosynthesis of the deoxysugar moiety of acarbose conforms to that of other deoxyhexoses which are found in many secondary metabolites (Piepersberg and Distler, 1997). It involves the formation of dTDP-D-glucose and the following conversion to dTDP-4-keto-6-deoxy-D-glucose through a stereospecific intramolecular hydride transfer catalyzed by the pyridine nucleotide-containing enzyme dTDP-glucose 4,6-dehydratase (**Fig.** 1.8). The corresponding two enzymes responsible for these reactions are identified as AcbA and AcbB (Stratmann, 1997; **Tab.** 1.4). Furthermore, L-glutamate was shown to be the primary source of the nitrogen in acarbose (Lee *et al.*, 1998) and the substrate for AcbV, an aminotransferase which catalyzes the synthesis of dTDP-4-amino-4,6-dideoxy-D-hexose from dTDP-4-keto-6-deoxy-D-glucose (Diaz-Gurdamino, 2000; **Fig.** 1.8).

The two terminal glucose moieties of acarbose are derived not from free glucose but from maltose and maltotriose in the culture medium (Degwert *et al.*, 1987). Using ^{3}H- and ^{2}H-labeled maltose and maltotriose, it was found that there are two metabolic routes from maltotriose to the

maltose unit of acarbose. About 60% of the acarbose is formed by attachment of maltose, produced by removing a glucose from the nonreducing end of maltotriose, to the pseudodisaccharide core unit. The other 40% of the acarbose is formed by direct attachment of maltotriose to the core unit, followed by loss of the terminal glucose from the reducing end (Lee *et al.*, 1997). But it remains unclear which enzymes are responsible for these reactions. An extracellular glycosyltranferase encoded by the *acbD* gene is shown to catalyze the transfer of the acarviosyl moiety of acarbose to glucose or malto-oligosaccharides (Hemker *et al.*, 2001). Therefore, the AcbD protein might be involved in the addition of the maltose moiety of acarbose. The *acbQ* gene encodes a protein with significant similarity to the amylomaltase MalQ, which is a typical constituent of the intracellular metabolism of maltose and other maltooligodextrins in *E. coli* (Boos and Shuman, 1998). It is also possible that the transfer of glucose or maltose residues to the reducing end of the acarviose is catalyzed intracellularly by AcbQ (**Fig.** 1.8).

In contrast, little was known about the mode of the formation of the valienol moiety of acarbose. Earlier feeding experiments with stable isotope-labeled precursors have established that this mC7-unit of acarbose is derived from the pentose phosphate pathway but not from the shikimate pathway (Degwert *et al.*, 1987). Recently, the transition from the primary- to the secondary-metabolism in the cyclitol pathway in *Actinoplanes* sp. SE50/110 is demonstrated by the characterization of a key enzyme AcbC, which catalyzes the formation of 2-*epi*-5-*epi*-valiolone using *sedo*-heptulose-7-phosphate as substrate (Stratmann *et al.*, 1999). Furthermore, ^{2}H and ^{13}C labeled 2-*epi*-5-*epi*-valiolone is shown to be significantly incorporated into acarbose in feeding experiments (Stratmann *et al.*, 1999; Mahmud *et al.*, 1999). These results provide strong evidence that 2-*epi*-5-*epi*-valiolone is indeed an intermediate in the biosynthesis of acarbose. Further feeding experiments have been intensively carried out to identify other intermediates using various stable isotope labeled cyclitols (**Fig.** 1.7). However, none of the tested putative precursors, valiolone, 5-*epi*-valiolone, valienone, 2-*epi*-valienone, 1-*epi*-valienol, valiolamine and valienamine, was shown to be incorporated into acarbose (Mahmud *et al.*, 1999; Dong *et al.*, 2000). In contrast, besides 2-*epi*-5-*epi*-valiolone, 5-*epi*-valiolone and valienone have also been identified as intermediates for the biosynthesis of validamycin, an antifungal antibiotic which consists of the same C7-cyclitol moiety as that of acarbose and is produced by *S. hygroscopicus* var. *limoneus* (Dong *et al.*, 2000). These results lead to the speculation that all of the steps from 2-*epi*-5-*epi*-valiolone to the putative pseudodisaccharidic intermediate dTDP-acarviose occur on

one enzyme or enzyme complex without any free intermediates during its conversion and condensation pathway (Mahmud *et al.*, 1999). However, such enzyme or enzyme complex was neither identified in *Actinoplanes* sp. nor suggested from the gene records of the *acb*-gene cluster. Furthermore, there is also no information available how and where (intracellularly or extracellularly) the dTDP-acarviose is further converted to acarbose.

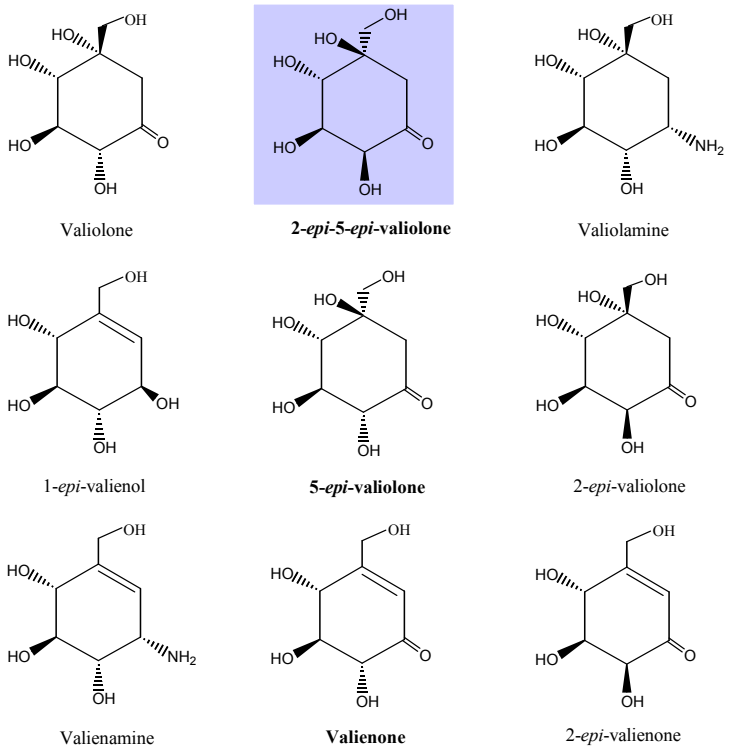


Fig. 1.7: C7-cyclitols examined in the feeding experiments with *Actinoplanes* sp. and *S. hygroscopicus* var *limoneus*. Only the shadowed cyclitol is incorporated into acarbose (after Mahmud *et al.*, 1999; Dong *et al.*, 2000).

The acarbose-7-phosphate, being a product of AcbK, is suggested to be an inactivation product of acarbose and can no longer inhibit the intracellular α-glucosidases or maltases of the producer *Actinoplanes* sp. (Drepper and Pape, 1996). This phenomenon is generally regarded as part of a self defense mechanism.

Based on the available information, a model of acarbose metabolism in *Actinoplanes* sp. is presented (**Fig.** 1.8). In this model, the starch is hydrolyzed to short-chain maltodextrins or maltose by the acarbose insensitive α-amylase (AcbE, AcbZ). The acarviosyl moiety is transferred to the resultant maltodextrins, catalyzed by the mixture of amylases (AcbE, AcbZ) and acarviosyl transferase AcbD to form acarbose and/or higher chain-length homologues of

acarbose. After uptake of acarbose or acarbose homologues by the specific ABC-importer AcbHGF into the cell, the glucose residues are removed by the amylomaltase AcbQ before or after the inactivation by the 7-phosphorylation of AcbK. The regenerated acarbose-7-phosphate is then transported outside the cell by the postulated ABC-exporter AcbWXY (**Fig.** 1.8).

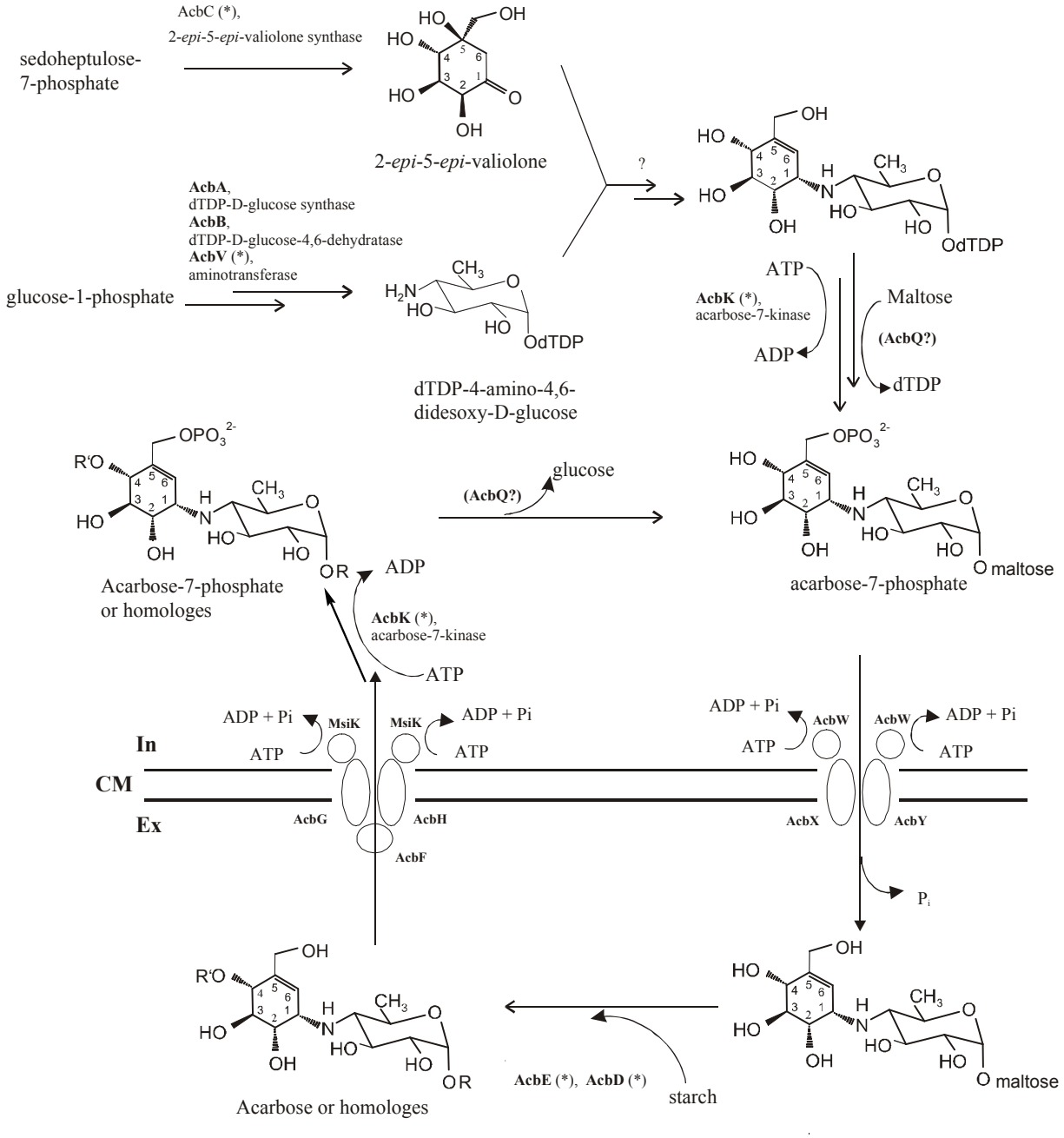


Fig. 1.8: Model of acarbose metabolism in *Actinoplanes* sp. SE50/110. The enzymes which have been functionally characterized *in vitro* are indicated with stars (*). R, R': glucose or maltooligosacchride; In: intracellular; Ex: extracellular; CM: cytoplasmic membrane. (after Thomas, 2001).

1.9: Aim of this work

The thesis falls into two, only distantly related parts since at the beginning of this work a different main focus was followed. Since it became apparent after the first year that the first approach could not be brought to an easy conclusion the goals were shifted to the main project of the group. Therefore, this dissertation falls into two parts (I) the "gene sponge" experiments and (II) the biosynthesis of acarbose.

I. The aims followed in the initial line of preliminary experiments for the establishment of *in situ* and *in vivo* gene transfer in microcosms ("gene sponge" approach) fell into the following parts:
   (i) to test the possibility of gene transfer (such plasmids and chromosomal markers) in sterile soil seeded with the "gene sponge".
   (ii) to characterize soil isolates which are similar to the seeded "gene sponge".
   (iii) to demonstrate the transfer of chromosomal markers to the "gene sponge" in mating experiments.

II. At the beginning of the second and main part of this work, little was known about the biosynthetic pathway leading to the cyclitol moiety of acarbose in *Actinoplanes* sp. SE50/110. Many cyclitols had been examined in feeding experiments. However only the AcbC reaction product, 2-*epi*-5-*epi*-valiolone, is shown to be significantly incorporated into acarbose. In order to find out the pathway to the valienol moiety of acarbose, the work was focused on the enzymatical conversion of 2-*epi*-5-*epi*-valiolone. The main aim of this work was therefore to elucidate the next steps in the biosynthesis of acarbose including the following milestones:
   (i) to experimentally characterize *in vitro* the activity of AcbK.
   (ii) to overproduce heterologously the Acb-proteins (AcbMLNOU) which are putatively involved in the pathway for the cyclitol moiety of acarbose and to characterize their functions *in vitro*.
   (iii) to identify and characterize eventually formed new intermediates.

2: MATERIALS AND METHODS

2.1: Chemicals and enzymes

Antibiotics

Ampicillin sodium salt	Roche Diagnostics, Mannheim
Apramycin	Serva, Heidelberg
Chloramphenicol	Serva, Heidelberg
Cycloheximide	Sigma, Deisenhofen
Erythromycin	Serva, Heidelberg
Gentamicin	Serva, Heidelberg
Hygromycin B	Serva, Heidelberg
Kanamycin	Serva, Heidelberg
Lincomycin	Serva, Heidelberg
Nystatin	BDH, Wesel
Rifampicin	Sigma, Deisenhofen
Spectinomycin	Serva, Heidelberg
Streptomycin	Serva, Heidelberg
Thiostrepton	Squibb and Sons, Princeton, USA

Chemicals

Acarbose	Bayer AG, Wuppertal
Agarose	Roche Diagnostics, Mannheim
	Roth, Karlsruhe
ATP	Amersham Bioscience, Freiburg
Blocking Reagent	Roche Diagnostics, Mannheim
Chemicals, p.a. Quality	Fluka, Buchs, CH
	Merck, Darmstadt
	Roth, Karlsruhe
	Serva, Heidelberg
	Sigma, Deisenhofen
Constituents of media	Difco, Detroit, USA
	Merck, Darmstadt
	Oxoid, Wesel
	Roth, Karlsruhe
	Bayer AG, Wuppertal
MD 50	Bayer AG, Wuppertal
NADPH, $NADP^+$, NADH, NAD^+	Sigma, Deisenhofen
Ni-NTA-Agarose	Qiagen, Hilden

Enzymes

Alkaline phosphatase (calfs intestine)	Roche Diagnostics, Mannheim
Klenow enzyme	Invitrogen, Eggenstein
Lysozyme	Serva, Heidelberg
Restriction endonucleases	Biolabs, Schwalbach

	Roche Diagnostics, Mannheim
	Invitrogen, Eggenstein
	Amersham Bioscience, Freiburg
Ribonuclease A	Sigma, Deissenhofen
Taq DNA polymerase	Perkin Elmer Cetus, Überlingen
	Amersham Bioscience, Freiburg
T4-DNA ligase	Invitrogen, Eggenstein
T4-DNA-polynucleotid-kinase	Roche Diagnostics, Mannheim
	Amersham Bioscience, Freiburg
Transketolase(EC: 2.2.1.1)	Sigma, Deissenhofen
Vent DNA polymerase	Biolabs, Schwalbach

Kits

Auto-Read-Sequencing-Kit	Amersham Bioscience, Freiburg
BioRad Protein Assay Kit	BioRad, München
BM Chromogenic Western Blotting Kit	Roche Diagnostics, Mannheim
Deaza T7-Sequencing-Kit	Amersham Bioscience, Freiburg
DNA-Preparations-Kit (Qiagen)	Qiagen, Düsseldorf
JetSorb	Genomed GmbH, Bad Oeyenhausen
Nick-Translation-Kit	Invitrogen, Eggenstein
Nucleo Spin Extract 2 in 1 Kit	Macherey-Nagel, Düren
PCR-Kit	Perkin Elmer Cetus, Überlingen
QIAprep Spin Miniprep Kit	Qiagen, Hilden
QIAquick Gel Extraction Kit	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
Ready-To-Go (RAPD analysis)	Amersham Bioscience, Freiburg
Rediprime	Amersham Bioscience, Freiburg

Radionuclides

α-^{32}P-dCTP	Amersham Bioscience, Freiburg
γ-^{32}P-ATP	Amersham Bioscience, Freiburg
^{14}C-ATP	Amersham Bioscience, Freiburg

2.2: Various materials

Biodyne B nylonmembranes	Pall, Dreieich
Hybond N^+ nylonmembranes	Amersham Bioscience, Freiburg
Membrane filters BA 85 (0,45 µm)	Schleicher & Schuell, Dassel
PVDF-membrane	Amersham Bioscience, Freiburg
TLC plates	Merck, Darmstadt
3MM Whatman	Whatman, Maidstone, GB
X-ray film Hyperfilm-MP	Amersham Bioscience, Freiburg
X-ray film Hyperfilm-β-max	Amersham Bioscience, Freiburg

2.3: Bacterial strains, vectors and recombinant plasmids

2.3.1: Bacterial strains

Tab. 2.1: Bacteria used in this study

Strain	Relevant products	Reference or strain no. [1]
Escherichia coli BL21 (DE3)	*ompT*, λ-prophage-T7-polymerase	Studier *et al.*, 1990
Escherichia coli BL21(DE3) pLysS	*ompT*, λ-prophage-T7-polymerase, pLysS, *cat*	Studier *et al.*, 1990
Escherichia coli DH5α	F^-, ϕ80Δ *lacZ*ΔM15, *recA1*, *endA1*, *thi*, *gyrA96*, *hsd*R17($r_k^-m_k^-$), *supE44*, *relA1*, *deoR*, *Δ*(*lacZYA-argF*)	Hanahan, 1983
Escherichia coli JM83	F^-, ϕ80Δ *lacZ*ΔM15, *ara*, *rpsL*, *Δ*(*lac-proAB*)	Yanisch-Perron *et al.*, 1985
Escherichia coli JM109	F^-[*traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15], *thi*, *recA1*, *endA1*, *gyrA96*, *hsdR17* ($r_k^-m_k^-$), *supE44*, *relA1*, *Δ*(*lac-proAB*)	Yanisch-Perron *et al.*, 1985
Escherichia coli JM110	F^-[*traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15], *thi*, *dam*, *dcm*, *leu*, *rpsL*, *lacY*, *hsdR17* ($r_k^-m_k^-$), *supE44*, *galK*, *galT*, *ara*, *tonA*, *thr*, *tsx*, *Δ*(*lac-proAB*)	Yanisch-Perron *et al.*, 1985
Escherichia coli LE392	F^-, *hsdR574*($r_k^-m_k^+$), *lacY1*, *supE44*, *supF58*, or *Δ*(*lacIZY*)*6*, *galK2*, *galT22*, *metB1*, *trpR55*	Promega, Mannheim
Escherichia coli XL1-Blue	F^-[*proAB*$^+$*lacI*^{q}*lacZ*ΔM15Tn*10*(*tet*^{r})], *thi*, *recA1*, *endA1*, *gyrA96*, *hsdR17*($r_k^-m_k^-$), *supE44*, *relA1*	Stratagene, Heidelberg
Actinoplanes sp. SE50/110	acarbose	ATCC31044
Actinoplanes sp. SN23/229	acarbose	
Micrococcus luteus		DSM 348
Streptomyces coelicolor M145	actinorhodin, prodigiosin	Hopwood *et al.*, 1985
Streptomyces galbus	streptomycin	DSM 40480
Streptomyces glaucescens GLA.0	OH-streptomycin	DSM 40716
Streptomyces griseus N2-3-11	streptomycin	Kaken Chem. Co., Tokyo
Streptomyces lincolnensis	lincomycin	NRRL 2936

Strain	Relevant products	Reference or strain no. [1]
Streptomyces lincolnensis 78-11	lincomycin	Zhang *et al.*, 1992
Streptomyces lividans 1326	actinorhodin, prodigiosin	John Innes Institute, Norwich, UK
Streptomyces lividans TK23	actinorhodin, *spc-1*	John Innes Institute, Norwich, UK
Streptomyces lividans TK64	*pro-2, str6*	Hopwood *et al.*, 1985
Streptomyces lividans Jni13C1	Sm^RCml^S, *str6, pro-2*	Volff *et al.*, 1997a
Streptomyces lividans Jni14C1	Sm^RCml^S, *str6, pro-2*	Volff *et al.*, 1997a
Streptomyces lividans WP	Sm^RCml^S, *argG, str6, pro-2*	Volff *et al.*, 1996
Streptomyces lividans AJ100	Sm^RCml^S, *argG, str6, pro-2*	J. Altenbuchner, Stuttgart
Streptomyces pseudogriseolus	lincomycin	NRRL 3985
Streptomyces lividans Strain 01-52	Sm^RCml^R	Transconjugants from soil, this work
E30d-1 (isolated from soil)	Sm^RCml^R	This work
E30d-2 (isolated from soil)	Sm^RCml^R	This work
E30d-3 (isolated from soil)	Sm^RCml^R	This work
E30d-4 (isolated from soil)	Sm^RCml^R	This work
E30d-5 (isolated from soil)	Sm^RCml^R	This work
F30d-10 (isolated from soil)	Sm^RCml^R	This work
F30d-12 (isolated from soil)	Sm^RCml^R	This work
F30d-19 (isolated from soil)	Sm^RCml^R	This work
E50d-1 (isolated from soil)	Sm^SCml^R	This work
E50d-3 (isolated from soil)	Sm^SCml^R	This work
F50d-2 (isolated from soil)	Sm^SCml^R	This work
F50d-3 (isolated from soil)	Sm^RCml^R	This work
F50d-6 (isolated from soil)	$Thio^R$	This work
F50d-7 (isolated from soil)	$Thio^R$	This work

[1]: ATCC: American Type Culture Collection; DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen; NRRL: Northern Regional Research Laboratories.

2.3.2: Vectors

Tab. 2.2: Vectors used for cloning

Plasmid	Description/properties	Reference
pBluescript II KS	*bla, lacZ-α, f1 ori*	Short *et al.*, 1988
pET11a	*bla, lacZ-α,*	Novagen, 1992
pET11aP	*bla, lacZ-α*	Novagen, 1992
pET16bP	*bla, lacZ-α*	Novagen, 1992
pIJ4123	*tsr, kan, tipAp*	Takano *et al.*, 1995
pIJ6021	*tsr, kan, tipAp*	Takano *et al.*, 1995
pIJ702	*tsr, melC*	Katz *et al.*, 1983
pIJ903	*bla, tsr*	Lydiate *et al.*, 1985
pJOE2702	*bla, rrnB, rhaP*	Volff *et al.*, 1996
pJOE2775	*bla, rrnB, rhaP*	Altenbuchner *et al.*, 1999
pPWW49	*bla, lacZ-α, tsr*	Doumith *et al.*, 2001
pPWW50	*bla, lacZ-α, tsr*	Doumith *et al.*, 2001
pUC18/19	*bla, lacZ-α*	Viera and Messing, 1982
pUCPU21	*bla, lacZ-α*	U. Wehmeier, Wuppertal
pUWL201	*bla, lacZ-α, tsr*	Doumith *et al.*, 2001
pUWL201RBSA	*bla, lacZ-α, tsr*	Doumith *et al.*, 2001
pUWL218/219	*bla, lacZ-α, tsr*	Wehmeier, 1995

2.3.3: Recombinant plasmids

Tab. 2.3: Recombinant plasmids or phages used in this study

Plasmid [1]	Description/properties	Reference
λGEM 54	20 kb genomic DNA from *Actinoplanes* sp. SE50/110	M. Jarling, Wuppertal
pAS6	13 kb *Bgl*II genomic DNA from *Actinoplanes* sp. in pBluescript II KS	Stratmann *et al.*, 1999
pAS8/7	1.26 kb *acbC* fragment in pIJ6021	Stratmann *et al.*, 1999

Plasmid [1]	Description/properties	Reference
pEFBA	*bla*, *aacC4* (aparamycin resistance cassette)	Fernandez, Oviedo
pJOE2424	4.3 kb *Xho*I Fragment in pIC20H (*Sal*I)	Volff *et al.*, 1997b
pJOE2568	1.6 kb *Bam*HI fragment containing chloramphenicol resistance gene from *S. lividans*	J. Altenbuchner, Stuttgart
pJOE801-1	15 kb *Eco*RI fragment with complete AUD1 in pEMBL8	J. Altenbuchner, Stuttgart
pHTW223	7.30 kb *Xho*I fragment in pBluescript KS	Thomas, 2001
pHTW233	1.5 kb *Nde*I/*Eco*RI *acbU* fragment in pUC57 (*Nde*I/*Eco*RI)	Thomas, 2001
pMJ6.6	5.7 kb *Sst*I fragment from pAS6 in pUC18 (*Sst*I)	M. Jarling, Wuppertal
pMJM1	1.07 kb *acbM* PCR fragment from *Actinoplanes* sp. in pBluescript SK$^-$ (*Eco*RV)	M. Jarling, Wuppertal
pMJL1	1.42 kb *acbL* PCR fragment from *Actinoplanes* sp. in pUC18 (*Sma*I)	M. Jarling, Wuppertal
pMJL6	1.42 kb *Nde*I/*Eco*RI fragment from pMJL1 in pIJ6021	M. Jarling, Wuppertal
pMJN1	0.86 kb *acbN* PCR fragment from *Actinoplanes* sp. in pBluescript SK$^-$ (*Eco*RV)	M. Jarling, Wuppertal
pMJN2	0.86 kb *Nde*I/*Bam*HI fragment from pMJN1 in pET11aP	M. Jarling, Wuppertal
pMJN4	0.86 kb *Nde*I/*Bam*HI fragment from pMJN1 in pPW49	M. Jarling, Wuppertal
pMJN5	0.86 kb *Nde*I/*Bam*HI fragment from pMJN1 in pPW50	M. Jarling, Wuppertal
pMJO1	0.87 kb *acbO* PCR fragment from *Actinoplanes* sp. in pBluescript SK$^-$ (*Eco*RV)	M. Jarling, Wuppertal
pMJO7	0.87 kb *Nde*I/*Bgl*II fragment from pMJO1 in pIJ4123 (*Nde*I/*Bam*HI)	M. Jarling, Wuppertal

[1] PCR primers for the inserts in the pMJ-series of plasmids are described in **App.** 7.2.

2.3.4: Recombinant plasmids constructed in this study

Tab. 2.4: Recombinant plasmids created in this study

Plasmid	Description
pWJC1	4.84 kb *Sph*I/*Bgl*II Fragment from pJOE801-1 in pUC18(*Sph*I/*Bam*HI)
pWJC2	5.65 kb *Bgl*II Fragment from pJOE801-1 in pUC18(*Bam*HI)
pWJC3	self-ligation of *Pst*I-digested pWJC1
pWJC4-5	1.5 kb *Pst*I Fragment (*aacC4*) from pEFBA in pWJC3
pWJC4-12	1.5 kb *Pst*I Fragment (*aacC4*) from pEFBA in pWJC3
pWJC201-5	4.8 kb *Eco*RI/*Hin*dIII fragment from pWJC4-12 in pUWL201 (*Eco*RI/*Hin*dIII)
pWJC201-12	4.8 kb *Eco*RI/*Hin*dIII fragment from pWJC4-12 in pUWL201 (*Eco*RI/*Hin*dIII)
pCSZ1	0.5 kb *rpsL* (PCR) from *S. lividans* Jni13C1 in pSK$^+$ (*Eco*RV)

pCSZ2	0.5 kb *rpsL* (PCR) from *S. lividans* TK23 in pSK$^+$ (*Eco*RV)
pCSZ3	0.5 kb *rpsL* (PCR) from *S. lividans* Strain-01 in pSK$^+$ (*Eco*RV)
pCSZ4	0.5 kb *rpsL* (PCR) from *S. lividans* Strain-13 in pSK$^+$ (*Eco*RV)
pCSZ6	0.5 kb *rpsL* (PCR) from *S. lividans* Strain-27 in pSK$^+$ (*Eco*RV)
pCSZ7	0.5 kb *rpsL* (PCR) from *S. lividans* Strain-31 in pSK$^+$ (*Eco*RV)
pCSZ8	0.5 kb *rpsL* (PCR) from *S. lividans* Strain-42 in pSK$^+$ (*Eco*RV)
pCSZ9	0.5 kb *rpsL* (PCR) from *S. lividans* Strain-53 in pSK$^+$ (*Eco*RV)
pCSZ12	0.5 kb *rpsL* (PCR) from F30d-19 in pUC18(*Hin*cII)
pCSZ13	0.5 kb *rpsL* (PCR) from E30d-2 in pUC18(*Hin*cII)
pCSZ14	0.5 kb *rpsL* (PCR) from E50d-1 in pUC18(*Hin*cII)
pCSZ15	0.5 kb *rpsL* (PCR) from E50d-3 in pUC18(*Hin*cII)
pCWK11	1.0 kb *acbK* PCR fragment (*Nde*I/*Bgl*II) from *Actinoplanes* sp. SE50/110 in pET11aP (*Nde*I/*Bam*HI)
pCWK16	1.0 kb *acbK* PCR fragment (*Nde*I/*Bgl*II) from *Actinoplanes* sp. SE50/110 in pET16bP (*Nde*I/*Bam*HI)
pCW21KM	1.98 kb *acbKM* PCR fragment from *Actinoplanes* sp. in pUCPU21 (*Eco*RV)
pCW21KM1	Self ligation of pCW21KM digested with *Pst*I
pCW21KM2	1.05 kb *Pst*I fragment from pCW21KM in pUCPU21
pCW21KL	3.07 kb *acbKL* PCR fragment from *Actinoplanes* sp. in pUCPU21 (*Eco*RV)
pCW21KL1	Self-ligation of pCW21KL digested with *Bam*HI
pCW21KL2	1.2 kb *Bam*HI fragment from pCW21KL in pUC18 (*Bam*HI)
pCW21KL3	Self ligation of pCW21KL digested with *Pst*I
pCW21KL4	1.23 kb *Pst*I fragment from pCW21KL in pUC18 (*Pst*I)
pCW21KL5	0.79 kb *Pst*I fragment from pCW21KL in pUC18 (*Pst*I)
pCW2072K	1.0 kb PCR *acbK* fragment (*Nde*I/*Bgl*II) from *Actinoplanes* sp. SE50/110 in pJOE2702 (*Nde*I/*Bam*HI)
pCW2775KM	1.98 kb *Nde*I/*Bgl*II fragment from pCW21KM in pJOE2775 (*Nde*I/*Bam*HI)
pCW201KM1	1.98 kb *Nde*I fragment from pCW21KM in pUWL201RBSA (*Nde*I)
pCW6021KM1	1.98 kb *Nde*I fragment from pCW21KM in pIJ6021 (*Nde*I)
pCW201KM6	1.98 kb *Nde*I/*Hin*dIII fragment from pCW2775KM in pUWL201RBSA (*Nde*I/*Hin*dIII)
pCW11a	0.79 kb *Nde*I/*Dra*I fragment from pJOE2775 in pET11a (*Nde*I/*Bam*HI..Klenow)
pCW11aKM	1.98 kb *Nde*I/*Bgl*II fragment from pCW21KM in pCW11a (*Nde*I/*Bam*HI)
pCW2775KL	3.07 kb *Nde*I/*Bgl*II fragment from pCW21KL in pJOE2775 (*Nde*I/*Bam*HI)
pCW11aKL	3.07 kb *Nde*I/*Hin*dIII fragment from pCW2775KL in pCW11a (*Nde*I/*Hin*dIII)
pCW201KL	3.07 kb *Nde*I/*Hin*dIII fragment from pCW2775KL in pUWL201RBSA (*Nde*I/*Hin*dIII)
pCWM16	1.08 kb *Nde*I/*Kpn*I fragment from pMJM1 in pET16bP (*Nde*I/*Kpn*I)
pCW21M	1.08 kb *acbM* PCR fragment from *Actinoplanes* sp. SE50/110 in pUCPU21 (*Eco*RV)
pCW2775M	1.08 kb *Nde*I/*Bgl*II fragment from pCW21M in pJOE2775 (*Nde*I/*Bam*HI)
pCW201M	1.08 kb NdeI/*Hind*III fragment from pCW2775M in pUWL201RBSA (*Nde*I/*Hin*dIII)
pCW4123M	1.08 kb *Nde*I/*Eco*RI fragment from pMJM1 in pIJ4123 (*Nde*I/*Eco*RI)
pCWL16	1.42 kb *Nde*I/*Sst*I fragment from pMJL1 in pET16bP (*Nde*I/*Sst*I)

pCW4123L	1.42 kb *Nde*I/*Eco*RI fragment from pMJL1 in pIJ4123 (*Nde*I/*Eco*RI)
pCW2775L	1.1kb *acbL* PCR fragment (*Nde*I/*Bgl*II) from genomic DNA of *Actinoplanes* sp. SE50/110in pJOE2775 (*Nde*I/*Bam*HI)
pCW21L	1.1 kb *Nde*I/*Hin*dIII fragment from pCW2775L in pUCPU21 (*Nde*I/*Hin*dIII)
pCW11aL	1.1 kb *Nde*I/*Hin*dIII fragment from pCW21L in pET11a (*Nde*I/*Hin*dIII)
pCW201L	1.1 kb *Nde*I/*Hin*dIII fragment from pCW21L in pUWL201RBSA (*Nde*I/*Hin*dIII)
pCWN16	0.86 kb *Nde*I/*Bam*HI fragment from pMJN1 in pET16bP (*Nde*I/*Bam*HI)
pCW2775N	0.76 kb *acbN* PCR fragment (*Nde*I/*Bgl*II) from genomic DNA of *Actinoplanes* sp. in pJOE2775 (*Nde*I/*Bam*HI)
pCW21N	0.76 kb *Nde*I/*Hin*dIII fragment from pCW2775N in pUCPU21 (*Nde*I/*Hin*dIII)
pCW11aN	0.76 kb NdeI/*Hin*dIII fragment from pCW21N in pET11a (*Nde*I/*Hin*dIII)
pCWO16	0.87 kb *Nde*I/*Bgl*II fragment from pMJO1 in pET16bP (*Nde*I/*Bam*HI)
pCW2775O	0.82 kb *acbO* PCR fragment (*Nde*I/*Bgl*II) from genomic DNA of *Actinoplanes* sp. in pJOE2775 (*Nde*I/*Bam*HI)
pCW21O	0.82 kb *Nde*I/*Hin*dIII fragment from pCW2775O in pUCPU21 (*Nde*I/*Hin*dIII)
pCW11aO	0.82 kb *Nde*I/*Hin*dIII fragment from pCW21O in pET11a (*Nde*I/*Hin*dIII)
pCW201O	0.82 kb *Nde*I/*Hin*dIII fragment from pCW21O in pUWL201RBSA (*Nde*I/*Hin*dIII)
pCW18P	1.252 kb *Pst*I fragment from pMJ6.6 in pUC18 (*Pst*I)
pCW218P	1.252 kb *Eco*RI/*Hin*dIII fragment from pCW18P in pUWL218 (*Eco*RI/*Hin*dIII)
pCW201P	1.252 kb *Eco*RI/*Hind*III fragment from pCW18P in pUWL201 (*Eco*RI/*Hin*dIII)
pCW218EB	4.76 kb *Eco*RI/*Bcl*I fragment from pMJ6.6 in pUWL218 (*Eco*RI/*Bam*HI)
pCW201P	1.252 kb *Eco*RI/*Hind*III fragment from pCW18P in pUWL201 (*Eco*RI/*Hin*dIII)
pCW218EB	4.76 kb *Eco*RI/*Bcl*I fragment from pMJ6.6 in pUWL218 (*Eco*RI/*Bam*HI)
pCW201EX	4.76 kb *Eco*RI/*Xba*I fragment from pCW218EB in pUWL201 (*Eco*RI/*Xba*I)
pCW201EH	4.76 kb *Eco*RI/*Hin*dIII fragment from pCW218EB in pUWL201 (*Eco*RI/*Hin*dIII)
pCW218PB	1.8 kb *Bam*HI fragment from Ωaac4 in pCW218P (*Bam*HI)
pCW223S1	Self-ligation of *Sst*I-digested pHTW223
pCW223S2	1.7 kb *Sst*I fragment from pHTW223 in pUCPU21 (*Sst*I)
pCW223K1	Self-ligation of *Kpn*I digested pHTW223
pCW223K2	3.1 kb *Kpn*I fragment from pHTW223 in pUCPU21 (*Kpn*I)
pCW4123U	1.5 kb *Nde*I/*Eco*RI fragment from pHTW233 in pIJ4123 (*Nde*I/*Eco*RI)
pCW54S1	2.1 kb *Sst*I fragment from λGEM54 in pUC18 (*Sst*I)
pCW54S1K1	1.2 kb *Kpn*I fragment from pCW54S1 in pUCPU21 (*Kpn*I)
pCW54S1K2	Self-ligation of *Kpn*I digested pCW54S1

2.4: Oligonucleotides

Tab. 2.5: Oligonucleotides used in this study

Primer	Nucleotide sequence [1]	Target
CZ1	5'- CCGGAGAAGTAGTGCCTACGATCC -3'	0.5 kb *rpsL* fragment of *S. lividans*
CZ2	5'- GGGGCCCTTACGAGGCATTC -3'	0.5 kb *rpsL* fragment of *S. lividans*
CZ3	5'- CCTCGGATCCACTCGATGCC -3'	1.3 kb *cmlR* fragment
CZ4	5'- AGTCGACTGGCGGTTTCACCGG -3'	of *S. lividans*
CZ5	5'- GTGCCCCGGTGGTCGACGTC -3'	0.9 kb terminal inverted repeats (TIR)
CZ6	5'- GGATCCTGCATCGTGCACGG -3'	at the chromosome ends of *S. lividans*
CZ7	5'- ATGACGTCACCGCCCATCGT -3'	1.7 kb left chromosome ends (*Ase*I-A)
CZ8	5'- AGGAATCCGACGCTGAGGGG -3'	of *S. lividans*
CZ9	5'- TAGACCGTGATGAGCCCGGC -3'	1.3 kb right chromosome ends (*Ase*I-H1)
CZ10	5'- AGATCCCGCACAGCACGTCC -3'	of *S. lividans*
CZ11	5'- GCGGCACATCTACGACGAGG -3'	1.2 kb fragment from AUD1
CZ12	5'- CCTTGGTCTGGGTGCACAGC -3'	of *S. lividans*
AcbK1	5'- CAAGGAGACATATGTCGGAGCAC -3'	1.0 kb *acbK*, *Nde*I
AcbK2	5'- GTGGTGAGATCTTCGCCCAGT -3'	1.0 kb *acbK*, *Bgl*II
2775M1	5'- GCCGGCCATATGAAGCGGC -3'	1.08 kb *acbM*, *Nde*I
2775M2	5'- CGGTGCCAGATCTTCGCCC -3'	1.08 kb *acbM*, *Bgl*II
2775L1	5'- TTGGTCGGCATATGAGCCGG -3'	1.10 kb *acbL*, *Nde*I
2775L2	5'- CCAGAGTCCAGATCTTCGATGATCC -3'	1.10 kb *acbL*, *Bgl*II
2775N1	5'- AGAGGATCACATATGAGCGGGACTC -3'	0.76 kb *acbN*, *Nde*I
2775N2	5'- ACCCGGCAGATCTCGTCCG -3'	0.76 kb *acbN*, *Bgl*II
2775O1	5'- GGTGCGCATATGACCTGCCG -3'	0.82 kb *acbO*, *Nde*I
2775O2	5'- CGTCTCGACACAGATCTCGTCAGCTT -3'	0.82 kb *acbO*, *Bgl*II
primer 1	5'-d[GGTGCGGGAA]-3'	RAPD analysis
primer 2	5'-d[GTTTCGCTCC]-3'	RAPD analysis
primer 3	5'-d[GTAGACCCGT]-3'	RAPD analysis
primer 4	5'-d[AAGAGCCCGT]-3'	RAPD analysis
primer 5	5'-d[AACGCGCAAC]-3'	RAPD analysis
primer 6	5'-d[CCCGTCAGCA]-3'	RAPD analysis

[1] : the sequence for restriction enzymes are underlined.

2.5: Media and buffers

2.5.1: Media for culturing *E. coli*

LB-Medium (Luria Bertani) (Miller, 1972)

Tryptone	10.00	g/l
Yeast extract	5.00	g/l
NaCl	10.00	g/l
(Agar)	15.00	g/l

SOB (Hanahan, 1983)

Tryptone	20.00	g/l
Yeast extract	5.00	g/l
NaCl	0.58	g/l
KCl	0.19	g/l
Add after autoclaving:		
$MgCl_2$ x 6 H_2O (1M)	10.0	ml/l
$MgSO_4$ x 7 H_2O (1M)	10.0	ml/l

SOC (Hanahan, 1983)

D-glucose	3.60	g/l
in SOB		

2.5.2: Media for culturing bacteriophage

LB-$MgSO_4$-Agar

LB-agar		
$MgSO_4$	10	mM

Top-Layer

Agarose	7	g/l
$MgSO_4$	10	mM

LB-maltose-Medium

LB-medium		
Maltose	2	g/l

2.5.3: Media for culturing actinomycetes

AGS (Arginine Glycerol Salts)

Glycerol	12.5	g/l
Arginine	1.0	g/l
NaCl	1.0	g/l
K_2HPO_4	1.0	g/l
$MgSO_4$ x $7H_2O$	0.5	g/l
Agar	15.0	g/l
Glucose	2.0	g/l
Casminoacid	0.1	g/l
pH 7.0		

AM-Agar (Hopwood *et al.*, 1985)

Glycerol	15.0	ml/l
L-arginine-HCl	5.0	g/l
L-leucine	1.0	g/l
L-methionine	0.3	g/l
KH_2PO_4	0.5	g/l
$MgSO_4$ x 7 H_2O	0.2	g/l
$FeSO_4$ x 7 H_2O	0.01	g/l
$CuSO_4$ x 5 H_2O	0.01	g/l
$ZnSO_4$ x 7 H_2O	0.01	g/l
$MnSO_4$ x 7 H_2O	0.04	g/l
Agar	15.0	g/l

AMT-Agar (Hopwood *et al.*, 1985)

AM-Agar		
L-tyrosine	1.0	g/l

MD-50 Medium (Hemker *et al.*, 2001)

Solution I: (add dd$H_2$0 to 400ml)

MD-50	70	g
$(NH_4)_2SO_4$	5	g
Yeast extract	2	g

Solution II: (add ddH20 to 400ml)

K_2HPO_4	1	g
KH_2PO_4	1	g
Tri-sodiumcitrate	5	g

Solution III: (add ddH20 to 400ml)

$MgCl_2$ x 6 H_2O	1	g
$FeCl_3$ x 6 H_2O	0.25	g
$CaCl_2$ x 2 H_2O	2	g

The above Solution I – III were mixed and sterilized by filtration

MM-Agar (Hopwood et al., 1985)

$(NH_4)_2SO_4$	1	g/l
K_2HPO_4	0.5	g/l
$MgSO_4$ x 7 H_2O	0.2	g/l
$FeSO_4$ x 7 H_2O	0.01	g/l
D-glucose	10	g/l

NBS-Medium (Bayer AG, Wuppertal, for *Actinoplanes* sp.)

Glucose	10	g/l
Peptone	4	g/l
Yeast extact	4	g/l
$MgSO_4$ x 7 H_2O	0,5	g/l
KH_2PO_4	2	g/l
K_2HPO_4	4	g/l

NBS-Agar (Bayer AG, Wuppertal, for *Actinoplanes* sp.)

NBS-Medium		
Agar	20	g/l

R2YE (Hopwood *et al.*, 1985)

Sucrose	103.00	g/l
D-glucose	10.00	g/l
$MgCl_2$ x 6 H_2O	10.12	g/l
K_2SO_4	0.25	g/l
Difco-casamino acids	0.10	g/l
Difco-Bacto yeast extract	22.00	g/l
Add after autoclaving:		
KH_2PO_4 (0.5%)	10.00	ml/l
$CaCl_2$ x 2 H_2O (3.68%)	80.00	ml/l
L-proline (20%)	15.00	ml/l
TES-buffer (5.73%, pH 7.2)	100.00	ml/l
Trace elements solution	2.00	ml/l
1 M NaOH	5.00	ml/l

Trace elements solution

$ZnCl_2$	40.00	g/l
$FeCl_2$ x 6 H_2O	200.00	g/l
$CuCl_2$ x 2 H_2O	10.00	g/l
$MnCl_2$	10.00	g/l
$Na_2B_4O_7$ x 10 H_2O	10.00	g/l
$(NH_4)_6Mo_7O_4$ x 4 H_2O	10.00	g/l

SNA (Soft Nutrient Agar) (Hopwood *et al.*, 1985)

Nutrient broth	8.00	g/l
Difco-Bacto agar	3.00	g/l

SMA (Distler *et al.*, 1985)

Soybean powder	20.00	g/l
Mannitol	20.00	g/l
Agar	20.00	g/l

SPMR (Babcock and Kendrick, 1988)

Sucrose	103.00	g/l
$MgCl_2$ x 6 H_2O	10.00	g/l
D-glucose	5.00	g/l
Yeast extract	5.00	g/l
TES, pH 7.2	4.58	g/l
Add after autoclaving:		
$CaCl_2$ x 2 H_2O (5 M)	2.00	ml/l
Trace elements solution	2.00	ml/l
Agar	15.00	g/l

TSB

Tryptone Soya Broth (Oxoid)	30.00	g/l

YEME (Hopwood *et al.*, 1985)

Yeast extract	3.00	g/l
Peptone	5.00	g/l
Malt extract	3.00	g/l
D-glucose	10.00	g/l
Sucrose	340.00	g/l
Add after autoclaving:		
$MgCl_2$ x 6 H_2O (1 M)	1.00	ml/l
Glycine	5.00	ml/l
(Agar)	15.00	g/l

2.5.4: Buffers

- for isolation of plasmid DNA

Alkaline Lysis Solution (Birnboim & Doly, 1979)

Solution I:

Glucose	50	mM
Tris-HCl, pH 8.0	50	mM
EDTA, pH 8.0	10	mM

Solution II:

SDS	1%	
NaOH	200	mM

Solution III:

Potassium acetate	3	M
Formic acid	1.8	M

STET Buffer (Sambrook *et al.*, 1989)

Sucrose	80	g/l
Triton X-100	50	ml/l
Tris-HCl, pH 8.0	50	mM
EDTA, pH 8.0	50	mM

Lysozyme solution (Hopwood *et al.*, 1985)

Sucrose	0.3	M
Tris-HCl, pH 8.0	25	mM
EDTA, pH 8.0	25	mM

- for preparation of competent *E. coli* cells (Hanahan, 1983)

FSB-buffer

Potassium acetate, pH7.0	10	mM
KCl	10	mM
$MnCl_2$ x 4 H_2O	45	mM
$CaCl_2$ x 2 H_2O	10	mM
Hexaminecobaltchloride	3	mM
Glycerol	100	g/l
pH 6.4		
Sterilized by filtration		

- for preparation of genomic DNA from *Streptomyces* (Pospiech & Neumann, 1995)

SET buffer

NaCl	75	mM
EDTA	25	mM
Tris	20	mM
pH 7.5		

TE buffer (Sambrook *et al.*, 1989)

Tris	10	mM
EDTA	1	mM
pH 8.0		

- for preparation of protoplasts from *Streptomyces* (Chater *et al.*, 1982)

P-buffer

Sucrose	103	g
K_2SO4	0.25	g
$MgCl_2$ x 6 H_2O	2.02	g
Add H_2O to 800 ml volume		
Autoclave in 80 ml aliquots and supplement with:		
K_2HPO_4 (0.5%)	1	ml
$CaCl_2$ x 2 H_2O (3.68%)	10	ml
TES (5.73%), pH 7.2	10	ml

- for transformation in *Streptomyces* (Babcock & Kendrick, 1988)

T-buffer:

Sucrose (10.3%)	25	ml
K_2SO_4 (2.5%)	1	ml
H_2O	75	ml
Trace elements	0.2	ml
$CaCl_2$ (0.25 M)	0.2	ml

- for enumeration of viable propagules from soil

One-fourth-strength Ringers solution

NaCl	2.25	g/l
KCl	0.1	g/l
$CaCl_2$	0.12	g/l
$NaHCO_3$	0.05	g/l

- for agarose gel electrophoresis of DNA fragments (Sambrook *et al.*, 1989)

DNA loading buffer (10 x)

Sucrose	0.5	g/ml
EDTA, pH 8.0	160	mM
Bromophenol Blue	0.5	mg/ml
Xylene cyanol	0.5	mg/ml

TAE (50 x)

Tris	242	g/l
Glacial acetic acid	57.1	ml/l
EDTA	0.4	g/l

TBE (10 x)

Tris	108	g/l
Boricacid	61	g/l
EDTA	0.4	g/l

TE 25 Suc (Leblond *et al.*, 1993)

Tris	25	mM
Sucrose	300	mM
EDTA	25	mM
Adjusted to pH 8.0 with 1 M HCl		

NDS (Leblond *et al.*, 1993)

Lauryl sarcosine	1.0%	
EDTA	50	mM
Tris, pH 9.5	10	mM

- for DNA-DNA hybridization (Southern, 1975)

Prehybridization solution

SSC (20 x)	300	ml/l
Sodium phosphate buffer (1 M), pH 6.8	10	ml/l
EDTA (0.5 M), pH 8.0	2	ml/l
SDS (10%)	50	ml/l
Blocking reagent	1	g/l

Hybridization solution

SSC (20 x)	330	ml/l
Sodium phosphate buffer (1 M), pH 6.8	10	ml/l
EDTA (0.5 M), pH 8.0	2	ml/l
SDS (10%)	50	ml/l
Blocking reagent	1	g/l

Wash solution (2 x)

SSC (20 x)	200	ml/l
SDS (10%)	10	ml/l

Wash solution (0.5 x)

SSC (20 x)	25	ml/l
SDS (10%)	10	ml/l

SSC (20 x)

NaCl	3	M
Sodium citrate	0.3	M

- for denatured polyacrylamide gel electrophoresis (Schägger and von Jagow, 1987)

Sample buffer:

SDS	4%	w/v
Glycerol	12%	w/v
Tris	50	mM
2-mercaptoethanol	2%	v/v
Serva blue G	0.01%	
Adjusted to pH 6.8 with 1 M HCl		

49.5%T/3%C PAA-Solution:

Acrylamide	480	g/l
N-N bisacrylamide	15	g/l

Gel-buffer:

Tris	3.0	M
SDS	0.3%	
Adjusted to pH 8.4 with HCl		

Cathode-buffer:

Tris	0.1	M
Tricine	0.1	M
SDS	0.1%	w/v
pH was not adjusted		

Anode-buffer:

Tris	0.2	M
Adjust to pH 8.9 with HCl		

Gel staining solution

Coomassie Brilliant Blue R250	1.5	g/l
Methanol	450	ml/l
Acetic acid	100	ml/l

Destaining solution

Methanol	250	ml/l
Acetic acid	100	ml/l

- for Western blotting and detection of proteins

TBS buffer

Tris-HCl (pH 7.5)	10	mM
NaCl	150	mM

TBS-T buffer

Tris-HCl (pH 7.5)	20	mM
NaCl	500	mM
Tween 20	0.5	g/l
Triton X-100	2	g/l

Blocking buffer

3% (w/v) BSA in TBS buffer

Blotting buffer

Tris-HCl (pH7.5)	48	mM
Glycine	39	mM
Methanol	20%	

- for disruption (sonication) of cells

Buffer 1 (for AcbK)

Tris-HCl, pH7.6	25	mM
$MgCl_2$	10	mM
2-mercaptoethanol	1.5	mM

Buffer 2 (for AcbM, L, N, O, U and 1-*epi*-valienol kinase)

Tris-HCl, pH7.6	25	mM
$MgCl_2$	10	mM
NH_4Cl	20	mM
2-mercaptoethanol	1.5	mM

Buffer A (for AcbC, Stratmann *et al.*, 1999)

K_2HPO_4	10	mM
KH_2PO_4	10	mM
NAD^+	0.2	mM
Dithiothreitol (DTT)	0.5	mM
Adjust to pH 7.5 with KOH		

- for purification of His-tag proteins

Starting buffer

Na_3PO_4	20	mM
NaCl	500	mM
NH_4Cl	20	mM
Imidazole	10	mM
2-mercaptoethanol	1.5	mM
pH 7.5		

Elution buffer

Na_3PO_4	20	mM
NaCl	500	mM
NH_4Cl	20	mM
Imidazole	500	mM
2-mercaptoethanol	1.5	mM
pH 7.5		

- for visualization of spots on the TLC

Cer-Reagent (Drepper *et al.*, 1996b)

Phosphomolybdic acid x H_2O	25	g/l
Cer (IV)-sulfate x 4 H_2O	10	g/l
H_2SO_4 (conc.)	60	ml/l
H_2O	940	ml/l

2.6: Antibiotics

The antibiotics used in this work were normally supplemented to the corresponding media at a concentration as shown in **Tab.** 2.6.

Tab. 2.6: Antibiotic concentrations for supplementation of media

Antibiotic	Final concentration (µg/ml)
ampicillin (Ap) [1]	100
apramycin (Apr) [1]	5
chloramphenicol (Cml) [2]	5
cycloheximide [1]	50
erythromycin (Ery) [1]	20
gentamicin (Gm) [1]	5
hygromycin B (Hyg) [1]	5
kanamycin (Km) [1]	50
lincomycin (Lm) [1]	30
lividomycin [1]	5
nystatin [3]	50
rifampicin (Rif) [4]	10
spectinomycin (Spc) [1]	5
streptomycin (Sm) [1]	50
tetracycline (Tet) [2]	5
thiostrepton (Thio) [5]	25
viomycin [1]	5

[1] : Dissolved in H_2O sterilized
[2] : Dissolved in ethanol
[3] : Dissolved in a small volume of sterile 0.1 N NaOH and made up to volume with H_2O
[4] : Dissolved in DMF
[5] : Dissolved in DMSO

2.7: Growth and maintenance of bacterial strains

2.7.1: Growth and maintenance of *E. coli*

E. coli strains were generally cultivated at 37°C on LB plates or in LB liquid medium. Strains harboring plasmids were grown on plates or in liquid media supplemented with the appropriate antibiotics. Strains were stored at - 70°C in 30% glycerol.

2.7.2: Growth and maintenance of *Streptomyces* cultures

Streptomyces strains were grown on SMA or TSB plates. The liquid cultures were generally grown at 28 - 30°C for 2 - 5 days in YEME medium. Strains harboring plasmids were cultivated on plates or in liquid media containing the appropriate antibiotics. Spore suspensions were generated according to Hopwood *et al.* (1985) by separating spores from a well sporulated plate by flooding it with 20% (v/v) glycerol and scraping the spores off the substrate mycelia with a sterilized inoculation loop. The spore suspension was filtered through sterile non-absorbant cotton wool to remove the fragments of mycelia. The spore suspensions were stored at -70°C.

2.7.3: Growth of *Actinoplanes* sp. cultures

Actinoplanes sp. strains SE50/110 and SN223/29 were grown on SMA plates. The liquid cultures were grown at 28°C in a two step fermentation procedure. A single colony of the strain was inoculated into 10 ml of MD50 (or NBS) medium on a rotary shaker for 96 - 120 hours at 28°C, and then 1 ml of this pre-culture was used to inoculate the main culture (100 ml of MD50 medium in 1,000 ml Erlenmeyer flasks). After 72 h at 28°C on a rotary shaker the culture was harvested by centrifugation at 4,000 rpm.

2.7.4: Crosses (mating) between *Streptomyces* species

Crosses (mating) between *Streptomyces* species were performed on SPMR agar using roughly equivalent numbers of spores of the two parental strains. Following one round of non-selective growth at 30°C for 5 – 7 days, spores were collected and dilutions were spotted onto TSB-agar containing antibiotics appropriate for the selection of the relevant cell phenotypes.

2.8: Environmental applications

2.8.1: Preparation of soil for use in microcosms

Shifnal type brown earth taken from a wheatfield site at Cryfield Hall, University of Warwick, Coventry, England and sand soil from a garden (Wuppertal, Dec. 1998) were used in soil microcosm experiments. The Warwick soil was air dried in the dark over a few months to approximate 1% moisture and stored at 22°C. Analysis particle size of the soil gave the following composition (percent, dry weight): 63.6% sand, 18.4% silt, 11.7% clay and 6.2% loss on ignition

(Herron *et al.*, 1990). Approximate 20% of the clay fraction was kaolin, 30% was mica, 14% was chlorite and 27% was an expansible phase (Soil Survey and Land Research Center; profile nr. SP 29/6909). To achieve standard experimental conditions, the Warwick soil used in microcosm experiments was pulverized using a pestle and mortar and passed through a 2 mm sieve. The garden soil was not examined so far. Sterile soil was prepared by autoclaving it twice at 121°C for 20 minutes on two consecutive days. The pH of the fresh Warwick soil was measured by mixing 20 g of soil with 50 ml distilled water and stirring for 15 min. The pH from nonsterilized soil ranged from 6.9 to 7.1, whereas the pH from sterilized soil was 6.5 to 6.8. For experiments on soil microcosms, normally 4 g soil from Warwick or garden was placed into a glass bottle (50 x 21 cm with lid, Scherf Praezision, Germany). Nutrient amendments were made by addition of 0.04 g of soluble starch and 0.04 g of chitin (crab shell) prior to sterilization and, for nonsterile soil, nutrient amendments were first autoclaved and added to soil just before inoculation.

2.8.2: Inoculation of soil microcosms

Normally, soil was wetted to 15% water content. Spore suspensions, fresh mycelia or protoplasts were used to inoculate the soil microcosms. After being heated at 50°C for 3 min, 600 µl spore suspensions (or 300 µl for each of the two parental strains) were inoculated into 4 g soil microcosms. For mycelia inoculation, strains were grown in 15 ml of YEME medium for 3 days, collected by centrifugation and washed twice with 10.3% sucrose, then resuspended in 5 ml of YEME, immediately used for inoculation. 4 g soil microcosms were inoculated with 600 µl (or 300 µl for each of the two parental strains) fresh mycelia to give a 15% water content. 600 µl protoplasts resuspended in P-buffer were inoculated into 4 g soil. After addition, liquid media with inoculants was penetrated into soil. To ensure that inoculants were averagely dispersed in soil, soil was stirred using sterile toothpicks. The inoculum levels at the start of each experiment were expressed as propagules recovered from the soil immediately after addition.

2.8.3: Enumeration of viable propagules from soil

On sampling day, spores and mycelia were extracted from soil by shaking about 200 mg of soil in 1.8 ml of One-fourth-strength Ringer solution (**Sect.** 2.5.4) for 30 min at 30°C. After 2 - 3 h, soil particles were naturally precipitated and then the supernatants were serially diluted in One-fourth-strength Ringer solution and plated on TSB plates supplemented with different antibiotics. When nonsterile soil was used, nystatin (50 µg/ml), nalidixic acid (25 µg/ml) and cycloheximide

(50 µg/ml) were added to plates for inhibition of the resident fungi. For counting viable cells, 200 µl of each dilution was spread on one TSB plate containing appropriate antibiotics. For each dilution, 3 plates were used. The colony forming units (c.f.u.) per 1 g of soil were calculated as following:

$$\frac{mx10^{n}}{y(\text{g soil})} \text{ x } \frac{1.8\text{ml}}{0.2\text{ml}}$$

If *m* colonies appeared on 10^{-n} diluting plates when extracted from *y* g of soil.

2.9: *In vitro* manipulation of DNA

2.9.1: Preparation of plasmid DNA from *E. coli*

Plasmid DNA from *E. coli* was prepared according to two methods. Plasmid DNA to be used for sequencing reactions was isolated from cells by using the Miniprep Plasmid Kit (Qiagen) according to the manufacturer's specifications. The boiling preparation method was used for rapid screening of transformants (Sambrook *et al*., 1989).

2.9.2: Preparation of plasmid DNA from streptomycetes

Plasmid DNA was generally extracted from streptomycete mycelia according to the method adapted from Birnboim and Doly (1979) with some modifications: Mycelia from streptomycete strains was harvested in the late logarithmic phase, washed twice with 10.3% sucrose prior to DNA preparation and were suspended in 150 µl of solution I (**Sect.** 2.5.4) containing 10 mg/ml of lysozyme. After the addition of 300 µl of solution II (**Sect.** 2.5.4), the cell mixture was mixed gently. Following an incubation for 30 – 60 min at 37°C, 225 µl of ice-cold solution III (**Sect.** 2.5.4) was added. The samples were centrifuged for 10 – 15 min and the supernatant was transferred to a fresh tube. The supernatant was extracted twice with an equal volume of phenol/chloroform (1:1). The supernatant was transferred to a new tube. The DNA was precipitated by the addition of 500 µl isopropanol with subsequent centrifugation for 30 min at 15,000 rpm, 4°C. The supernatant was discarded and the DNA was washed in 70% cold ethanol and dried. DNA was then dissolved in 50 µl distilled water.

2.9.3: Extraction of genomic DNA from actinomycetes

Chromosomal DNA was isolated from mycelia of actinomycetes species according to the protocol of Pospiech and Neumann (1995) with minor modifications. The following steps were used. Strains were inoculated in 15 ml of YEME medium in a 10 x 22 cm glass tubes with a short metal spring. The culture was grown at 28°C on a shaker (about 200 rpm) for 3 - 4 days to the late logarithmic phase. The mycelia were harvested by centrifugation (10 min, 4,000 rpm) and washed twice with 10.3% sucrose. Approximate 0.5 g of cells were resuspended in 5 ml SET buffer (**Sect.** 2.5.4) containing 1 mg/ml of lysozyme and incubated at 37°C for 0.5-1 h. 1/10 volume of 10% SDS (500 µl) and 0.5 mg/ml of proteinase K (2.5 mg) were added and incubated at 55°C with occasional inversion for 1-2 h. Then 1/3 volumes of 5M NaCl (1.7 ml) and 1 volume of phenol (5 ml) were added and incubated at room temperature for 30 min with frequent gentle inversion. The flocculant precipitate was removed by centrifugation at 4°C, 5,000 rpm for 15 min. The aqueous phase was transferred to a new tube using a blunt-ended pipette tip and extracted twice with 1 volume of chloroform (5 ml). The chromosomal DNA was precipitated by adding 1 volume of isopropanol, mixing by gentle inversion, incubation at room temperature for 30 min, followed by centrifugation for 30 min at 4°C, 15,000 rpm. The precipitate was washed with 70% cold ethanol, air dried at room temperature and dissolved in TE buffer (200 µl) with 2 µl of 10 mg/ml of RNase.

2.9.4: Preparation of bacteriophage DNA

The preparation of phage DNA of λGEM54 from *E. coli* LE392 was performed according to the plate-lysis-method (Sambrook *et al.*, 1989).

2.9.5: Restriction endonuclease digestion of DNA

DNA restriction analyses were carried out with 2 - 5 units enzyme per 1 µg DNA in the reaction mixture. The volume was made up to 20 µl with distilled water and enzyme buffer according to recommendations of the manufacturer, and incubated at 37°C for one to two hours. An aliquot of the digestion was checked on an agarose gel.

2.9.6: Recovery of DNA fragments from agarose gels

DNA fragments were subjected to agarose gel electrophoresis. The fragments were excised and subsequently purified either by using a QIAquick Gel Extraction Kit (Qiagen, Hilden), or by a Nucleo Spin Extract 2 in 1 Kit (Macherey-Nagel, Düren) according to the manufacture's specifications.

2.9.7: Removal of phosphate groups from DNA

Linearized DNA was treated with calf intestine alkaline phosphatase (CIAP) in order to remove 5' phosphate groups from DNA. DNA was incubated at 37°C for 30 min following the addition of 1 - 2 U CIAP. CIAP was either heat inactivated by an incubation of 15 min at 68°C or by a phenol/chloroform extraction.

2.9.8: Generation of blunt ends using the Klenow fragment of DNA polymerase I

In order to generate blunt ends necessary for cloning or for generating shifts of a reading frame, DNA fragments were treated with Klenow fragment according to the manufacture's recommendations prior to ligation. The enzyme was either heat inactivated by an incubation of 15 - 30 min at 70°C or by a phenol/chloroform extraction. The DNA was precipitated, washed in 70% ethanol and dissolved in an appropriate amount of water.

2.9.9: Ligation of DNA fragments

Ligation reactions were carried out in 20 µl volume with a 4:1 molar ratio of insert to vector. The reaction mixture was heated to 50°C for 5 min prior to the addition of T4-DNA ligase and ligation buffer. Ligation of blunt ended fragments was carried out with 2 U T4-DNA ligase for a minimum of 4 h at 12 - 14°C, whereas the ligation of fragments with cohesive ends was conducted with 1 U T4-DNA ligase at room temperature for at least 1 h.

2.9.10: Quantitation of DNA

DNA concentrations were quantified photometrically at a wavelength of 260 nm as described by Sambrook *et al.* (1989). An OD_{260} of 1.0 is equivalent to approximate 50 µg/ml DNA. The OD of the DNA solution was also measured at 280 nm to check for contamination with phenol or proteins. The ratio of OD_{260}/OD_{280} of pure DNA solutions should range from 1.8 - 2.0.

2.9.11: DNA sequencing

DNA was subjected to a sequence reaction based on the method described by Sanger *et al.* (1977) using the Thermosequenase Cycle-Sequencing kit (Amersham Bioscience, Freiburg) and primers labeled with Cy5 according to manufacturer's specifications on an automatic DNA sequencer (A.L.F. express, Amersham Bioscience, Freiburg).

2.9.12: Southern capillary blotting

Southern blotting was carried out according to Reed *et al.* (1985). Following electrophoresis of DNA agarose gels were placed in 0.25 M HCl until the dye of the loading buffer had changed color and left a further 10 min. Gels were then placed in 0.5 M NaOH/1.5 NaCl solution for 30 min to denature DNA. Gels were rinsed in distilled water for 30 min with shaking. DNA was capillary transferred from agarose gels to positively charged Hybond N^+-membranes using 0.4 N NaOH for a minimum of 6 hours. Membranes were rinsed in 2 x SSC, wrapped in Saran-Wrap and stored at 4°C until use.

2.9.13: Labeling of DNA fragments with [^{32}P]

DNA fragments were labeled with [^{32}P] using the *Redi*prime DNA labeling kit (Amersham) according to manufacturers specifications. In general, 5 µl of α-[^{32}P]-dCTP with a specific activity of 3,000 Ci/mmol was used in the labeling reaction of 50 µl volume .

2.9.14: DNA-DNA hybridization

Membranes were prehybridized in 200 - 400 ml prehybridization solution (**Sect.** 2.5.4) in a shaking water bath for a minimum of 1 h. The labeled probe was denatured by boiling for ten minutes, then immediately placed on ice. Membranes were transferred to 100 ml hybridization solution and the denatured radioactive probe then added. Following incubation at the appropriate temperature (68°C) for a minimum of 8 h, membranes were washed once in 5 x SSC (**Sect.** 2.5.4) for a total of at least 45 min. Stringency washes were carried out in 0.50 - 2 x SSC (**Sect.** 2.5.4) at the proper temperatures (68°C) for 15 minutes with gentle shaking.

2.9.15: Autoradiography

Membranes or TLC plates were placed in a film cassette with X-ray film (Amersham Typ Hyperfilm MP^{TM}) along with intensifying screens (DuPont Typ Quanta III). The cassette was sealed in and left at -70°C or at room temperature for a sufficient time to obtain the best exposure.

2.10: Preparation of competent *E. coli* cells

Cells were rendered competent for transformation by either the $CaCl_2$-method (Sambrook *et al.*, 1989) or the Hanahan-method (Hanahan, 1983). The $CaCl_2$-method commenced with the inoculation of 50 ml LB with 0.5 ml of an overnight culture of the appropriate strain. Cells were ready to be harvested at an OD_{600} of 0.5 to 0.7. The cells were cooled on ice for at least 10 min and subsequently spun for 10 min at 3,000 rpm at 4°C. All the following steps were carried out on ice or at 4°C. The cells were suspended in 10 mM ice-cold $CaCl_2$ and left on ice for 20 min. The cells were resuspended in 50 mM ice-cold $CaCl_2$ followed by centrifugation at 3,000 rpm for 10 min at 4°C. Cells were left on ice until use or stored at -70°C following the addition of glycerol to a total concentration of 25%.

All manipulations to make cells competent using the Hanahan method other than cell cultivation were carried out at 4°C or on ice. Cells from 1 colony of *E. coli* strains were used to inoculate 50 ml of SOB. Cells were grown at 37°C and harvested at an OD_{600} of 0.4 - 0.5. The cell suspension was chilled on ice for a minimum of 10 min. The cell suspension was then centrifuged at 3,000 rpm for 10 min. The cells were suspended in 17 ml ice-cold FSB-buffer and incubated on ice for 15 min. Cells were resuspended in 4 ml FSB-buffer following centrifugation at 3,000 rpm, 4°C for 10 min. Cells were chilled on ice for 10 min and 2 x 140 µl DMSO were added in a 5 min interval. Cells were aliquoted in microfuge tubes and stored at -70°C.

2.11: Transformation of *E. coli* strains

E. coli cells were transformed according to Sambrook *et al.* (1989). In general, 7 µl of a ligation mixture containing 0.01 - 1 µg DNA were added to 100 µl competent cells and stored on ice for 10 min. Following a heat shock (2 min, 42°C), cells were allowed to regenerate in 800 µl SOC for 45 - 60 min at 37°C to allow phenotypic expression. The cell suspension was plated out on LB agar plates containing the appropriate antibiotics and/or X-Gal as a selective medium.

2.12: Preparation of protoplasts from streptomycetes

Protoplasts were prepared according to the method of Chater *et al.* (1982). Mycelium was grown in 25 ml of YEME supplemented with 34% sucrose and, in the case of *S. lividans*, with 0.5% glycine. Media was inoculated with 2 ml of a culture previously grown to the stationary phase incubated at 30°C for 36 h. The mycelium was sedimented by centrifugation at 3,000 rpm, washed twice in 10.3% sucrose and suspended in P-buffer containing 1 mg/ml lysozyme. The cell suspension was incubated at 30°C for 45 - 90 min or until microscopic examination showed that the cells were protoplasted. Following the addition of 5 ml P-buffer, the mycelium was passed through sterile cotton wool. The protoplasts were sedimented by spinning at 3,000 rpm for 4 min at 4°C and resuspended in 4 ml P-buffer. Cells were stored at -70°C in aliquots of 1 µl or used directly for transfomations**.**

2.13: Transformation of streptomycete protoplasts with plasmid DNA

Protoplasts were transformed according to Babcock und Kendrick (1988). Frozen protoplasts were quickly thawed and centrifuged at 3,500 rpm for 7 min. Approximately 100 ng of plasmid DNA and 100 µl of T-buffer/PEG were added to the protoplasts and gently mixed by pipetting up and down three times. After 900 µl P-buffer had been added, the cells were spun off at 3,500 rpm for 7 min and plated out on predried R2YE or SPMR agar. Plates were incubated at 30°C for 24 h and the regenerated protoplasts overlaid with SNA agar containing 300 µg/ml thiostrepton or kanamycin. Plates were checked for transformants after 3 - 7 days.

2.14: Gel electrophoresis of DNA

2.14.1: Agarose gel electrophoresis

Agarose gel electrophoresis was performed essentially as described by Sambrook *et al.* (1989). DNA molecules were separated on 0.7 - 1.5% agarose gels containing 0.1 µg/ml ethidium bromide. Approximate 1/10 volume loading buffer was added to the DNA solutions prior to loading samples into the wells. Electrophoresis was carried out at 5 - 10 V/cm. DNA was visualized by illumination with long wavelength UV-light (302 nm). Fragment sizes were determined by comparison to conventionally used or commercially available DNA size markers.

2.14.2: Pulsed field gel electrophoresis (PFGE)

PFGE was performed as described by Leblond *et al.* (1993) with some variations. *Actinoplanes* sp. SE50/110 (also used for streptomycetes) was cultivated in 10 ml MD50 (or YEME with 34% sucrose) at 30°C for 4 days. The cells were harvested and washed twice in 10.3% sucrose. The washed cells were resuspended in 5 ml TE25 Suc (**Sect.** 2.5.4). To make plugs, 0.8 ml of the suspension was mixed with 0.8 ml of 1% low-melting-point agarose (Incert; FMC, Rockland, Maine) in TE25 Suc at 37°C. The resulting mixture was pipetted into a prechilled plug mold. After setting, the plugs were carefully pushed out of the molds into 5 ml of lysozyme (1 mg/ml) in TE25 Suc. After incubation at 37°C for 2 h the plugs were transferred to NDS (**Sect.** 2.5.4) incubated at 50°C for 1 h and after addition of proteinase K (1 mg/ml) further incubated at 50°C for 20 to 30 h. After cooling on ice for 1 h, the plugs were incubated in 5 ml TE containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for 2 h at 4°C for inactivation of the protease and then washed 3 times for at least 1 h each with 5 ml of TE at 4°C.

The plugs were cut into pieces (2 x 5 x 10 mm) soaked in 1 x restriction enzyme buffer for 15 min (for *Dra*I at least 4 h at 4°C). The buffer was replaced by fresh buffer to cover the plugs (total volume, ca. 250 ml), 5 µl of bovine serum albumin (50 mg/ml) and 20 to 30 U of the restriction enzymes were added and the plugs were incubated at 37°C for 4 h (*Dra*I) or 6 h (all other enzymes). The electrophoresis was performed with 1.2% agarose gels (SeaKeam; FMC) in 0.5 x TBE buffer, using a hexagonal array of electrodes with the Pulsaphor unit and Controller Plus from Amersham Bioscience. PFGE were run at 180 V for 16 – 24 h with changing pulse time.

2.15: Polymerase chain reactions (PCR)

2.15.1: General PCR conditions

Approximate 50 - 500 ng of genomic template DNA were used with 100 pmol of each primer per 100 µl reaction volume. In general, 0.2 mM dNTP solution obtained from Biolabs were used in assays using Vent-DNA polymerase (New England Biolabs) or Boehringer Mannheim when using *Taq*-DNA polymerases (Amersham Bioscience). To improve the denaturation of the DNA, 10% DMSO was added to the reaction mixture. A few drops of mineral oil were layered over the reaction mixture to prevent evaporation. To ensure that no contaminating DNAs would give false positive results, one sample lacking a template was included in each series of reactions.

Amplifications were performed in a Biometra Personal Cycler (Biometra, Göttingen, FRG) using *Taq*-DNA polymerase. The annealing temperature of each primer (with mismatches T_1°C, without mismatches T_2°C, see also **Tab.** 2.7 and 2.8) was calculated using the computer program PrimerFind 3.0. The amplification profile usually was designed as follows (**Tab.** 2.7):

Tab. 2.7 Standard reaction conditions for PCR

	Step	Temperature	Time
	1	98°C	5 min
	2	95°C	1 min
5 x	3	T_1°C	1 min
	4	72°C	1 min/kbp
	5	95°C	1 min
25 x	6	T_2°C	1 min
	7	72°C	1 min/kbp
	8	72°C	7 min

Polymerase was added at step 2

Tab. 2.8 Annealing temperatures for specific primer pairs

Primer pairs [1]	Amplified genes	Temperatures	
		T_1°C	T_2°C
CZ1/CZ2	0.5 kb *rpsL*	61	61
CZ3/CZ4	1.3 kb *cmlR*	56	65
CZ5/CZ6	0.9 kb TIR	63	63
CZ7/CZ8	1.7 kb left chromosome end	62	62
CZ9/CZ10	1.3 kb right chromosome end	62	62
CZ11/CZ12	1.2 kb fragment of AUD1	60	60
AcbK1/AcbK2	1.0 kb *acbK*	41	57
2775L1/2775L2	1.1 kb *acbL*	45	61.5
2775M1/2775M2	1.08 kb *acbM*	39	63
2775N1/2775N2	0.76 kb *acbN*	46	61
2775O1/2775O2	0.82 kb *acbO*	45	63
AcbK1/2775M2	1.98 kb *acbKM*	41	58
AcbK1/2775L2	3.07 kb *acbKML*	41	58

[1] For sequences see **Tab.** 2.5.

2.15.2: PCR amplification of specific gene fragments

Some specific gene fragments were amplified in this work. Conditions (the annealing temperatures) for the amplifications were summarized in **Tab.** 2.8.

2.15.3: Random amplified polymorphic DNA (RAPD) PCR amplification of specific gene fragments

PAPD-PCR were performed by using the Ready-To-Go Kits (Amershma Bioscience, Freiburg) according to the instructions of the manufactures. For each assay, 25 pmol of a single RAPD primer (**Tab.** 2.5) and 5 - 50 ng template DNA were used. Each reaction was performed in a total volume of 25 µl. The following cycle profile was used: 1 cycle at 95°C for 5 min followed by 25 cycles at 95°C for 1 min, 36°C for 1 min and 72°C for 2 min.

2.16: SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were electrophoresed in vertical slab gels in a discontinuous buffer system according to Schägger and von Jagow (1987). The composition of the PAA mixtures is defined by the letters T, denoting total percentage of monomers and C, designating the percentage concentration of crosslinker. Resolving gels were composed of 16.5%T/3%C PAA (**Sect.** 2.5.4), whereas the stacking gels contained 4.0%T/3%C PAA (**Sect.** 2.5.4). Resolving gels also contained 13.3% glycerol. Ammonium persulfate and TEMED added to final concentrations of 0.05% and 0.005%, respectively, were used to initiate polymerization. An equivalent volume of sample buffer was added to the protein solution prior to electrophoresis and the proteins denatured by heating to 90°C for 5 min. Electrophoresis runs started at 80 V for 1 h after which the voltage was adjusted to a constant 120 V for the remainder of the run. Proteins were fixed in staining buffer (**Sect.** 2.5.4) for 10 - 30 min. Gels were destained in destaining buffer (**Sect.** 2.5.4) for 60 – 120 min at 50°C or overnight at room temperature with gentle shaking. The protein standard VII-L (Sigma), BSA (Mr = 66 kDa), ovalbumin (Mr = 45 kDa), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (Mr = 36 kDa), carbonic anhydrase from bovine erythrocytes (Mr = 29 kDa), trypsinogen from bovine pancreas (Mr = 24 kDa), trypsin inhibitor from soybean (Mr = 20 kDa) and α-lactalbumin (Mr = 14 kDa) was applied to the gels in order to determine the molecular weight (Mr) of the proteins.

2.17: Heterologous gene expression in *E. coli*

2.17.1: Heterologous gene expression under the control of the *T7* promoter

The method used was described by Studier *et al.* (1990). A single colony of *E. coli* BL21 (DE3) or *E. coli* BL21 (DE3) pLysS harboring a plasmid derived from pET plasmids was used to inoculate 5 ml of LB medium containing 50 µg/ml of ampicillin (plus 25 µg/ml of chloramphenicol if the cells carried pLysS). The cells were grown overnight or until they had reached the stationary phase. Appropriate amounts of this culture were used to inoculate fresh LB medium (or LB supplemented with 1 M sorbitol and 2.5 mM betaine) to an OD_{600} of approximate 0.1. The cells were grown at 37°C to an OD_{600} of 0.5 - 0.7. T7-RNA polymerase production was induced by the addition of 0.4 mM isopropyl-ß-thiogalactopyranoside (IPTG). Samples (1 ml) were taken prior to induction and at the following intervals after the addition of IPTG: 30 min, 60 min, 90 min, 120 min and 150 min (or 2 h, 6 h, 12 h and 24 h, when sorbitol and betaine were added). The cells were harvested by centrifugation at 4,000 rpm for 5 min and resuspended in 50 - 100 µl of the appropriate loading buffer (**Sect.** 2.5.4). The proteins were subjected to electrophoresis in the PAGE-gels as described in **Sect.** 2.16.

2.17.2: Heterologous gene expression under the control of the *rha* promoter

The vectors pJOE2702 and pJOE2775 were used for the expression of genes under the control of the *rha* promoter. In general, *E. coli* JM109 was used as a host. L-rhamnose was added between OD_{600} = 0.001 and 0.5, gene products become detectable at the onset of the stationary growth phase, and the course of induction was not much affected by the total induction time.

2.18: Heterologous gene expression in *S. lividans* 66

2.18.1: Heterologous gene expression under the control of the *ermE*-up promoter

Recombinant plasmids (derivative of pUWL201 or pUWL201RBSA, under the control of the *ermE*-up promoter) were transformed into *S. lividans* TK23 and a single transformant was inoculated into 10 ml YEME medium supplemented with 30 µg/ml of thiostrepton. After growth at 28°C for 3 days, a sample of 0.5 ml of the preculture was inoculated into 50 ml of YEME medium with 30 µg/ml of thiostrepton and incubated at 28°C for another 2 – 3 days. Cells were harvested by centrifugation and washed twice with 10.3% sucrose. Gene products were analyzed by SDS-PAGE.

2.18.2: Heterologous gene expression under the control of the *tipA* promoter

The expression of genes inserted in pIJ6021 or pIJ4123 (under the control of the *tipA* promoter) in *S. lividans* 1326 (or TK64) was performed as described by Takano *et al.* (1995) with some modifications. A single colony was inoculated into 10 ml of YEME medium with 50 µg/ml kanamycin and grown at 28°C for 3 days. A sample of 0.5 ml of the precultue was then inoculated into 50 ml of YEME medium with 50 µg/ml kanamycin. After about 36 h of cultivation at 28°C, 10 µg/ml of thiostrepton was added to induce the *tipA* promoter. The cultivation was continued for another 24 - 36 h. The cells were harvested and the gene products were analyzed by SDS-PAGE.

2.19: Cell-free extracts of *E. coli* and *Streptomyces*

The *E. coli* or *Streptomyces* cells harboring the expression-plasmids were harvested by centrifugation and washed twice with a cold buffer 1, buffer 2 or buffer A (**Sect.** 2.5.4). The pellet was resuspended with the same buffer in a ratio of 5.0 ml buffer/g for *E. coli* cells and 1.5 ml/g for *Streptomyces*. The cells were disrupted by sonication (3 times, each time for 1 min following a 30 sec interval at 60 watt). Cell debris was sedimented by centrifugation (15,000 rpm, 60 min, 4°C). The supernatants were dialyzed against 5 l of the appropriate buffer for 12 h at 4°C.

2.20: Cell-free extracts of *Actinoplanes* sp. SE50

The harvested *Actinoplanes* sp. cells were washed twice and resuspended in the double volume (v/w) of buffer 2 (**Sect.** 2.5.4) and disrupted by sonication. Cell debris were sedimented by centrifugation (15,000 rpm, 30 min, 4°C). The supernatants were dialyzed against 5 l of buffer 2 for 12 h at 4°C. The cell-free extracts were used directly for enzyme assays or aliquoted (120 µl per microtube) and stored at –70°C.

2.21: Determination of protein concentration

The protein concentration of the cell-free extracts was measured according to Bradford (1976) using the Protein Assay Kit (Bio-Rad) and BSA as a standard at a wavelength of 595 nm.

2.22: Western blotting and immuno-detection of proteins

The His-tag proteins produced in *E. coli* and *Streptomyces* were separated by SDS-PAGE. The proteins were transferred onto a PVDF membrane (Hybond P, Amersham) using a Semi-Dry Transfer Cell (Bio-Rad). For Western blotting a standard protocol (Winston *et al.*, 1987) was followed and the method of immuno-detection of proteins was adapted here as recommended by Qiagen's protocol using an Anti-His-tag antibody.

2.23: Thin-layer chromatography (TLC)

Samples of the enzyme reactions were chromatographed on silica thin-layer sheets (Merck) using solvent I (isobutric acid / 1 N NH_3, v/v, 5/3) or solvent II (butanol / ethanol / water, v/v/v= 9/7/4). The substrates were detected as brown spots after heating or as blue spots with the Cer-reagent (**Sect.** 2.5.4). In assays with [γ-^{32}P] ATP, the plates were autoradiographed with X-ray film (Amersham Typ Hyperfilm MP^{TM}) at room temperature for 1 - 12 h.

2.24: Purification of His-tag AcbM proteins

Samples of 10 ml cell-free extracts of *S. lividans* TK64/pCW4123M were applied to a Ni^{2+} HiTrap chelating column (Amersham Bioscience, Freiburg). The process was performed according to the specifications of the manufactures. The column was first washed with 10 - 20 ml of starting buffer (**Sect.** 2.5.4), then washed with a linear gradient of 10 - 500 mM imidazole in 10 ml starting buffer (**Sect.** 2.5.4) and 10 ml elution buffer (**Sect.** 2.5.4). The elution fractions were collected as 1 ml of each. The various fractions were analyzed by SDS-PAGE. The His-tag AcbM protein was eluted at approximately 200 - 300 mM imidazole from the column. The fractions containing partially purified protein was pooled and dialyzed for 24 h against 5 l of buffer 2 (see **Sect.** 2.5.4).

2.25: Determination of the activity of AcbK

The activity of AcbK was determined as described by Goeke *et al.* (1996), both non-radioactively and radioactively. The reactions for the analysis of AcbK were performed as follows:

Tris-HCl, pH7.6	25 mM
$MgCl_2$	10 mM
ATP	10 mM
([γ-^{32}P] ATP)	(2 µCi)
Acarbose	10 mM
AcbK extracts	variable

ATP stock solution (100 mM) was made in 100 mM Tris-buffer (natural pH), resulting in pH 7.5. The mixture was incubated at 30°C for 2 - 3 h. The reaction was stopped by heating at 95°C for 3 min. The products were analyzed by TLC (**Sect.** 2.23).

2.26: Determination of a 1-*epi*-valienol kinase activity

The activity of a 1-*epi*-valienol kinase in the *Actinoplanes* sp. crude extracts was determined as described for AcbK with minor modifications. NH_4Cl was found to be essential for the activity.

Tris-HCl, pH7.6	25 mM
$MgCl_2$	10 mM
NH_4Cl	20 mM
ATP	10 mM
([γ-^{32}P] ATP)	(2 µCi)
1-*epi*-valienol	10 mM
Actinoplanes sp. extracts	variable

The reaction mixture was incubated at 30°C for 2 - 6 h. The reaction was stopped by heating at 95°C for 3 min. The products were analyzed by TLC (see **Sect.** 2.23).

2.27: Enzymatical synthesis of 2-*epi*-5-*epi*-valiolone

Sedo-heptulose-7-phosphate was formed from hydoxypyruvate and ribose-5-phosphate by transketolase (EC 2.2.1.1) reaction according to Schärken (1997). *Sedo*-heptulose-7-phosphate was converted to 2-*epi*-5-*epi*-valiolone by AcbC (Stratmann *et al.*, 1999). The AcbC proteins was prepared from *S. lividans* 1326/pAS8/7 (**Tab.** 2.3). The cells were disrupted by sonication in buffer A (**Sect.** 2.5.4). The resulting cell-free extracts were dialyzed against 5 l of buffer A at 4°C for 12 h. The coupled enzyme assays were performed as follows:

Hydroxypyruvate	10 mM
Ribose-5-phosphate	10 mM
Thiaminpyrophosphate (TPP)	0.5 mM
$MgCl_2$	1 mM
$CoCl_2$	0.025 mM
NaF	2 mM
Transketolase	variable
AcbC extracts	variable

The analytical scale was performed in a total volume of 20 µl with 0.025 units of transketolase and the preparative scale was carried out in a total volume of 30 ml with 5 units of transketolase.

The mixture was incubated at 30°C for 12 - 20 h and stopped by heating at 90°C for 3 min. The reaction was monitored by TLC.

2.28: Determination of the activity of AcbM

The AcbM activity was determined as described in **Sect.** 2.25 for AcbK with minor modifications. The dialyzed cell-free extracts from *S. lividans* TK64/pCW4123M were incubated with ATP and 2-*epi*-5-*epi*-valiolone (or other cyclitols which were tested). The enzyme assays were carried out as follows:

Tris-HCl, pH7.6	25 mM
$MgCl_2$	10 mM
NH_4Cl	20 mM
ATP	10 mM
([γ-^{32}P]-ATP)	(2 µCi)
2-*epi*-5-*epi*-valiolone	10 mM
AcbM extracts	variable

Normally, the analytical assay was performed in a total volume of 15 µl at 30°C for 2 – 12 h. In preparative scale, the 20 mg purified 2-*epi*-5-*epi*-valiolone (**Sect.** 2.30) was incubated with 5 ml of the partially purified His-tag AcbM protein (**Sect.** 2.24) and 1 ml of 100 mM ATP solution (in 100 mM Tris) in an end volume of 10 ml at 30°C for 2 - 12 h. The reaction was monitored by TLC.

2.29: Determination of the activity of AcbO

For the assays with AcbO a mixture of cells from S. *lividans*/pCW4123M and *S. lividans*/pMJO7 (1:1) was used. The cells were mixed first and then the cell-free extracts containing both AcbM and AcbO were prepared as described for the AcbM tests, the test conditions and the detection of the reaction products were identical to those described for AcbM. Co-enzymes (NAD, NADH, NADP or NADPH, respectively) were used in final concentrations of 10 mM in the tests.

2.30: Purification of 2-*epi*-5-*epi*-valiolone

The preparative scale volumes of 30 ml of the AcbC reaction solutions (**Sect.** 2.27) were heated at 90°C for 5 min, centrifuged (5,000 rpm, 20 min) and then were applied to an ultrafiltration Amicon cell with a YM-10 ultrafiltration membrane (cut-off 10,000 Da, Amicon, Witten, Germany). The flow-through was collected. After freeze-drying around 250 mg yellow powder

was acquired. The product was dissolved in 3 ml of Milli-Q water and then was applied to an anion-exchange chromatography with Dowex 1 x 8, Cl^--form ("mesh" = 100 – 200, Serva, Heidelberg) in a SR 25/50 column (Amersham Bioscience, Freiburg). The column was washed with water (flow rate 2 ml/min) and the fractions containing 2-*epi*-5-*epi*-valiolone were pooled. After lyophilization, 20 mg of 2-*epi*-5-*epi*-valiolone was obtained as a light yellow powder.

2.31: Purification of 2-*epi*-5-*epi*-valiolone-7-phosphate

The whole 5 ml of the partially purified AcbM proteins (**Sect.** 2.24) were used to convert the 20 mg of purified 2-*epi*-5-*epi*-valiolone (**Sect.** 2.30) to give the product, 2-*epi*-5-*epi*-valiolone-7-phosphate. The reaction solution was applied to an ultrafiltration Amicon cell with a YM-10 ultrafiltration membrane (cut-off 10,000 Da, Amicon, Witten, Germany). The 10 ml of flow-through was collected, concentrated to an end volume of about 3 ml by freeze-drying and then was applied to an anion-exchange chromatography with Dowex 1 x 8, Cl^--form ("mesh" = 100 – 200, Serva, Heidelberg) in a SR 25/50 column (Amersham Bioscience, Freiburg). The column was washed with plenty of water (flow rate 2 ml/min). 2-*epi*-5-*epi*-valiolone-phosphate was eluted with a linear gradient of 0 - 600 mM of NaCl (flow rate 2 ml/min). The elution fractions were collected as 2 ml of each and analyzed by thin-layer chromatography. Fractions containing the desired product were pooled (a total volume of 48 ml) and concentrated to an end volume of around 5 ml by freeze-drying. The product was desalted at 4°C with a Sephadex G-10 column (5.0 x 81cm, SR 25/100 column, Amersham Bioscience, Freiburg). The product was eluted with water (flow-rate, 1.5 ml/min). The fractions were analyzed by thin-layer chromatography and those containing 2-*epi*-5-*epi*-valiolone-7-phosphate were pooled. After lyophilization, 12 mg of white powder was obtained.

2.32: Ion chromatography and mass spectrometry (IC-MS)

The IC-MS analyses were carried out by R. Brückner (Institue of Biotechnology, Research Center Jülich) under the following conditions:

Ion chromatography. The chromatographic part consisted of a Dionex DX-500 ion chromatography (IC) system (Dionex, Idstein, Germany), equipped with a gradient pump (GP40), an eluent generator (EG40) with an EGC-KOH cartridge, a 25-µl injection loop, and an

electrochemical conductivity detector (ED40). Chromatographic separation was performed on a Dionex Ion Pac AS11 column (250 × 2 mm) provided with a guard column (AG11). An anion trap column (ATC-1) was placed in line with the GP40 and the injection valve in order to remove anionic contaminants from the eluent. Micro filtered (0.2 µm) water with a specific resistance of at least 18 mΩ was used. The gradient was run as shown in **Tab.** 2.9. The Dionex ASRS-II self regenerating chemical suppressor was housed in a LC30 chromatography oven at 35°C, and operated in the external water mode at 100 mA. The samples were analyzed in triplicate with a flow rate of 0.25 ml/min.

Tab. 2.9: The following gradient profile was used:

Time (min)	KOH (mM)
0 - 2.00	100
2.01 – 9.00	1.00
9.00 (Inject.)	1.00
11.0	1.00
15.0	19.0
18.0	20.0
25.0	100
26.0	100

Mass spectrometry. The experiments were performed on a PE SCIEX API 2,000 triple quad instrument (Applied Biosystems, Langen, Germany) equipped with a Turbolon ion spray and heated nebulizer. In order to avoid leakage current the connection tube between the ion chromatograph and the mass spectrometer had to be earthed directly behind the ED40. All measurements were preceded in the negative scan mode. Hyphenation was realized with the original flow rate of 0.25 ml/min at a heater temperature of 380°C, and a Turbo ion spray voltage of –4,000 V. Optimal gas (N_2) pressures were found as follows: nebulizer gas (GS1) at 30 psi, heater gas (GS2) at 90 psi, curtain gas (CUR) at 45 psi. Especially for MRM measurements collision added dissociation (CAD) gas rate was set at 3 psi.

2.33: Determination of optical rotations of 2-*epi*-5-*epi*-valiolone-7-phosphate

The Determination of the rotation was performed in a Perking-Elmer 24 1 Polarimeter. A tempered quartz cuvette (sample volume: 1 ml, length 10 cm) was used. Optical rotation was calculated according to following formula:

$$[\alpha]_{\lambda}^{T} = \alpha \frac{100}{c \cdot l}$$

$[\alpha]_{\lambda}^{T}$ =	specific rotation [10^{-1} Grad cm^2 g^{-1}]
T	temperature [°C]
λ	Wavelength [nm], D-line of sodium lamp
α	rotation [10^{-1} Grad]
l	layer thickness [dm]
c	concentration [g/100ml solution]

2-*epi*-5-*epi*-valiolone-7-phosphate: $[\alpha]_{D}^{20}$ = + 4.9° (c = 0.35, H_2O).

2.34: NMR spectrometry

NMR (Nuclear Magnetic Resonance) spectrometric analyses were carried out at organic chemistry department of the BUGH Wuppertal by M. Podeschwa. All NMR-spectra were recorded on a Bruker ARX 400 (400 MHz) spektrometer. ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz, respectively. Besides ^{1}H- and ^{13}C- experiments, 2D COSY- (^{1}H-^{1}H, ^{1}H-^{13}C as well as ^{1}H-^{31}P) and DEPT spectra for the unequivocal correlation of the hydrogen-, carbon- an phosphorous-atoms were also recorded. Chemical shifts were given in ppm, related to the solvents as internal standard. The multiplicity is given through the following symbols: s (singulet), d (dublet), t (triplet), q (quartet), m (multiplet), ψt (pseudotriplet) and br (broad). The coupling constant *J* is given in Hz. For interpretation of ^{1}H spectra ψt (pseudo triplet) for unresolved dd was used. Peak assignments were derived from DEPT and two-dimensional NMR-experiments.

NMR spectra for 2-epi-5-epi-valiolone (2SR,3SR,4SR,4RS)-5-Hydroxymethyl-cyclhexanone-2,3,4,5-tetrol). ^{1}H-NMR (d_4-MeOH, 400 MHz): δ = 2.33 (dd, 1 H, *J* = 13.7 Hz, *J* = 1.7 Hz, H-$6_{ax.}$), 2.84 (d, 1 H, *J* = 13.7 Hz, H-$6_{eq.}$), 3.43 (d, 1 H, *J* = 11.3 Hz, H-7A), 3.64 (d, 1 H, *J* = 11.3 Hz, H-7B), 4.03 (m, 1 H, H-4), 4.27 (ψt, 1 H, *J* = 4 Hz, H-3), 4.59 (d, *J* = 4.0 Hz, H-2). ^{13}C-NMR (d_4-MeOH, 101 MHz): δ = 46.0 (C-6), 67.7 (C-7), 70.9, 76.1, 79.7 (C-2, C-3, C-4), 81.5 (C-5), 209.8 (C-1).

NMR spectra for2-epi-5-epi-valiolone-7-phosphate. ^{1}H-NMR (D_2O, 400 MHz): δ = 2.38 (d, 1 H, *J* = 13.8 Hz, H-$6_{ax.}$), 2.89 (d, 1 H, *J* = 14.2 Hz, H-$6_{eq.}$), 3.58 (dd, 1 H, *J* = 6.6 Hz, *J* = 11.7 Hz, H-7A), 3.99 (dd, 1 H, *J* = 9.4 Hz, *J* = 11.5 Hz, H-7B), 4.18 (m, 1 H, H-4), 4.38 (ψt, 1 H, *J* = 3.8 Hz, H-3), 4.71 (d, under HDO, *J* = 4.0 Hz, H-2). ^{13}C-NMR (D_2O, 101 MHz): δ = 46.38 (C-6), 70.17 (d, *J* = 5.1 Hz, C-7), 71.19 (C-4), 76.74 (C-2), 79.62 (C-3), 82.86 (C-5), 101.23 (C-1). ^{31}P{^{1}H}-NMR (D_2O, 162 MHz): δ = 5.55 (PC-7)

2.35: Computer programs

Restriction enzyme analyses and conversion of DNA sequences into protein sequences were done using the DNA-STRIDER™ 1.1 (Marck, 1989). The BRUJENE II program and the FramePlot (Ishikawa and Hotta, 1999; http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl) were used to determine open reading frames and G+C contents. PCR amplification temperatures were computed using the PRIMER-FIND 3.0 program (Fröbel Labor-Geräte, Lindau). Multiple sequence alignments were computed using the Clustal V 3.0 program (Higgins, 1991) or Clustal W (Thompson *et al.*, 1994; http://www2.ebi.ac.uk/clustalw/). Comparisons of DNA- and protein-sequences to the data banks, EMBL, GENBANK and SWISSPORT were done using FASTA 3 (Pearson and Lipman, 1988; http://www2.ebi.ac.uk/fasta3/) and BLAST (Altschul *et al.*, 1990; http://www.ncbi.nlm.nih.gov/BLAST/).

3: Result I - Gene Sponge

3.1: An new approach for exploring of natural products (bioactive secondary compounds) from soil – Use of *in vivo* "genetic sponges" for uptake of expressible genetic materials

As mentioned in the introduction (**Sect.** 1.4), a new frontier is emerging that unites biology and chemistry – the exploration of natural products from previously uncultured soil microorganisms (Handelsman *et al*., 1998). The question arises how the still unknown resources ("biodiversity") in the natural gene pool, e.g. in soil samples, can be made accessible for production. One approach involves directly accessing the genomes of soil microorganisms by isolating and/or cloning of gene clusters for new secreted, bioactive metabolites. A major disadvantage to this approach is that currently few methods are available which would extract most of the DNA quantitatively from soil and in sufficient length of fragments. An alternative approach for the enhancement of chances to isolate a complex metabolic trait encoded by a gene cluster from soil samples (and other material) is *in situ* gene transfer into a seeded recipient ("gene sponge"). The task of this research work is to find or construct such strains ("gene sponge"). Four *S. lividans* strains, namely Jni13C1, Jni14C1, WP and AJ100 (Volff *et al.*, 1996, 1997a, 1997b), were studied for the possibility to serve as "gene sponges". They have large deletions at the chromosomal ends so as that they might have much more potential to take up DNA pieces of lengths up to 1 Mb. Among the four putative "gene sponges", *S. lividans* Jin13C1 and WP were investigated more intensively in this work.

3.2: Characterization of four deletion strains

3.2.1: Description of four *S. lividans* 66 strains with large deletions at the chromosomal ends

In *S. lividans,* mutants sensitive to chloramphenicol (Cml^s) are observed at a frequency of above 1% (Altenbuchner and Cullum, 1985). These mutants themselves give rise to arginine auxotrophic (Arg^-) mutants at a frequency of 25% of spores (Altenbuchner and Cullum, 1984). Both mutant phenotypes are due to deletion of the corresponding chromosomal markers, *cmlR* and *argG* (Betzler *et al.*, 1987; Dittrich *et al.*, 1991). Extremely large chromosomal deletions (up to 1 Mb) are very frequently found in mutants, leading to the irreversible loss of the unstable

traits (Volff *et al.*, 1996). It has been found that the chromosome of *Streptomyces* is linear (Lin *et al.*, 1993), but the Cml^s mutants of *S. lividans* so far tested have a circular chromosome. The selected putative recipient strains ("gene sponges"), Jni13C1, Jni14C1, WP and AJ100, are all derivatives of *S. lividans* TK64 due to this kind of genetic instability. They have large deletions at the chromosomal ends and all are sensitive to chloramphenicol due to loss of the *cmlR* gene (encoding an antibiotic exporting transporter causing the resistance to chloramphenicol) at the ends (**Fig.** 3.1). The deletions in WP are even extended to the AUD1 element and the *argG* gene (**Fig.** 3.1).

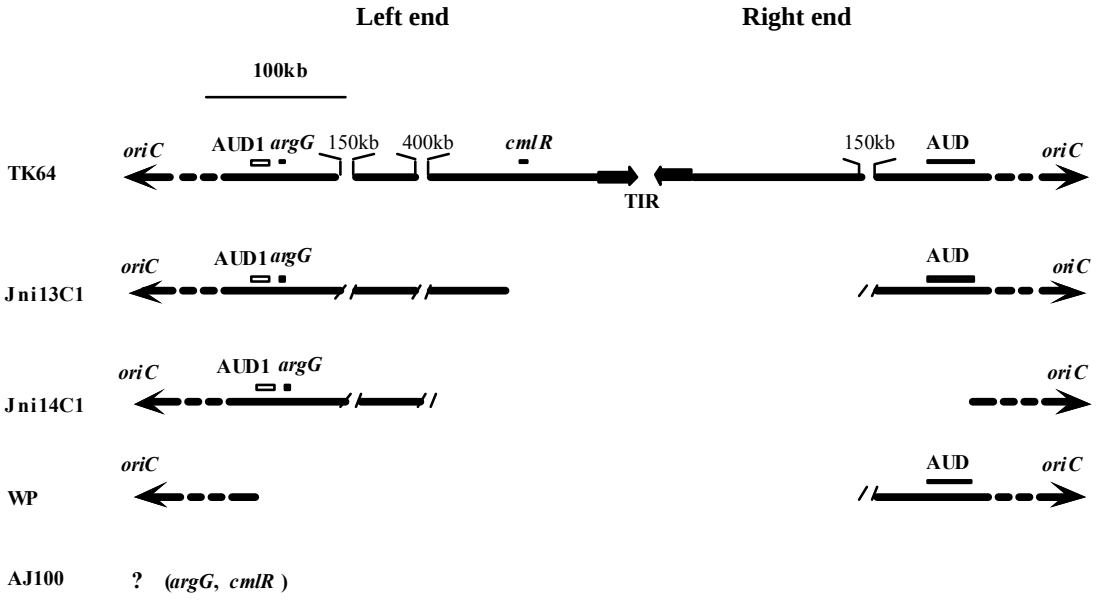


Fig. 3.1: Structure of the unstable regions of the wild-type strain TK64 and its four Cml^s derivatives. The absence of a line indicates the extent of the deletion. Thick arrows show the terminal inverted repeats (TIR). AUD, amplifiable unit of DNA. The locations of AUD1, AUD and *cmlR* relative to the chromosomal ends are indicated by rectangles.

3.2.2: Phenotypic behavior of the 4 selected recipient strains in plate cultures

The four strains, Jni13C1, Jni14C1, WP and AJ100, were first cultured on different plates and their phenotypic behavior was compared with that of the parental strain TK64 (**Tab.** 3.1). Strains Jin13C1 and Jin14C1 produced diffusible blue pigments on SPMR-agar. The growth of Jni13C1 and Jni14C1 required proline on MM-agar. Arginine and proline were essential for the growth of stains WP and AJ100 on MM-agar.

Tab. 3.1: Growth behavior of 4 strains on different plates

	Strains	TK64	Jni13C1	Jni14C1	WP	AJ100
Growth rate [1]	TSB	+	+	+	+	+
	YEME	+	+	+	+	+
	SPMR	+	+	+	+	+
	SMA	+	+	+	±	±
Presence of spores [2]	TSB	±	±	±	-	-
	YEME	-	-	-	-	-
	SPMR	+	+	+	-	-
	SMA	+	+	+	+	+
Production of diffusible pigments [3]	TSB	-	-	-	-	-
	YEME	-	-	-	-	-
	SPMR	+	+	+	-	-
	SMA	-	-	-	-	-
Auxotrophy		Pro	Pro	Pro	Pro, Arg	Pro, Arg

Note: [1]: +, quickly growing on the solid medium; ±, slowly growing on the solid medium.
[2]: +, rich in spores; ±, poor in spores; -, no presence of spores.
[3]: +, producing dark blue pigments; -, producing no pigments.

3.2.3: Regeneration and transformation of protoplasts of the selected recipient strains

Cell lysis in soil can release free DNA which is rather stable in soil particles (Lorenz and Wackernagel, 1994). In order to test the capacity of the selected recipient strains to take up naked DNA, plasmid DNA was transformed into the protoplasts of these strains.

The regeneration frequencies of protoplasts of the strains Jni13C1, Jni14C1, WP and AJ100 were tested both on SPMR and R2YE plates and compared to those of TK23 and TK64. The protoplasts from TK23, TK64, Jni13C1 and WP regenerated well both on SPMR and R2YE plates with a frequency about 10^{10} ml^{-1}. But regeneration rates of protoplasts from Jni14C1 and AJ100 were only about 10^{7} ml^{-1}. When the protoplasts were transformed with methylated DNA (pUWL218 isolated from *E. coli* DH5α) or non-methylated DNA (pUWL218 isolated from *E. coli* JM110), no differences were detected between the four strains and the wild type TK64. These results showed that there were no enhanced restriction barriers for these recipient strains to take up naked plasmid DNA.

3.2.4: Antibiotic resistance pattern of the recipient strains

A "gene sponge" should be easily reisolated from soil after being seeded into soil. Therefore, the antibiotic resistance patterns of the strains Jni13C1, Jni14C1, WP and AJ100 were determined to find some selective markers. The antibiotic resistance patterns are presented in **Tab.** 3.2. The

strains Jni13C1, Jni14C1, WP and AJ100 were resistant to streptomycin at a high concentration (more than 100 μg/ml) due to the point mutation in *rpsL* (*str-6*), but highly sensitive to chloramphenicol, gentamicin, kanamycin, neomycin, lividomycin, and thiostrepton. Strain TK23 was highly resistant to spectinomycin due to the *rpsE* mutation (*spc-1*).

Tab. 3.2 : Antibiotic resistance patterns of six *S. lividans* strains

Antibiotic (μg/ml)	Strains					
	TK23	**Jni13 C1**	**Jni14 C1**	**TK64**	**AJ100**	**WP**
chloramphenicol	+	-	-	+	-	-
erythromycin	-	20	15	-	-	-
gentamicin	-	-	-	-	-	-
hygromycin	25	25	20	30	-	5
kanamycin	-	-	-	-	-	-
lincomycin	100	20	30	30	-	5
lividomycin	2.5	5	5	5	2.5	2.5
nalidix acid	›100	50	50	50	100	100
neomycin	-	-	-	-	-	-
rifampicin	5	5	-	-	-	5
spectinomycin	›100	20	20	20	5	5
streptomycin	-	›100	›100	›100	›100	›100
tetracyclin	20	30-50	10-20	5	-	-
thiostrepton	-	-	-	-	-	-
viomycin	15	20	15	20	5	20

Note: +, growth on TSB plates containing 5 μg/ml of the respective antibiotic; -, no growth on TSB plates supplemented with 5 μg/ml of the respective antibiotic. Numbers in the table indicate the minimum inhibition concentration (μg/ml) on TSB plates.

3.3: Survival of *S. lividans* 66 strains in sterile soil

The survival of the strains Jni13C1 and WP was examined in soil first using an unamended sterile Warwick soil microcosm. First, mycelia of strains Jini13C1 and WP, pregrown in YEME, were used for the inoculation into the soil microcosms. WP was still detectable after 30 days incubation and Jni13C1 was still viable in the soil microcosm after 60 days (**Fig.** 3.1). When spore suspensions of Jni13C1 and WP were inoculated into the sterile soil, they survived with the same rates as mycelia (data not shown). But when the protoplasts of the two strains were inoculated into the sterile soil, no viable propagules could form on the selective TSB plates during the 30 days incubation period (data not shown). Therefore, the protoplasts might be destroyed directly after they had contacted with the soil particles.

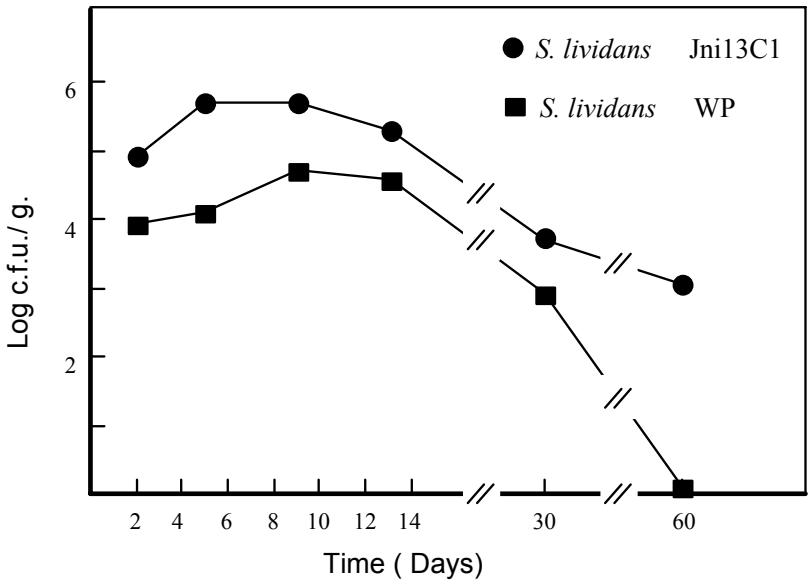


Fig. 3.2: Survival of *S. lividans* Jni13C1 and WP in unamended sterile soil. The Warwick soil microcosm was prepared as described in **Sect.** 2.8. Fresh mycelia of both strains were inoculated into the soil microcosms. Samples were taken at day 2, 4, 9, 13, 30 and 60. The survival of the strains was expressed as the colony forming unit (c.f.u.) per g soil using TSB Sm plates. Sm, see **Tab.** 2.6.

3.4: Transfer of a non-conjugative plasmid pIJ702 in sterile soil

3.4.1: Detection of plamid transfer in sterile amended soil

To investigate the transfer of the non-conjugative plasmid pIJ702 ($Thio^R$) in sterile soil, the fresh mycelia of Jni13C1 and TK23/pIJ702 were mixed (at a ratio of 2:1) and inoculated into sterile Warwick soil amended with 1% soluble starch and 1% chitin (**Sect.** 2.8). The transfer of plasmid pIJ702 was determined by selecting transconjugants that were resistant to streptomycin and thiostrepton (cf. **Tab.** 3.2). The first transconjugant was found at the 9th day of the incubation. The number of transconjugants increased with the incubation time and maintained at a constant level after 13 days (**Fig**. 3.3). This result indicated that the transfer of the nonconjugative plasmid pIJ702 from the donor strain *S. lividans* TK23 to the recipient strain *S. lividans* Jni13C1 occurred in the amended sterile Warwick soil. In an equivalent experiment with *S. lividans* WP as the recipient strain, no transfer of the plasmid pIJ702 was detected (data not shown).

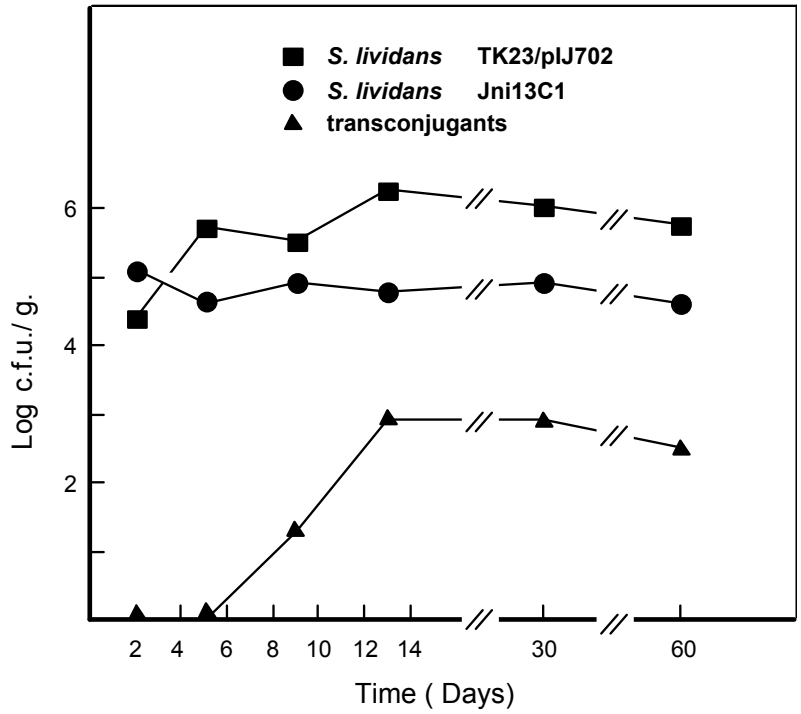


Fig. 3.3: Survival of *S. lividans* Jni13C1 and TK23/pIJ702 and the transfer of plasmid pIJ702 in the amended sterile soil. The soil microcosm was prepared as described in **Sect.** 2.8. Fresh mycelia of both strains were inoculated into the soil microcosms. Samples were taken at day 2, 5, 9, 13, 30 and 60. The survival of the strains was expressed as colony forming units (c.f.u.) per g soil. Parent strains and transconjugants were selected using TSB plates containing the antibiotics as follows: Jni13C1, Sm; TK23/pIJ702, Thio, Cml; transconjugants, Thio, Sm. Cml, Sm, Thio, see **Tab.** 2.6.

3.4.2: Characterization of transconjugants from the amended sterile soil

The putative transconjugants (*S. lividans* Jni13C1 derivatives, strains 01 – 52) isolated from the sterile Warwick soil (**Fig.** 3.3) were analyzed and compared with those of the parent strains *S. lividans* TK23/pIJ702 and Jni13C1. Normally, TK23 was $Sm^{S}Cml^{R}Pro^{+}$, Jni13C1 was $Sm^{R}Cml^{S}Pro^{-}$. The presence of plasmid pIJ702 led to the resistance to thiostrepton ($Thio^{R}$) and the production of melanin (Mel^{+}). Interestingly, 14 out of the 52 (26.9%) transconjugants were $Thio^{R}Sm^{R}Cml^{R}$, and all of the 14 strains were also Pro^{+} (**Tab.** 3.3). Among all transconjugants 28 strains (53.8%) had a Mel^{-} phenotype. Plasmids isolated from Mel^{-} strains, such as Strains 11, 18, 24, and 42, were used to transform protoplasts of strain Jni13C1. All plasmids led to a Mel^{+} phenotype. This showed that the plasmids were still intact and the Mel^{-} phenotype was due to a second-site mutation of the receptor strain, suppressing the Mel^{+} phenotype.

Tab. 3.3: Characterization of 52 transconjugants isolated from sterile soil

Transconjugants	$Thio^{R}Sm^{R}Cml^{S}Pro^{-}$ [1]		$Thio^{R}Sm^{R}Cml^{R}Pro^{+}$	
	Mel^{+}	Mel^{-}	Mel^{+}	Mel^{-}
Amounts	21	17	3	11
Percentage	40.4%	32.7%	5.8%	21.1%
Strains No.	Strain 01-10, 19, 20, 30, 34, 35, 40, 41, 43, 47, 50, 51	Strain 11, 12, 14, 15, 22, 24-26, 28, 29, 33, 36-39, 44, 48	Strain 21, 31, 52	Strain 13, 16, 17, 18, 23, 27, 32, 42, 45, 46, 49
Total percentage	73.1%		26.9%	

Note: [1]: R/S, resistant/sensitive to the corresponding antibiotic.
+/-, prototroph/auxotroph relative to the corresponding nutrient.

For further investigation of some transconjugants, chromosomal DNA was isolated and subjected to Southern blotting analysis. A 2.1 kb *Bam*HI fragment from pJOE2424 (**Tab.** 2.3) was used as a probe for the detection of the right chromosomal end of *S. lividans*. Results revealed that the selected transconjugants, strains 01, 02, 03, 04, 24 (cf. **Tab.** 3.3) and Jni13C1 gave no positive signal, but strains 13 and 31 (cf. **Tab.** 3.3), as well as TK23 and TK64 had the desired hybridizing band of 2.9 kb (data not shown). This meant that the strains with a phenotype of $Thio^{R}Sm^{R}Cml^{S}Pro^{-}$ (e.g., strains 01 - 04 and 24) were probably derivatives of Jni13C1. They had no chromosomal end and were sensitive to chloramphenicol. These data indicated that a natural transfer of the plasmid pIJ702 from TK23 to Jni13C1 occurred in sterile Warwick soil. To our knowledge, this is the first observation of a natural transfer of a non-conjugative (Tra^{-}) plasmid in soil without help of other conjugative (Tra^{+}) plasmids.

Some putative transconjugants were resistant both to streptomycin and chloramphenicol (**Tab.** 3.3). Southern blotting analysis showed that they still had chromosomal ends and their phenotypes were more similar to that of TK23 ($Cml^{R}Pro^{+}$). It was known that a point mutation in the *rpsL* gene of *S. lividans* TK21 could confer the resistance to streptomycin (Shima *et al.*, 1996). Therefore, the *rpsL* gene was amplified using primer pairs CZ1 and CZ2 (**Tab.** 2.5 and 2.8) from Jni13C1, TK23, strains 01 and 31, cloned, sequenced and compared (**Fig.** 3.4). Sequence analysis revealed a point mutation (A to G at position 262) in the *rpsL* gene. The resultant mutation could confer streptomycin resistance and in some case, could induce the production of a pigmented (blue) antibiotic, actinorhodin. Normally *S. lividans* does not produce this antibiotic though the *act*-cluster is present in this strain (Shima *et al.*, 1996). Thus, the

streptomycin resistance of Jni13C1, strains 01 and 31 was probably caused by the point mutation in the *rpsL* gene. The strains with a phenotype of Thio^{R}Sm^{R}Cml^{R}Pro^{+} (e.g., the strains 13 and 31), therefore, were either the *rpsL* mutants of TK23 or they originated by larger recombination events between the two mixed strains in soil (cf. **Sect.** 3.7.4).

```
          215                                                  262         274
     TK21: CACAACCTGCAGGAGCACTCCATCGTGCTCGTGCGCGGCGGCCGTGTGAAGGACCTGCCG
     TK23: CACAACCTGCAGGAGCACTCCATCGTGCTCGTGCGCGGCGGCCGTGTGAAGGACCTGCCG
  Jni13C1: CACAACCTGCAGGAGCACTCCATCGTGCTCGTGCGCGGCGGCCGTGTGGAGGACCTGCCG
Strain 01: CACAACCTGCAGGAGCACTCCATCGTGCTCGTGCGCGGCGGCCGTGTGGAGGACCTGCCG
Strain 31: CACAACCTGCAGGAGCACTCCATCGTGCTCGTGCGCGGCGGCCGTGTGGAGGACCTGCCG
```

Fig. 3.4: Point mutation in the *rpsL* gene of *S. lividans* (partial sequence). Part of the *rpsL* gene sequence from *S. lividans* TK21, TK23, Jni13C1, strains 01 and 31 (cf. **Tab.** 3.3) were compared. The point mutation (A to G, in shadow) located at the position 262 of the *rpsL* gene, leading Lys-88 (AAG) to Glu (GAG), compared to the sequence of the *rpsL* gene (D83746) from *S. lividans* TK21.

3.5: Possibility of transfer of chromosomal markers in sterile soil

The above experiments have confirmed that the transfer of a non-conjugative plasmid pIJ702 to the selected recipient strain Jni13C1 was possible in sterile soil. In order to find out whether the exchange of chromosomal markers could also occur in sterile soil, some strains with known traits were used as donor strains and introduced into sterile soil together with the recipient strain Jni13C1. *S. lincolnensis* 78-11 is a lincomycin-producing strain. It was found that, the non-adjacent gene cluster for the production of lincomycin and melanin (*melC*) both are duplicated in *S. lincolnensis* 78-11 (Peschke *et al.*, 1995). The *melC* operon is responsible for the production of melanin and can serve as a good indicator for the detection of the gene transfer. The strains *S. lincolnensis* 78-11 and Jni13C1 were inoculated together into the sterile Warwick soil microcosm, as spore suspensions (**Fig.** 3.5, A) or as fresh mycelia (**Fig.** 3.5, B). No detectable transfer of the *melC* operon occurred during the 20 days incubation. Two derivatives of *S. lincolnensis* NRRL 2936, namely Mu1 and Mu2, which contained the *aacC4* gene cassette (Apr^{R}) inserted in the *lmbY* gene by homologous gene replacement (Neusser, 1999), were also used as potential donor strains. The two strains survived very well in soil (**Fig.** 3.5, C and D), but no genetic exchanges (*melC* or *aacC4*) between Jni13C1 and mutants of *S. lincolnensis* NRRL 2936 were detected. Also no transfer of the plasmid pIJ702 was detected from the two mutants of *S. lincolnensis* NRRL 2936 to Jni13C1 (**Fig.** 3.5, E and F).

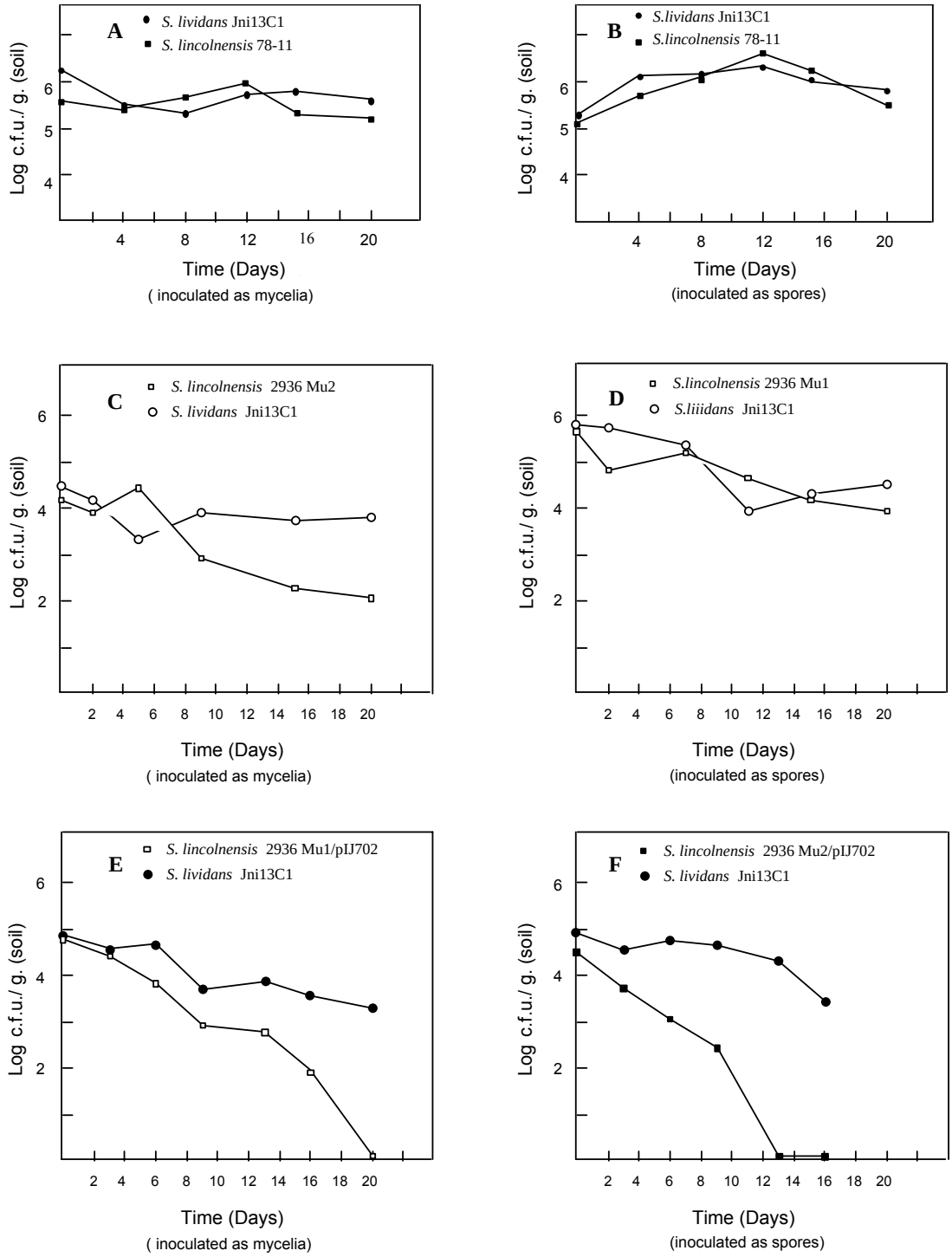


Fig. 3.5: Survival of *S. lividans* Jni13C1 and some strains of *S. lincolnensis* in sterile soil. The soil microcosm was prepared as described in **Sect.** 2.8. Spore suspensions or fresh mycelia were inoculated into the soil microcosms. The strains were selected from soil using TSB plates containing the following selection: *S. lividans* Jni13C1 (A-F), Sm; *S. lincolnensis* 78-11 (A and B), Lm; *S. lincolnensis* NRRL 2936 Mu1 and Mu2 (C and D), Apr; *S. lincolnensis* NRRL 2936 Mu1 and Mu2 harboring plasmid pIJ702 (E and F), Thio, Apr; transfer of the plamsid pIJ702 (E and F) was selected on TSB (Thio, Sm); TSB plates supplemented with Sm and Apr were used in the selection of the transfer of *aacC4* gene cassette (C, D, E and F). Apr, Lm, Sm, Thio, see **Tab.** 2.6.

3.6: Possibility of gene transfer in nonsterile soil

3.6.1: Survival of *S. lividans* Jni13C1 in nonsterile soil

The goal of this work was the isolation of new genes from indigenous bacteria (cultivable or unculturable) in nonsterile soil through natural gene transfer to a selected recipient strain (gene sponge). Two types of soil were used. The Warwick soil has been well characterized but the composition of the soil from a Wuppertal garden plot has not been analyzed yet (**Sect.** 2.8). *S. lividans* Jni13C1 was introduced into nonsterile soil both as spores and fresh mycelia. The survival and reisolation of Jni13C1 was studied. Results revealed that Jni13C1 could survive rather long time in both types of nonsterile soil (**Fig.** 3.6).

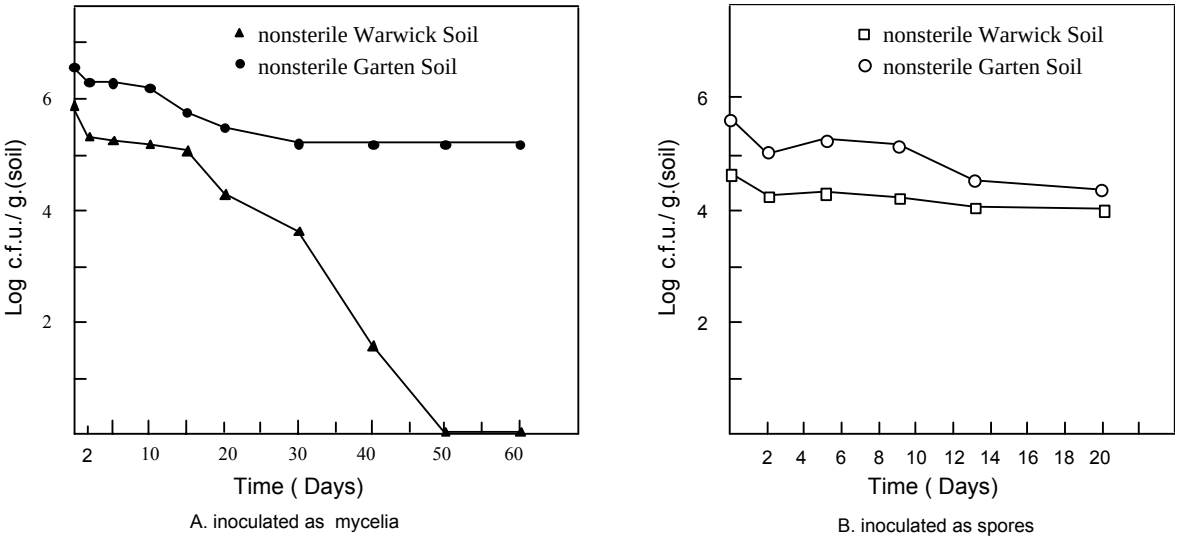


Fig. 3.6: Survival of *S. lividans* Jni13C1 in amended nonsterile soil. The soil microcosms were prepared as described in **Sect.** 2.8 using nonsterile Warwick or Garden soil amended with 1% chitin and 1% soluble starch. Fresh vegetative mycelia (**A**) and heat-shocked (50°C, 15min) spore suspensions (**B**) were used as inoculants. Viable counts of the recipient strain of Jni13C1 was carried out on TSB-agar containing nalidixic acid, nystatin, cycloheximide and streptomycin (**Tab.** 2.6).

When spore suspensions were used as the inoculum (**Fig.** 3.6 B), the survival rates were similar for the two types of soil. The culture in Warwick soil maintained relatively constant viable populations over the entire incubation period (20 days). The culture in the Wuppertal garden soil showed a little fluctuation during incubation, but there were no significant differences from the culture in Warwick soil (**Fig.** 3.6 B). Survival patterns were different when vegetative mycelia

were introduced (**Fig.** 3.6 A). The viable counts in the Wuppertal garden soil were nearly constant during the 60 days experiments; but when Warwick soil was used, the viable counts decreased with incubation time and the inoculants (Jni13C1) could not be detected after 50 days of incubation.

3.6.2: Characterization of some *Streptomyces* species isolated from nonsterile soil

During the incubation of the microcosm based on nonsterile soil inoculated with vegetative mycelia (**Fig.** 3.6 A), some streptomycetes-like strains were isolated. The isolated soil strains were preliminarily assigned to the genus *Streptomyces* on the basis of morphology on TSB-agar. These strains were further characterized (**Tab.** 3.4).

Tab. 3.4: Characterization of some strains isolated from nonsterile soil on SPMR-agar

Strains	spores [1)]	color of aerial spore mass	pigmentation of substrate mycelium	production of diffusible pigments [2)]	antibiotic resistance	selection pressure [3)]
Jni13C1	+	gray	red/blue	blue	Sm^RCml^S	N_2CS
E30d-1	+	white	brown	-	Sm^RCml^R	N_2CS+Cml
E30d-2	+	gray	red/orange	blue	Sm^RCml^R	N_2CS+Cml
E30d-3	+	gray	blue	blue	Sm^RCml^R	N_2CS+Cml
E30d-4	+	light green	brown	-	Sm^RCml^R	N_2CS+Cml
E30d-5	+	white	brown	-	Sm^RCml^R	N_2CS+Cml
F30d-6	+	light green	brown	-	Sm^RCml^R	N_2CS+Cml
F30d-10	+	gray	yellow	-	Sm^RCml^R	N_2CS+Cml
F30d-11	+	white-gray	brown-yellow	-	Sm^RCml^R	N_2CS+Cml
F30d-12	+	white-green	brown	-	Sm^RCml^R	N_2CS+Cml
F30d-13	+	gray	brown	-	Sm^RCml^R	N_2CS+Cml
F30d-14	+	yellow	brown	-	Sm^RCml^R	N_2CS+Cml
F30d-15	+	white	brown-yellow	-	Sm^RCml^R	N_2CS+Cml
F30d-19		gray	brown	-	Sm^RCml^R	N_2CS+Cml
E50d-1	+	gray	red/orange	blue	Sm^SCml^R	N_2C+Cml
E50d-3	+	gray	red/orange	-	Sm^SCml^R	N_2C+Cml
F50d-2	+	white	yellow	-	Sm^SCml^R	N_2C+Cml
F50d-3	+	white	yellow	-	Sm^RCml^R	N_2C+Cml
F50d-4	+	white	red	-	Sm^R	N_2C+Thio
F50d-5	+	white	red	-	Sm^R	N_2C+Thio
F50d-6	+	gray	yellow	-	$Thio^R$	N_2C+Thio
F50d-7	+	gray	brown	green	$Thio^R$	N_2C+Thio

[1]: Soil isolates were named by the soil type (E or F), the sampling day (x) and the number (y) as Exd-y or Fxd-y, 'E' refers to Warwick soil and 'F' means Wuppertal garden soil. +/-, presence/absence of spores.
[2]: -, no production of pigments. The strains exhibiting similar growth behavior as Jni13C1 are shadowed.
[3]: N_2C, TSB-agar supplemented with nystatin, nalidixic acid and cycloheximide; N_2CS, TSB-agar supplemented with N_2C and Sm. Cml, Sm, Thio, see **Tab.** 2.6.

Most of the soil native strains were isolated on TSB (Cml). The original purpose was to try to isolate Cml^R recombinant derivatives of Jni13C1. Some isolated Cml^R strains were found to have similar phenotypes as *S. lividans*. Four strains, namely E30d-2, E30d-3, E50d-1 and E50d-3, had phenotypes similar to that of Jni13C1 (**Tab.** 3.4, in shadow). Among them, two (E30d-2 and E30d-3) were Sm^RCml^R while the other two (E50d-1 and E50d-3) were Sm^SCml^R. In order to get some information on the four soil isolates a molecular biological analysis by PCR, random amplified polymorphic DNA (RAPD) analysis, comparison of the restriction fragments length polymorphisms (RFLPs) generated from 16S rRNA genes and pulsed field gel electrophoresis (PFGE) analysis of the chromosomal DNA was carried out.

3.6.2.1 Strain characterization by PCR

Three primer pairs CZ5/CZ6, CZ7/CZ8, and CZ9/CZ10 (**Tabs.** 2.5 and 2.8) were designed according to the known sequences for the detection of the chromosomal ends (**Fig.** 3.7). Based on the *cmlR* sequence from *S. lividans* 1326 (EMBL accession number X59968), CZ3/CZ4 (**Tabs.** 2.5 and 2.8) were designed to amplify the complete 1.3 kb *cmlR* gene. Primer pairs CZ11/CZ12 (**Tabs.** 2.5 and 2.8) were designed to amplify a 1.2 kb fragment of the AUD1 element according to the known sequence (accession no. U22894) from *S. lividans* TK64 (Volff *et al.*, 1996).

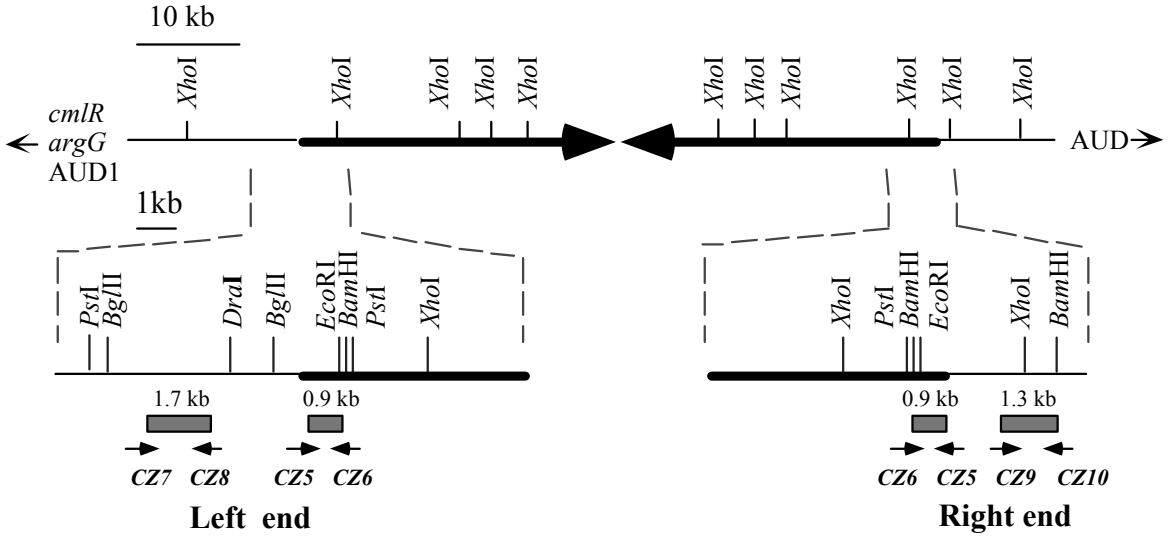


Fig. 3.7: Physical map of the chromosomal ends of *S. lividans* TK64 and the location of the primers for PCR. The end relative to the *cmlR* and *argG* genes was designated as "left end" and the end relative to AUD regions was designated as "right end". Black arrows mark the 30 kb terminal inverted repeats (TIRs). Location of the PCR primers was also indicated as shadowed rectangles. Directions of primers are indicated with small arrows. Some restriction sites (not all) are given.

The PCR amplification results are summarized in **Tab.** 3.5. The 1.2 kb AUD1 fragment could be amplified from all six strains. In the four soil isolates, *cmlR* and the TIR could also be amplified. But they had no amplifiable left end (CZ7/CZ8) and the amplified right end fragment (CZ9/CZ10) was larger (2.2 kb) than expected (1.3 kb).

Tab. 3.5: Amplification of chromosomal ends, *cmlR* and AUD1 from different strains

Primer pairs	TK23	Jni13C1	E30d-2	E30d-3	E50d-1	E50d-3
CZ3/CZ4 (*cmlR*)	1.3 kb	-	1.3 kb	1.3 kb	1.3 kb	1.3 kb
CZ5/CZ6 (TIR)	0.9 kb	-	0.9 kb	0.9 kb	0.9 kb	0.9 kb
CZ7/CZ8 (left end)	1.7 kb	-	-	-	-	-
CZ9/CZ10 (right end)	1.3 kb	-	2.2 kb	2.2 kb	2.2 kb	2.2 kb
CZ11/CZ12 (AUD1)	1.2 kb	1.2 kb	1.2 kb	1.2 kb	1.2 kb	1.2 kb

Note: -, no PCR product. The numbers indicated the size of the PCR products.

3.6.2.2 Random amplified polymorphic DNA (RAPD) analysis

Genomic fingerprinting assays using random amplified polymorphic DNA (RAPD) have already been shown to be useful for the differentiation of bacterial strains (Welsh & McClelland, 1990; Makino *et al.*, 1994; Mehling *et al.*, 1995b; Mehling, 1996). This method is based on the amplification of distinct genomic DNA sequences under low stringy conditions during annealing using an oligonucleotide of arbitrary sequence. The primer is not directed at any specific sequence within the template, making previous sequence knowledge on at least part of the genomic DNA non-essential. The efficiency of the amplification procedure is primarily dependent on sufficient sequence similarity at the 3' end of the oligonucleotides to allow adequate priming. The resulting pattern of amplification products of varying size can subsequently be used as a genetic fingerprint of the organisms tested. Experiments were carried out to study the possibility of using RAPD to differentiate the soil isolates. The PAPD experiments were performed as described in **Sect.** 2.15.3. All tested *S. lividans* strains TK64, Jni13C1, Jni14C1, WP, AJ100 and TK23, created a similar and typical four-major-bands pattern when primer 4 (**Tab.** 2.5) was used. Further experiments showed these results were reproducible. This led to the use of only primer 4 to differentiate the other *Streptomyces* species from *S.*

lividans. The RAPD patterns generated from all other primers tested (**Tab.** 2.5) were not reproducible.

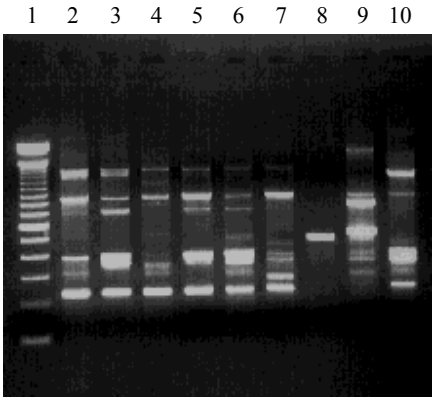


Fig. 3.8: Electrophoretic analysis of a typical RAPD assay with primer 4. Electrophoresis was run on a 2.0 % agarose gel. *lane* 1, 100 bp ladder as a size marker; *lanes* 2-10, RAPD patterns generated from: *S. lividans* TK23, Jni13C1, E30d-2, E30d-3, E50d-1, E50d-3, F30d-19 (**Tab.** 3.4), *S. lincolnesis* 78-11 and *S. celicolor* A(3)2.

The RAPD band patterns generated from the four isolates E30d-2, E30d-3, E50d-1 and E50d-3 were similar to that of Jni13C1, especially the four-major-bands, but quite different from another soil isolate (F30d-19; **Tab.** 3.4) and *S. coelicolor* A(3)2, *S. lincolnensis* 78-11 (**Fig.** 3.8). This result gave hints that these four soil isolates might be derivatives of *S. lividans*.

3.6.2.3: Identification of 16S rRNA operons and comparison of the restriction fragment length polymorphisms (RFLPs)

The variations in rRNA gene restriction fragment length polymorphisms (RFLPs) of streptomycete species could also be used as a rapid method for differentiating streptomycete strains (Clarke *et al.*, 1993; Mehling *et al.*, 1995a; Mehling, 1996). The ribosomal DNA restriction patterns from *S. lividans* TK23, Jni13C1 and the soil isolates E30d-2, E30d-3, E50d-1 and E50d-3 were compared. Genomic DNAs hydrolyzed with *Bam*HI and *Nco*I were immobilized on a nylon membrane and subjected to Southern hybridization using a probe consisting of the internal *Kpn*I/*Hin*cII fragment of the 16S rRNA gene of *S. galbus* DSM40480 (cloned in pAMW28; Mehling, 1996). The result revealed that the four soil isolates contained six distinct signals with a pattern similar to that of *S. lividans* (data not shown).

3.6.2.4: Genomic analysis by pulsed field gel electrophoresis (PFGE)

Low-frequency restriction fragment analysis of DNA by pulsed-field gel electrophoresis (PFGE) as a relatively new technique for analyzing large DNA fragments is useful for identifying the genetic variations of microorganisms (Leblond *et al.*, 1990; Ichiyama *et al.*, 1991). The physical map of the *S. lividans* 66 ZX7 chromosome (Zhou *et al.*, 1988) has been well constructed (Leblond *et al.*, 1993). It provided us a good basis for identifying the genomes of the four soil isolates E30d-2, E30d-3, E50d-1 and E50d-3 by comparing the respective PFGE patterns. The two isolates E30d-2 and E30d-3 (lanes 3 and 4 in **Fig.** 3.9 A) gave a different *Ase*I pattern from that of *S. lividans* (lanes 1 and 2 in **Fig.** 3.9 A). The four soil isolates (lanes 3-6 in **Fig.** 3.9 B) showed totally different *Hin*dIII patterns in comparison with those of *S. lividans* TK23 and Jni13C1 (lanes 1 and 2 in **Fig.** 3.9 B). This meant that these four strains were either not derivatives of strain Jni13C1 or that large chromosomal rearrangements had occurred.

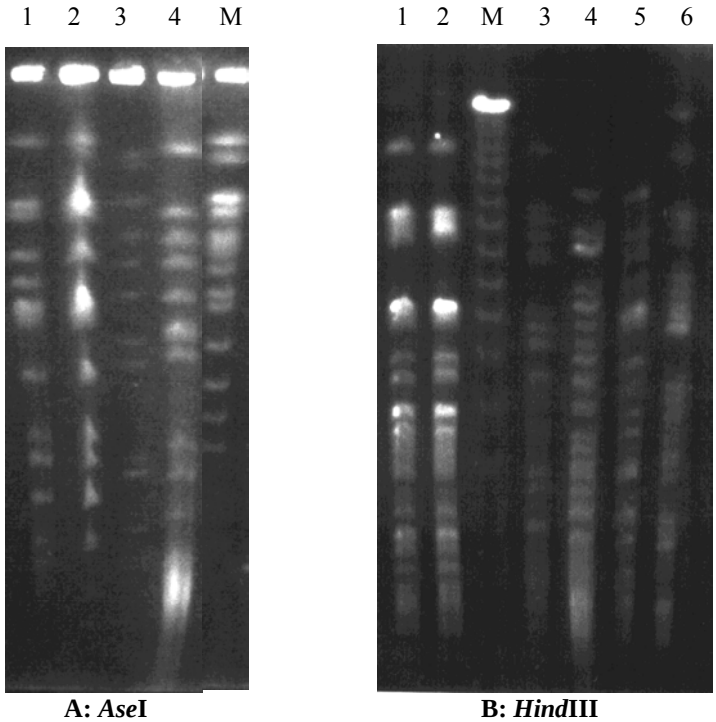


Fig. 3.9: PFGE analysis and comparison of the genomes of the soil isolates and *S. lividans*. A: The genomic DNA was digested with *Ase*I and the PFGE was performed at 180 V for 24 h with changing pulse time: 20 s for 4 h, 30 s for 4 h, 45 s for 4 h, 60 s for 4 h, 100 s for 4 h and 120 s for 4 h. *Lanes* 1-4, TK23, Jni13C1, E30d-2 and E30d-3, *lane* M, Yeast PFGE marker. **B**: The genomic DNA was digested with *Hin*dIII and the PFGE was performed at 180V for 22 h with changing pulse time: 5 s for 4 h, 10 s for 4 h, 20 s for 4 h, 30 s for 4 h, 40 s for 4 h and 50 s for 2 h. *Lanes* 1 and 2, TK23, Jni13C1; *lane* M, Lamda PFGE marker; *lanes* 3 - 6, E30d-2, E30d-3, E50d-1 and E50d-3.

3.7: Mobilization of chromosomal markers by the conjugative plasmid pIJ903

3.7.1: The conjugative plasmid pIJ903

Conjugative plasmids with the ability to mobilize chromosomal genes (fertility plasmids) are common in streptomycetes (Hopwood *et al.*, 1986). Most of the conjugative streptomycetes plasmids have chromosome-mobilizing ability (Cma) with recombination frequencies between 10^{-6} and 10^{-3}. For example, SCP2*, a 31 kb low-copy-number circular plasmid of *S. coelicolor* A3(2), mobilizes chromosomal markers randomly without the involvement of known integrated forms or transferable elements (Lydiate *et al.*, 1985; Xiao *et al.*, 1994). The conjugative plasmid pIJ903 (**Fig.** 3.10) is a bifunctional plasmid, derived from SCP2* and pBR327. It contains all the genes for transfer, pock formation and stability and it has the *tsr* gene ($Thio^R$) for selection in *Streptomyces*, and the origin of replication and the *bla* gene (Ap^R) of pBR327 for replication and selection in *E. coli* (Lydiate *et al.*, 1985).

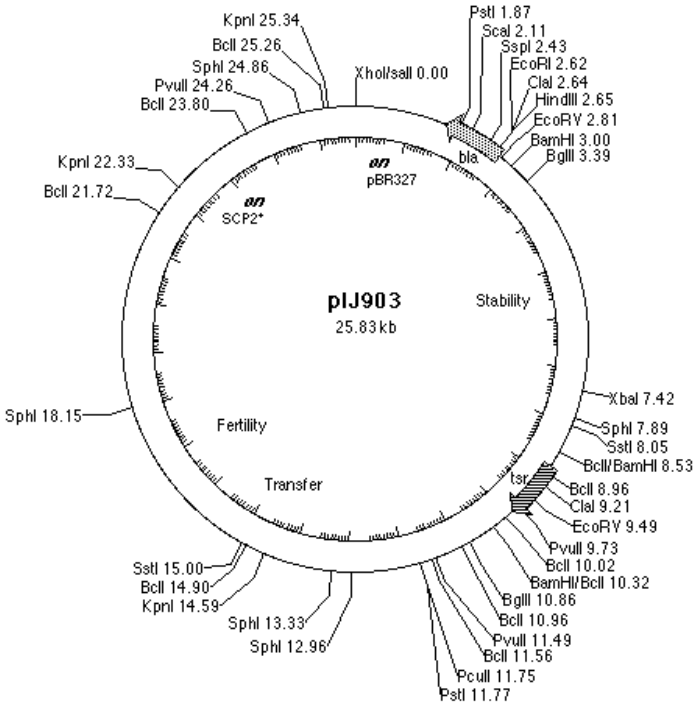


Fig. 3.10: Physical map of the conjugative plasmid pIJ903. The regions responsible for stability, fertility and transfer are indicated. *tsr*, thiostrepton resistance gene; *bla*, ampicilin resistance gene.

3.7.2: Mating experiments with *S. lividans* 66 strains

Natural gene exchange is very widespread in *Streptomyces* sp. In the laboratory it requires the mixed culture of pairs of strains on agar ("matings"). Conjugation occurs non-synchronously during several hours of mixed growth, involving successive rounds of recombination, as can be deduced from the progeny of triparental matings (Bibb and Hopwood, 1981). Quantitative analysis of recombinant progeny from matings has been the main means of genetic mapping in *Streptomyces* sp. In this work, matings were carried out on SPMR-agar to demonstrate the transfer frequency of the conjugative plasmid pIJ903 and to test the possibility that chromosomal markers are transferred. The first matings were performed with different *S. lividans* strains. The results were summarized in **Tab.** 3.6. When *S. lividans* TK23/pIJ903 was used as the donor strain, a transfer of the plasmid pIJ903 to the strains Jni13C1, Jni14C1 and WP occurred at high frequencies (M1-M3, **Tab.** 3.6). When Jni13C1/pIJ903 served as the donor, the plasmid pIJ903 was transferred to TK23 with a higher frequency (M6, **Tab.** 3.6).

Tab. 3.6: Transfer of plasmid pIJ903 between *S. lividans* strains

Mating Pair	Parental Genotypes (ml^{-1})		Exconjugants (ml^{-1})			Frequency of plasmid transfer	Frequency of Sm^R Mutation
	Sm^R	$Thio^RCml^R$	$Thio^RSm^R$	Sm^RCml^R	$Thio^RSm^RCml^R$		
M1	$1.27x10^{10}$	$2.58x10^{10}$	$3.10x10^7$	$6.90x10^3$	$4.90x10^3$	$2.44x10^{-3}$	$2.67x10^{-7}$
M2	$1.46x10^5$	$1.23x10^7$	$2.20x10^4$	$3.00x10^1$	0	0.151	$2.44x10^{-6}$
M3	$1.20x10^4$	$9.13x10^9$	$3.75x10^3$	$5.00x10^2$	$2.70x10^2$	0.312	$0.55x10^{-7}$
	$Thio^RSm^R$	Cml^R	$Thio^RCml^R$	Sm^RCml^R	$Thio^RSm^RCml^R$		
M6	$2.10x10^7$	$1.37x10^9$	$7.57x10^8$	$5.60x10^3$	$4.30x10^3$	0.553	$4.09x10^{-7}$

Note: Spores of each parental type were mixed and plated on SPMR medium, following one cycle of non-selective growth, spores were collected and quantified for parental type or exconjugants by plating dilutions on TSB-agar containing different antibiotics. M1, TK23/pIJ903 x Jni13C1; M2, TK23/pIJ903 x Jni14C1; M3, TK23/pIJ903 x WP; M6, Jni13C1/pIJ903 x TK23. Sm, Cml, Thio, see **Tab.** 2.6. The plasmid transfer frequency was expressed as the fraction of the recipient phenotype that acquired the plasmid. Sm^R mutation frequency was expressed as the fraction of Sm^RCml^R genotypes relative to TK23.

From these matings, some putative chromosomal recombinant ($Sm^RCml^RThio^R$) were found. These strains were of great interest. The question was whether the chromosomal marker *cmlR* was really mobilized by the plasmid pIJ903 and transferred from TK23 to the recipient strains Jni13C1 and WP.

3.7.3: Characterization of some Sm^RCml^R strains from matings between *S. lividans* 66 strains

Some $Sm^RCml^RThio^R$ strains were found after mating the pair of strains TK23/pIJ903 x Jni13C1. These $Sm^RCml^RThio^R$ derivatives (**Sect.** 3.7.2) fell into 3 main groups (Group I – III) based on their resistance to spectinomycin and their requirement for proline, as summarized in **Tab.** 3.7. Normally, colonies of TK23 were $Sm^SCml^RSpc^RPro^+$ and could not produce blue pigments (actinohordin) on SPMR plates, colonies of Jni13C1 were $Sm^RCml^SSpc^SPro^-$ and could produce diffusible blue pigments (actinohordin). Of the 108 tested colonies, all Pro^- colonies (25.9%) were also Spc^S (Group I). Some colonies, e.g. M1-17, 19, 71, were Spc^R but also could produce blue pigments. Normally, TK23/pIJ903 produced yellow colonies on TSB plates while colonies of Jni13C1/pIJ903 were white. About 18.5% of the exconjugants were white. All of them belonged to Group I and could produce blue pigments on SPMR-agar (**Tab.** 3.8). Some of these white colonies, e.g., M1-14, 23, 48, 106 and 107, had identical phenotypes to Jni13C1 except that Jni13C1 was Cml^S.

Tab. 3.7: Phenotypic differentiation of Sm^RCml^R derivatives from a mating between *S. lividans* Jni13C1 and TK23/pIJ903

Exconjugants	Group I $Sm^RCml^RSpc^SPro^-$	Group II $Sm^RCml^RSpc^SPro^+$	Group III $Sm^RCml^RSpc^RPro^+$
Amounts	28	29	51
Percentage	25.9%	26.8%	47.2%
Strains No.	M1-1, 14, 21, 23, 48, 54, 58, 59, 86, 87, 88, 89, 90, 92, 93, 94, 95, 96, 97, 98, 99, 100, 102, 103, 104, 105, 106, 107	M1-3, 5, 6, 7, 10, 20, 24, 26, 34, 35, 36, 38, 41, 44, 46, 51, 52, 57, 67, 70, 72, 74, 75, 76, 81, 82, 83, 84, 85,	M1-2, 4, 8, 9, 11, 12, 13, 15, 16, 17, 18, 19, 22, 25, 27, 28, 29, 30, 31, 32, 33, 37, 39, 40, 42, 43, 45, 47, 49, 50, 53, 55, 56, 60, 61, 62, 63, 64, 65, 66, 68, 69, 71, 73, 77, 78, 79, 80, 91, 101, 108

Note: The underlined strains could produce diffusible blue pigments (actinohordin) on SPMR plates.

From the mating pair TK23/pIJ903 x WP, some $Sm^RCml^RThio^R$ colonies were obtained. Among them, one white colony, namely M3-1, could not sporulate on SPMR-agar, was sensitive to spectinomycin (80 μg/ml) and required proline and arginine for growth. These markers were characteristic for the strain WP, except for Cml^R.

The strain M3-1 and some exconjugants from mating 1 were further analyzed. The presence of the chromosomal end regions, including the *cmlR*, the AUD1 element and the ends of these strains were studied by PCR using the primer pairs CZ3/4, CZ5/6, CZ7/8, CZ9/10 and CZ11/12

(**Fig.** 3.7; **Tabs.** 2.5 & 2.8). The PCR results were summarized in **Tab.** 3.8. The strain M3-1 possessed the left chromosomal end, the TIR region and the *cmlR* gene but lost the right end and the AUD1 element. All these regions were lost in the strain WP (**Tab.** 3.8; **Fig.** 3.1).

Tab. 3.8: Characterization of the selected exconjugants and comparison with the parental strains

Strains	M1 [a]		M1(I) [b]			M1(II) [b]	M1(III) [b]	M3 [a]	
Phenotypes [c]	**TK23**	**Jni13C1**	**M1-48**	**M1-106**	**M1-107**	**M1-5**	**M1-49**	**M3-1**	**WP**
Aerial spores	+	+	+	+	+	+	+	-	-
Blue pigment	-	+	+	+	+	-	+	-	-
ArgG	+	+	+	+	+	+	+	-	-
Sm^R	-	+	+	+	+	+	+	+	+
Spc^R	+	-	-	-	-	-	+	-	-
Pro	+	-	-	-	-	-	+	-	-
Cml^R	+	-	+	+	+	+	+	+	-
PCR amplification [d]									
TIR (CZ5/6)	+	-	+	+	+	+	+	+	-
Left end (CZ7/8)	+	-	+	+	+	+	+	+	-
Right end (CZ9/10)	+	-	-	-	-	+	+	-	-
AUD1 (CZ11/12)	+	+	+	+	+	+	+	-	-
cmlR (CZ3/4)	+	-	+	+	+	-	+	+	-

Note: [a]: M1, M3, see **Tab.** 3.6.
[b]: M1(I), M1(II), M1(III) refer to the Group I – III in **Tab.** 3.7, respectively.
[c]: Phenotype: +, prototroph; -, auxotroph.
[d]: PCR amplification: +, PCR possible; -, PCR negative.
The identical properties between the recombinant strains and the parental receptor strains Jni13C1 and WP are shadowed.

Southern hybridization was also carried out to detect the chromosomal ends using a 2.1 kb *Bam*HI fragments containing part of the TIRs from pJOE2424 as a probe. The results showed that there were two hybridizing bands, 7.5 kb (left end) and 2.9 kb (right end) in TK23, M1-5, M1-49, one band (7.5 kb) in M3-1, M1-48, M1-106 and M1-107, but no bands were obtained with WP and Jni13C1 (data not shown). These results were in good accordance with that of the PCR.

In order to get more information, DNA of some recombinant strains (e.g. M3-1, M1-48 and M1-107) and, for comparison, of TK23, TK64, Jni13C1 and WP, were digested with some low-frequency restriction enzymes (e.g. *Ase*I and *Dra*I). The resulting fragments were analyzed by PFGE and subsequent Southern hybridizations using specific probes for the location of the chromosomal ends and the *cmlR* gene. The results of the PFGE patterns and the corresponding

hybridizations are shown in **Fig.** 3.11, **Fig.** 3.12 and summarized in **Tabs.** 3.9 and 3.10. In comparison with TK23 and TK64, Jni13C1 had lost the 615 kb *Ase*I-D, 225 kb *Ase*I-H1 and 25 kb *Ase*I-K fragments (**Tab.** 3.9). The chromosomes of the strains M1-48 and M1-107 (Group I of M1, **Tab.** 3.7) showed large differences to TK23, Jni13C1 and from each other (**Fig.** 3.11 and 3.12; **Tab.** 3.9 and 3.10).

In *Ase*I digestion of chromosomal DNA of TK23, the end probe (cf. **Tab.** 3.10) hybridized to the 2,600 kb A (left end) and 225 kb H1 (right end) fragments as expected (cf. **Fig. 3.13**). In the strain M1-107, the end probe hybridized with 3 *Ase*I fragments, namely the 2,600 kb A, the 975 kb B and the 630 kb newly-generated (comparing to TK23) fragments but not with the 225 kb H1 fragment (lane 4 in **Fig.** 3.11 I.A, I.C). Furthermore, only a single 100 kb *Hin*dIII fragment from M1-107 hybridized with the end probe (lane 3 in **Fig.** 3.12 II). In contrast, there were two hybridizing *Hin*dIII fragments (190 kb and 100 kb) in TK23 (lane 1 in **Fig.** 3.12 II). These results suggested that M1-107 did not have the right chromosomal end, represented in TK23 by the 225 kb *Ase*I-H1 and the 190 kb *Hin*dIII fragments, respectively, but possessed 3 copies of the left chromosomal end. Notably, the *cmlR* probe (cf. **Tab.** 3.10) gave similar hybridizing results in M1-107 (lane 4 in **Fig.** 3.11 I.B; **Tab.** 3.10). All these data indicated that the 100 kb *Hin*dIII fragment containing the left chromosomal end together with the 340 kb *Hin*dIII fragment containing the *cmlR* gene (**Tab.** 3.10) was duplicated to 3 copies in M1-107, resulting in the above described three *Ase*I fragments, which hybridized with both probes (cf. **Tab.** 3.10).

Hybridization data showed that the strain M1-48 also did not have the right chromosomal end (lane 3 in I. C, **Fig.** 3.11) but had 3 copies of the left end, located on the 55 kb, 50 kb *Dra*I fragments, and a huge *Dra*I fragment (larger than approximately 3,000 kb) of which the size could not be resolved by the PFGE program used, respectively (lane 3 in II. C and II. D, **Fig.** 3.11). The two end-containing *Dra*I fragments in M1-48 (55 kb and 50 kb) were both larger than the smaller one in TK23 (30 kb *Dra*I-VII fragment, lane 1 in II. C, **Fig.** 3.11). Similarly, in strain M1-48, the end probe hybridized with a single 150 kb *Hin*dIII fragment (lane 2 in **Fig.** 3.12 II), which was larger than the left (100 kb) but smaller than the right end (190 kb) of TK23 (lane 1 in **Fig.** 3.12 II). Surprisingly, triplication of the left end was not reflected in the hybridizing pattern of *Ase*I-digested chromosomal DNA of M1-48 (lane 3 in I.C, **Fig.** 3.11) and no duplication of the *cmlR* gene was detected in M1-48 (**Tab.** 3.10).

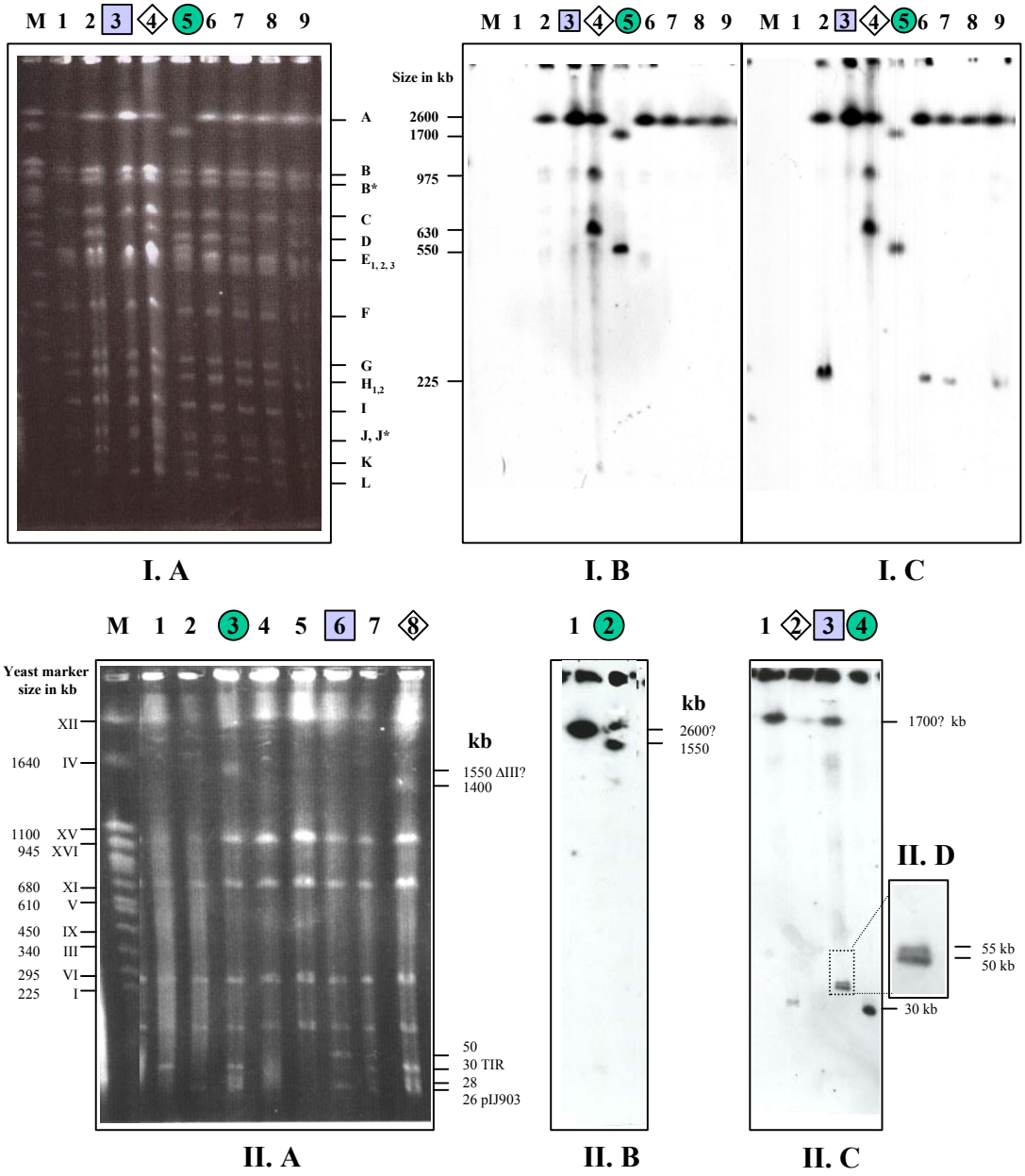


Fig. 3.11: PFGE analysis of the chromosomes digested with *Ase*I or *Dra*I of different *S. lividans* strains and the corresponding Southern hybridization analysis. I. A, *Ase*I digestion, the PFGE was performed at 180 V for 24 h with changing pulse time: 20 s for 4 h, 30 s for 4 h, 45 s for 4 h, 60 s for 4 h, 100 s for 4 h and 120 s for 4 h. The corresponding Southern hybridization analysis using probe 1 (**I. B**) and probe 2 (**I. C**) was shown. *Lane* M: Yeast PFGE marker; *lanes* 1-9: Jni13C1, TK23, M1-48, M1-107, M3-1, M1-108, M1-49, M1-41, M1-5. **II. A:** *Dra*I digestion, PFGE was performed at 180 V for 24 h with changing pulse time: 25 s for 4 h, 50 s for 4 h, 100 s for 8 h and 120 s for 8 h. *Lane* M: Yeast PFGE marker; *lanes* 1-8: TK23, Jni13C1, M3-1, WP, Jni14C1, M1-48, M1-106, M1-107. **II. B:** Southern hybridization analysis of *Dra*I fragments separated by PFGE (the same condition as I. A) using probe 1. *Lane* 1: TK23; *lane* 2, M3-1. **II. C:** Southern hybridization analysis of *Dra*I fragments separated by PFGE (the same condition as II. B) using probe 2. *Lane* 1: TK23; *lanes* 2-4: M1-107, M1-48, M3-1. **II. D:** amplification of part of II. C. Probe 1: 0.9 kb *Nco*I fragment from pJOE2568 for detecting the *cmlR* gene; probe 2: 2.1 kb *Bam*HI fragment from pJOE2424 for detecting chromosomal ends. The lanes representing the 3 putative recombinant strains were indicated: M1-48, ■; M1-107, ◇; M3-1, ●.

Tab. 3.9: Comparison of PFGE patterns of different putative recombinant strains

I: *Ase*I digestion

Fragment (size in kb)	**TK64**	**TK23**	**Jni13C1**	**M1-48**	**M1-107**	**M3-1**	**WP**
A	2,600	2,600	2,600?	2,600?	2,600?	**1,700**	**1,200?**
B	975	975	975	975	975	975	975
B*	900	900	900	900	900	900	900
C	710	710	710	710	710	710	710
D	615	615			**630**	615; **550**	615
E_1	525	525	525	525	525	525	525
E_2	515	515	515	515	515	515	515
E_3	485	485	485	485	485	485	485
F	355	355	355	355	355	355	355
G	270	270	270	270	270	270	270
H_1	225	225					
H_2	225	225	225	225	225	225	225
I_1	145	145	145	145	145	145	145
I_2	145	145	145	145	145	145	145
J*	80	80	80	80	80	80	80
J	70	70	70	70	70	70	70
K	25	25			25	25	25
L	15	15	15	15	15	15	15

II: *Dra*I digestion

Fragment (size in kb)	**TK64**	**TK23**	**Jni13C1**	**M1-48**	**M1-107**	**M3-1**	**WP**
I	2,600	2,600	2,600?	2,600?	2,600?	2,600?	2,600?
II	2,500	2,500	2,500?	2,500?	2,500?	2,500?	2,500?
III	1,700	1,700	?	?	?	?	?
					1400	**1550**	
IV*	980	980	980	980	980	980	980
IV	700	700	700	700	700	700	700
V	300	300	300	300	300	300	300
VI	150	150	150	150	150	150	150
				55+50			
VII	30	30			30	30	
					28	**28**	

Note: The new fragments from the putative recombinants are in bold. The presence or the sizes of fragments which could not be resolved in the corresponding PFGE are indicated with a question mark. The free boxes mean that the corresponding fragments were absent. The *Ase*I fragments (A-L) and the *Dra*I fragments (I-VII) were named after Leblond *et al.* (1993).

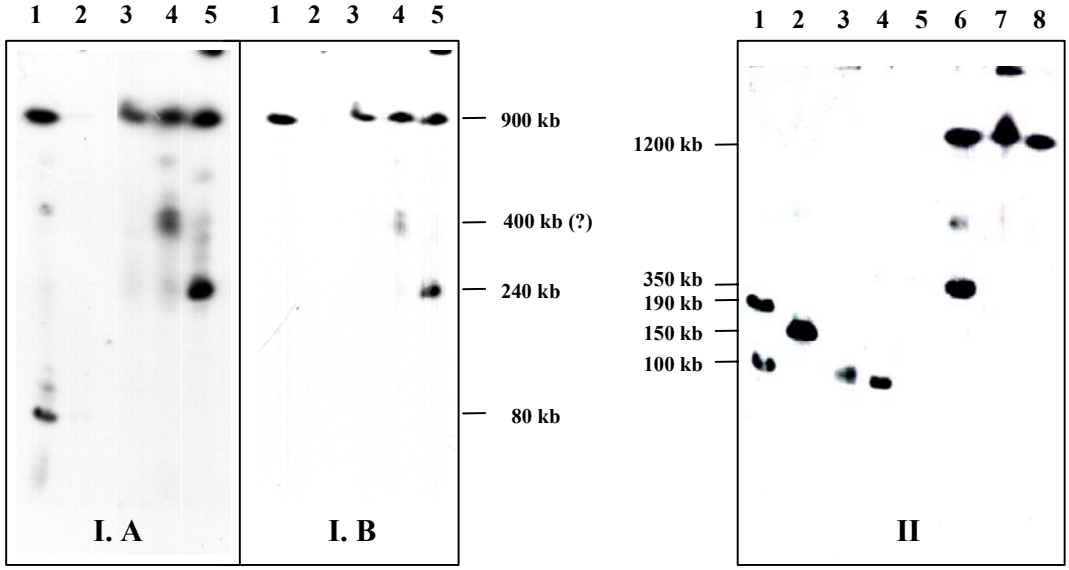


Fig. 3.12: Southern hybridization analysis of the chromosomes of some recombinants separated by PFGE. I: *Bfr*I digestion, the PFGE was performed at 180 V for 24 h with changing pulse time: 5 s for 4 h, 10 s for 4 h, 20 s for 4 h, 30 s for 4 h, 40 s for 4 h and 50 s for 4. The corresponding southern analysis using probe 1 (**I. B**) and probe 2 (**I. A**) is shown. *Lane* 1-5: TK23, Jni13C1, M1-48, M1-107, M3-1. **II:** chromosomes were digested with *Hin*dIII (*lanes* 1-4) and *Ssp*I (*lanes* 5-8) and separated by PFGE (180 V for 24 h with changing pulse time: 5 s for 4 h, 10 s for 4 h, 20 s for 4 h, 30 s for 4 h, 40 s for 4 h and 50 s for 4 h), the corresponding Southern hybridization was done using probe 2. *Lanes* 1-4: TK23, M1-48, M1-107, M3-1; *lanes* 5-8: Jni13C1, TK23, M1-48, M1-107. Probe 1: 0.9 kb *Nco*I fragment from pJOE2568 for detecting the *cmlR* gene; probe 2: 2.1 kb *Bam*HI fragment from pJOE2424 for detecting chromosomal ends.

Tab. 3.10: Summary of the hybridizing fragments from PFGE of putative recombinant strains using two different probes

Hybridizing bands (size in kb)		**TK64**	**TK23**	**M1-48**	**M1-107**	**M3-1**
Probe1 (*cmlR*)	*Ase*I	2,600	2,600	2,600?	2,600?; **975; 630**	**1,700; 550**
	*Dra*I	2,600	2,600	2,600?	2,600?	2600**?; 1,550**
	*Bfr*I	900	900	900?	900?; **400**?	900?; **240**
	*Hin*dIII	340	340	340	340	340
	*Ssp*I	1,200	1,200	1,200?	1,200?	**900**?
Probe 2 (end)	*Ase*I	2,600; 225	2,600; 225	2,600?	2,600?; **975; 630**	**1,700**
	*Dra*I	1,700; 30	1,700; 30	1,700?; **55; 50**	1,700?; 30	30
	*Bfr*I	900; 80	900; 80	900?	900?	900?; **240**
	*Hin*dIII	190; 100	190; 70	**150**	100	100
	*Ssp*I	1200; 350	1200; 350	1200?	1200?	1200?

Note: Probe 1, 0.9 kb *Nco*I fragment from pJOE2568 for detecting the *cmlR* gene; probe 2: 2.1 kb *Bam*HI fragment from pJOE2424 for detecting chromosomal ends. The new bands are indicated in bold. The fragment sizes which could not be resolved in the corresponding PFGE are indicated with a question mark.

The PFGE analysis showed that the chromosome of the strain M3-1 had large scale differences to those of the parental strains TK23 and WP (**Tab.** 3.9 and 3.10). In comparison with TK23, a smaller *Ase*I-A fragment of about only 1,700 kb (1200 kb in strain WP, **Tab.** 3.9) and an additional 550 kb *Ase*I fragment were detected in the strain M3-1 (lanes 2 and 5 in I. A, **Fig.** 3.11). Unlike the strains Jni13C1, M1-107 and M1-48, the 615 kb *Ase*I-D fragment was present in the strains M3-1 and WP (Tab. 3.9). The *Dra*I pattern of the strain M3-1 was also different from that of TK23 (lane 3 and lane 1 in II. A, **Fig.** 3.11; **Tab.** 3.9 II). Relative to TK23, the strain M3-1 had three additional *Dra*I fragments (1,550 kb, 28 kb and 26 kb, respectively; lane 3 in II. A, **Fig.** 3.11). By available evidence, the 26 kb *Dra*I fragment was interpreted to represent the linearized plasmid pIJ903 (data not shown).

The following Southern hybridization analysis showed that the strain M3-1 had no right end but two copies of the left end, which were located on the 1,700 kb *Ase*I-ΔA fragment and the 550 kb new *Ase*I fragment (lane 5 in I. C, **Fig.** 3.11; lane 5 in I. A, **Fig.** 3.12), respectively, probably derived from duplication of the single hybridizing 100 kb *Hin*dIII fragment (lane 4 in **Fig.** 3.12 II). M3-1 had also two copies of the *cmlR* gene, located on the two *Ase*I fragments containing the left end (lane 5 in I. B, **Fig.** 3.11). Similarly, two copies of the *cmlR* gene were also detected in the 2,600 kb and the 1,550 kb *Dra*I fragments (lane 2 in II. B, **Fig.** 3.11), but notably neither of them contained the chromosomal end sequence. Only the 30 kb *Dra*I fragment hybridized with the end probe (lane 4 in II.C, **Fig.** 3.11). The presence of the two copies of the left end and the *cmlR* gene were also revealed by the hybridization with the *Bfr*I-digested chromosomal DNA of M3-1 (lane 5 in I.A and I.B, **Fig.** 3.12). The detailed comparisons of the chromosomes of TK23, M3-1 and WP were summarized in **Tab.** 3.9 and **Tab.** 3.10.

These data indicated that recombination and large rearrangements had occurred in the instable regions of the chromosome of the strains M3-1, M1-107 and M1-48.

3.7.4: Chromosome organization of the recombinant strains

As an immediate interpretation of the structural data on the chromosome organization in the three recombinant exconjugants, M3-1, M1-107 and M1-48, here their putative genome maps are presented.

The first circular physical map of the linear chromosome of *S. lividans* was constructed using the strain ZX7 by ordering the *Ase*I and *Dra*I macrorestriction fragments (Leblond *et al.*, 1993; Lin

et al., 1993). In later research, two additional *Ase*I fragments (900 kb B* and 80 kb J*) and one additional *Dra*I fragment (980 kb IV*) were found in the multipurpose, laboratory strain *S. lividans* TK64 (Zhou *et al.*, 1994; Volff and Altenbuchner, 1997; **Tab.** 3.9). These latter fragments had not been mapped so far. The most convincing and immediate interpretation is that presented in **Fig.** 3.13, in which the two additional *Ase*I fragments were assembled together in unknown order between the *Ase*I-A and *Ase*I-J fragments. An additional *Dra*I site located on either of the AseI-B* or *Ase*I-J* fragments would result in the 980 kb *Dra*I-IV* fragment and all the other *Ase*I- and *Dra*I-fragments would not be affected (**Fig.** 3.13).

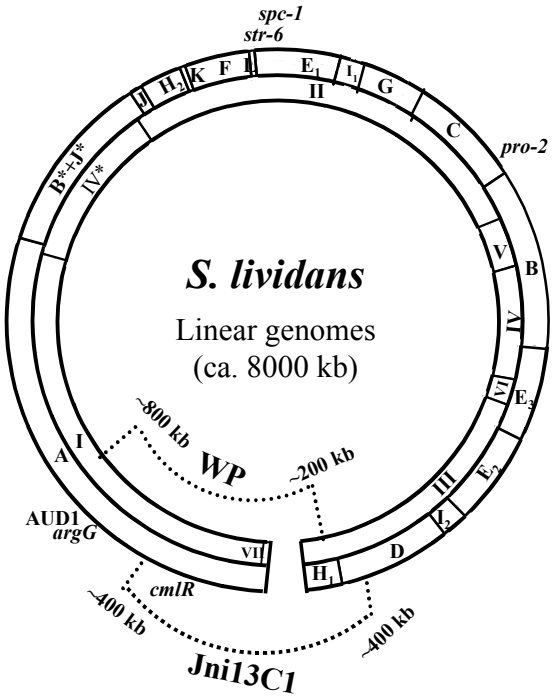


Fig. 3.13: Physical map of the linear genome of *S. lividans* for the enzymes *Ase*I (outer circle) and *Dra*I (inner circle). For the sizes of the corresponding *Ase*I and *Dra*I fragments see **Tab.** 3.9. The relative positions of the genetic markers (*cmlR*, *argG*, AUD1, *str-6*, *spc-1* and *pro-2*) are indicated. The deletions in the strains WP and Jni13C1 are indicated by dotted lines and the estimated deletion sizes are given, relative to the chromosome end.

The data from this study are in good accordance with the estimated deletion sizes (around 1,000 kb) in the strain WP (Volff *et al.*, 1996; **Fig.** 3.13; cf. **Tab.** 3.9). In strain Jni13C1 (Volff *et al.*, 1997a; 1997b; cf **Fig.** 3.13), the mode of disappearance of the *Ase*I-K fragment could be either explained (i) by a point mutation removing one of the flanking *Ase*I sites or (ii) by deletion of most of its material. The first event would increase the size of one of the neighboring fragments, *Ase*I-H2 or *Ase*I-F, which was not detected (cf. **Fig.** 3.11 I.A).

Based on the data collected from this work (**Tabs.** 3.9 and 3.10) and the literature (Leblond *et al.*, 1993; Lin *et al.*, 1993; Redenbach *et al.*, 1993; Zhou *et al.*, 1994; Rauland *et al.*, 1995; Volff *et al.*, 1996; Volff and Altenbuchner, 1997; Volff *et al.*, 1997a; 1997b), restriction maps of both end regions of the strains *S. lividans* TK23, M3-1 and M1-107 were constructed (**Fig.** 3.14). The construction of the chromosome maps was based on the following interpretations and hypotheses:

(i) In wild type *S. lividans* strains like TK23, there exists a very instable end region ("500 kb mobile element", cf. **Fig.** 3.14). Large-scale deletions and recombinations occur frequently in this region. The region is also a kind of highly "mobile element", which can be mobilized and transferred into closely-related other strains, e.g. with the help of conjugative plasmids.

(ii) The formation of the strains WP and Jni13C1 was in part due to illegitimate recombination. The fusion regions seem to be hot spots for recombination. Thus, in the conjugation experiments described in **Sect.** 3.7.2, two copies of the "500 kb mobile element" from TK23 were inserted near the fusion sites in WP and Jni13C1, resulting in the linear chromosomes of M3-1 and M1-107 (**Fig.** 3.14), respectively.

(iii) Mutations could have additionally occurred in the 500 kb "mobile element" during recombination. The D* mutation (**Fig.** 3.14 B and C) formed an assumed *Dra*I site in both strains M3-1 and M1-107. The B* mutation led to an assumed *Bfr*I site in the strain M3-1 and the A* mutation gave the *Ase*I site in M1-107 (**Fig.** 3.14 B and C).

In these interpretations, strains WP and Jni13C1 were assumed to play the role of recipients in a gene transfer event. The resultant strains M3-1 and M1-107 had identical central chromosomal regions, which carried the genetic markers like *pro-2*, *str-6*, *spc-1*, and *argG*, as their parental strains WP and Jni13C1, respectively (cf. **Tab.** 3.8). Furthermore, the integration of the hypothetical "500 kb mobile element" was assumed to be due to an event of illegitimate recombination, as it was demonstrated in two deletion mutants of *S. griseus* (Kameoka *et al.*, 1999). The results indicated that the chromosomal circularization of the of *S. griseus* mutants was caused by such a process without concomitant amplification.

The recombinant strains M3-1 and M1-107 probably had linear chromosomes (cf. **Fig.** 3.14). The putative hybridizing patterns using the two probes (*cmlR* and end probes), generated from these maps were consistent with the data from the actual results (**Tab.** 3.10). The 975 kb *Ase*I fragment

in M1-107, which hybridized with both probes, was likely to be created by the partial digestion of the A* (*Ase*I) site. Variations in the fragments, *Ase*I-A, *Dra*I-I and *Dra*I-III fragments (cf. **Fig.** 3.13) were due to the limitation of PFGE to resolve such huge fragments.

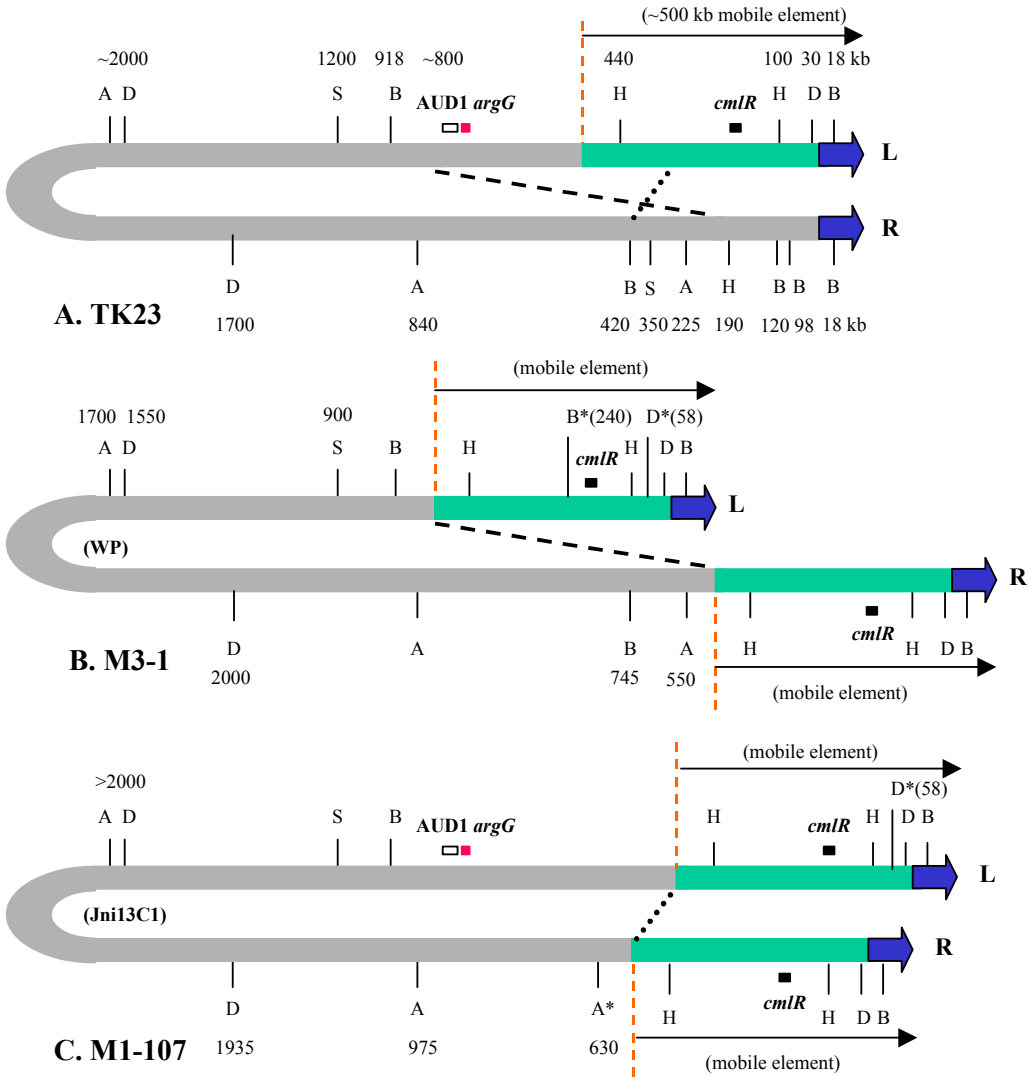


Fig. 3.14: Postulated maps of the organization of the linear chromosomes of strains TK23, M3-1 and M1-107. The schematic diagrams are not drawn to scale. **A**, the physical map of the chromosomal end regions of TK23. **B**, the map for M3-1, formed by the insertion of two copies of the "500 kb mobile element" including *cmlR* and TIR from the left end regions of TK23 into WP. **C**, the map for M1-107, formed by the insertion of two copies of the "500 kb mobile element" from TK23 into Jni13C1. The chromosomes are symbolized with gray boxes and the "500 kb mobile element" is symbolized with a green one. The 25 kb TIRs are symbolized by the blue broad arrows. L and R refer to the left and right chromosomal arms. The positions of AUD1, *argG* and *cmlR* are indicated by small rectangles) and the restriction sites, relative to the respective chromosomal end terminus are given in kb. The dashed line indicates the putative illegitimate recombination sites in the strains WP and Jni13C1. A, *Ase*I; B, *Bfr*I; D, *Dra*I; H, *Hind*III; S, *Ssp*I; * indicates that the restriction sites are assumed to be created by mutations.

In contrast, the recombinant strain M1-48 was interpreted to have a circular chromosome. This could be deduced from the fact that M1-48 lacked free chromosomal ends. Its putative structure was shown in **Fig.** 3.15. After integration of the "500 kb mobile element" from the donor strain TK23 into the recipient strain Jni13C1, the 25 kb TIR obviously was amplified to 3 copies. The partial digestion of the new *Dra*I site (D*) led to the weaker hybridizing 55 kb *Dra*I fragment and the largest hybridizing *Dra*I fragments with the end probe (**Fig.** 3.11 II.C and II.D). Another interpretation of this phenomenon could be that there was a clonal heterogeneity in the culture of strain M1-48 concerning the D* site, with part of the chromosomes lacking this site. The single hybridizing 150 kb *Hin*dIII fragment was interpreted to be due to the original *Hin*dIII site near the end of the left arm and a possible original *Hin*dIII site on the other chromosomal arm downstream of the illegitimate recombination site (called fusion site in **Fig.** 3.15).

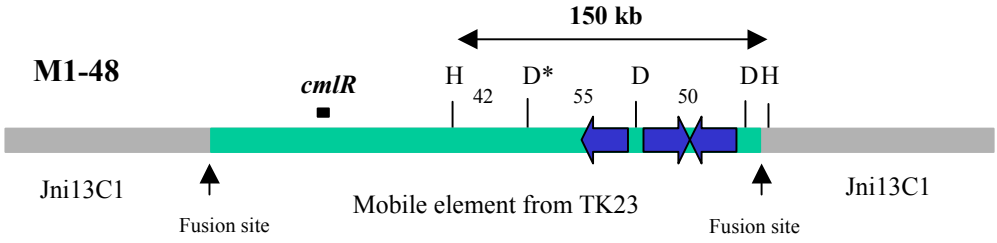


Fig. 3.15: Model of the organization of the putatively circular chromosome of strain M1-48 around the end fusion site. The schematic diagrams are not drawn to scale. The strain was formed by insertion of one copy of the "500 kb mobile element" from TK23 into Jni13C1, which was accompanied by two times amplification of the TIR. The symbols refer to **Fig.** 3.14. The numbers between the restriction sites indicate the size of the DNA fragment in between. The small arrows indicate the fusion sites of the "mobile element" and the chromosome of Jni13C1.

Further studies would be needed to confirm these interpretative chromosome maps. PFGE experiments should be performed to determine the exact topology of the chromosomes (linear/circular structure) of different strains like M3-1, M1-107 and M1-48. The experiments with the enzymes *Ssp*I and *Bfr*I also could be repeated under use of another PFGE program to get a better resolution in large fragments. Another important and feasible experiment would be to hybridize the *Dra*I fragments generated from these strains again by the use of a newly-designed end probe which contains the *Dra*I site (near the end of the left arm) but not the TIR, to find out which *Dra*I fragments were linked with the 30 kb *Dra*I fragment in the strains M3-1 and M1-107 and the *Dra*I-linkage in the strain M1-48.

4: RESULTS II – Acarbose Biosynthesis

4.1: Analysis of the genome of *Actinoplanes* sp. SE50/110 by PFGE

The biosynthetic gene cluster for the production of acarbose (*acb*-cluster) in *Actinoplanes* sp. SE 50/110 has been identified, cloned and sequenced (Stratmann, 1997; Diaz Guardamino, 2000; Thomas, 2001; M. Jarling, p.c.; U. Wehmeier, p.c.). It covers about 35 kb of chromosomal DNA and encompasses 25 ORFs (**Fig.** 1.6). Two strategies were employed in order to clone the whole *acb*-cluster on a suitable plasmid vector. One strategy was to clone the whole cluster directly by random shot-gun cloning. The genomic DNA of *Actinoplanes* sp. SE50/110 was partially digested with *Sau*3AI and then cloned into an *E. coli* - *Streptomyces* shuttle cosmid pOJ446 (Bierman *et al*., 1992). Various gene probes were used to screen the cosmid bank for clones which may contain the whole biosynthesis cluster (Thomas, 2001). The second strategy used in this work was to locate the whole cluster on the genome of *Actinoplanes* sp. SE50/110 by pulsed field gel electrophoresis (PFGE). Then a desired DNA fragment of a manageable size could be cloned, using specific restriction endonucleases, into a suitable vector.

In order to locate the *acb*-cluster on the genome of *Actinoplanes* sp. SE50/110, Southern hybridization was carried out against PFGE patterns created by various restriction endonucleases (**Fig.** 4.1 A) and probed with the *acbC* gene (**Fig.** 4.1 B). The size of *Ase*I and *Dra*I fragments is summarized in **Tab.** 4.1. The length of these fragments was evaluated from several independent PFGE experiments using different running programs (data not shown). From these data, the genome size of *Actinoplanes* sp. SE50/110 was estimated to be of about 6.5 Mb, less than that of *Streptomyces lividans* (about 8 Mb, Leblond *et al.*, 1993) and *Streptomyces coelicolor* A3(2) M145 (8.67 Mb, http://www.sanger.ac.uk/Projects/S_coelicolor/). The hybridization results are summarized in **Tab.** 4.2. These data indicated that only one copy of the *acb*-cluster was present in *Actinoplanes* sp. SE50/110, unlike that the *lmb*/*lmr*-cluster was duplicated in the industrial strain *S. lincolnensis* 78-11, resulting in enhanced lincomycin production (Peschke *et al.*, 1995).

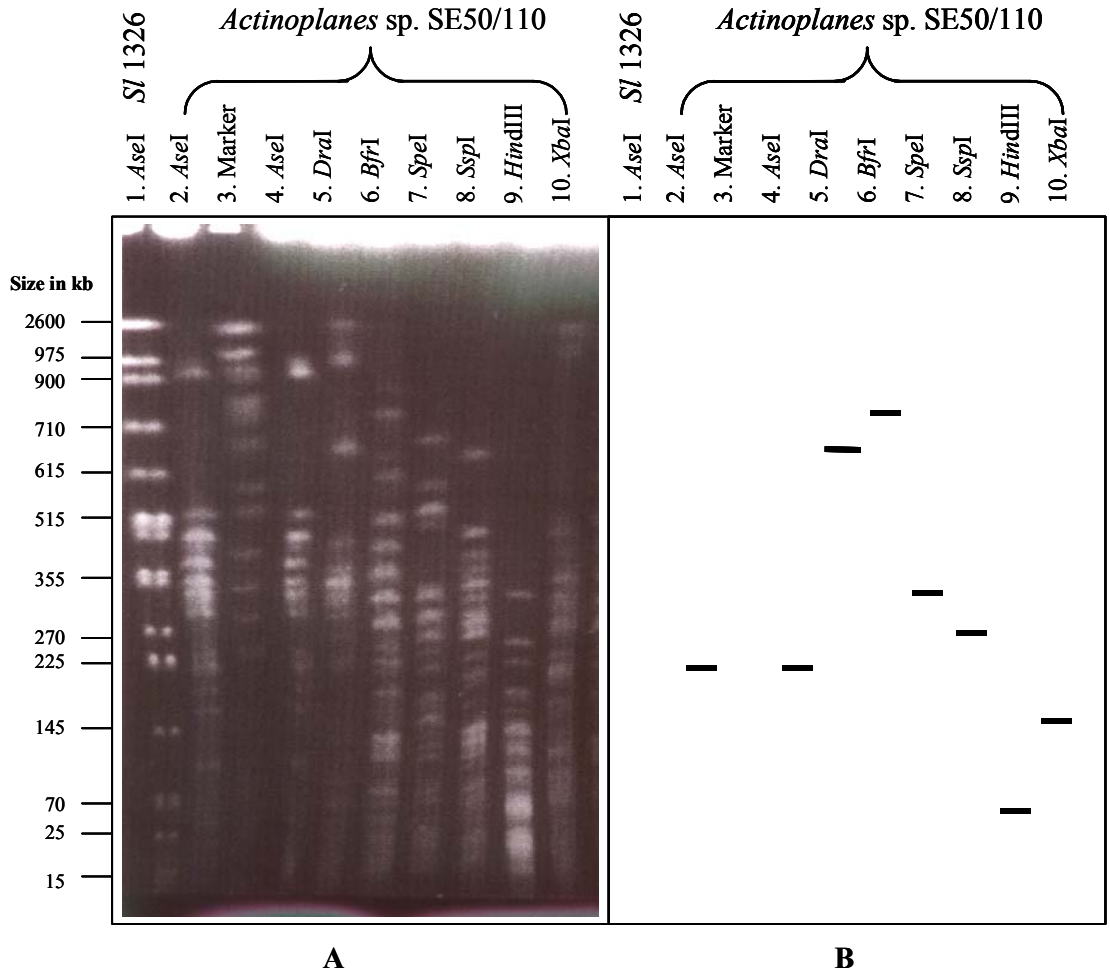


Fig. 4.1: PFGE analysis of the genome of *Actinoplanes* sp. SE50/110 hydrolyzed with various restriction endonucleases. A: The PFGE was performed at 180 V for 24 h with changing pulse times: 10 s for 6 h, 40 s for 6 h, 60 s for 6 h and 100 s for 6 h. **B:** Schematic diagram of the corresponding southern analysis using the *acbC* probe (1.3 kb *Eco*RI/*Hin*dIII fragment from pAS8/5.1, Stratmann *et al.*, 1999). *Lane* 3 is the Yeast DNA-PFGE marker. The *Ase*I pattern generated from *S. lividans* 1326 (*lane* 1) served also as a size marker (after Leblond *et al.*, 1993).

Tab. 4.1: Sizes of *Ase*I and *Dra*I fragments of the genome of *Actinoplanes* sp. SE50/110

*Ase*I		*Dra*I	
No.	Size (kb)	No.	Size (kb)
A	930	1	2200?
B	530	2	1025
$C_{1,2}$	470	3	650
D	450	4	470
$E_{1,2}$	405	5	395
$F_{1,2}$	363	6	365
$G_{1,2}$	338	7	365
H	308	8	320
I	230	9	264
J	210	10	225
K	180	11	140
L	150	12	90
M	100	13	65
N	53		
O	28		
Total	**6,321 kb**		**6,574 kb**

Note: The size of the fragments was estimated by comparison with the yeast chromosome PFGE marker and the *Ase*I fragments of *S. lividans* 1326. The error range was about +/- 10%. Some bands may consist of two different fragments according to the intensity of the fluorescence.

Tab. 4.2: Genomic fragments hybridizing with *acbC* and *acbKML* probes

Enzymes	**Single digestion (size in kb)**	**Double digestion in combinations with**	
		***Hin*dIII (size in kb)**	***Xba*I (size in kb)**
***Dra*I**	650	60	150
***Bfr*I**	750	60	150
***Ssp*I**	270	-	-
***Spe*I**	350	60	150
***Ase*I**	210	30	45
***Xba*I**	150	-	150
***Hin*dIII**	60	60	-

Note: Genomic DNA of *Actinoplanes* sp. SE50/110 was digested with the indicated enzymes and separated by PFGE. In the following hybridizations, the two probes gave identical results. The *acbC* probe was a 1.3 kb *Eco*RI/*Hin*dIII fragment from pAS8/5.1 (Stratmann *et al.*, 1999) and the *acbKML* probe was a 3.1 kb *Eco*RI/*Hin*dIII fragment from pCW21KL (**Tab.** 2.4). -, indicates the double digestion was not tested

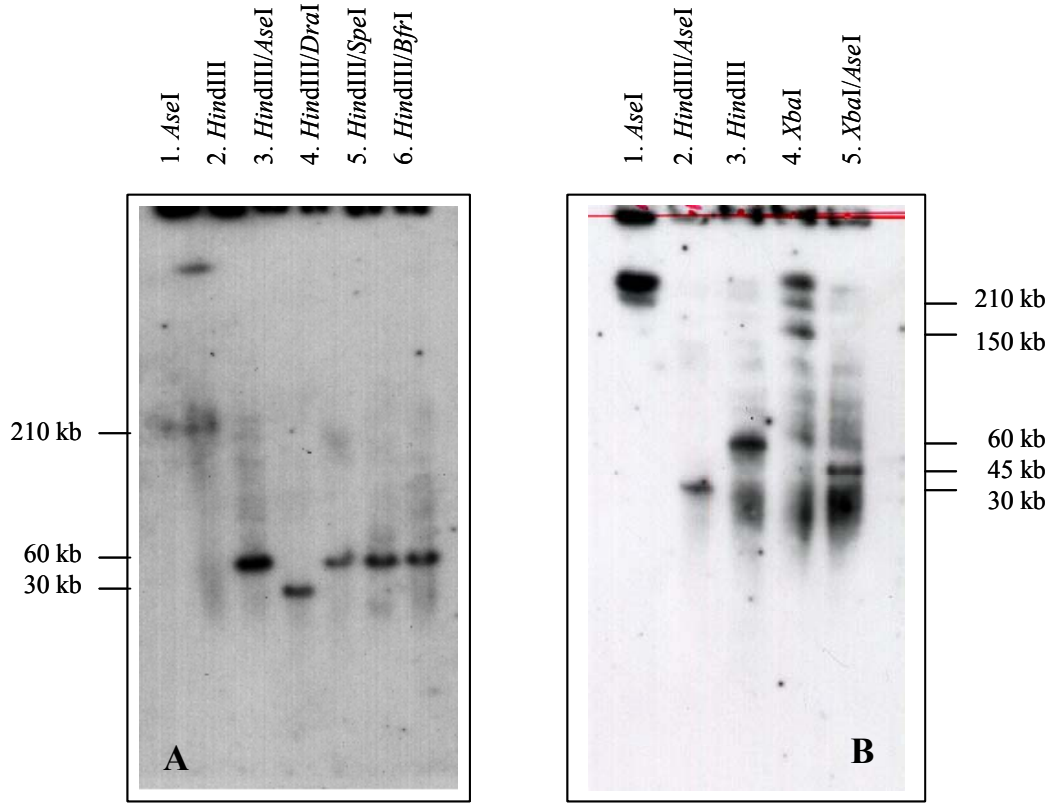


Fig. 4.2: Southern analysis against PFGE patterns created by various restriction endonucleases and probed with *acbKML*. A, PFGE was performed at 180 V for 20 h with changing pulse time: 10 s for 6 h, 20 s for 6 h, 30 s for 6 h and 40 s for 2 h; **B,** PFGE was carried out at 180 V for 12 h: 5 s for 6 h, 10 s for 6 h. The 3.0 kb *Eco*RI/*Hin*dIII DNA fragment from pCW21KL which contained the genes *acbKML* served as a probe.

The sequence contained no *Ase*I, *Xba*I, *Dra*I, *Bfr*I, and *Spe*I sites inside the whole *acb*-cluster, however, a *Ssp*I site and a *Hind*III site were found in the *acb*-cluster (Thomas, 2001; M. Jarling, p.c.; cf. **Fig.** 1.6). The whole *acb*-cluster was located on a 210 kb *Ase*I fragment and a 150 kb *Xba*I fragment (**Tab.** 4.2). These two fragments were too large to be cloned. Therefore, the chromosome of *Actinoplanes* sp. SE50/110 was digested in combinations with two restriction endonucleases and separated by PFGE. A 3.0 kb *Eco*RI/*Hin*dIII DNA fragment from pCW21KL containing the *acbKML* gene (cf. **Tab.** 2.4) was used as a probe in the following Southern hybridization. It was found that *Ase*I hydrolyzed both the 60 kb *Hind*III and the 150 kb *Xba*I fragments, which resulted in newly hybridizing 30 kb *Hin*dIII/*Ase*I (lane 3 in **Fig.** 4.2 A) and 45 kb *Xba*I/*Ase*I fragments (lane 5 in **Fig.** 4.2 B), respectively. In the *acb*-cluster, there is a *Hin*dIII site 27 kb downstream of the *acbZ* gene (cf. **Fig.** 1.6). Therefore, the *Ase*I site should lie about 3

kb upstream of the start of the *acbZ* gene. The *Xba*I site was assumed to be about 6 kb downstream of the *acbH* gene. In fact, this *XbaI* site was found in the sequence in that area (U. Wehmeier, p.c.). This meant that the whole *acb*-cluster was located on a 45 kb *Xba*I/*Ase*I fragment in *Actinoplanes* sp. SE50/110, as depicted in **Fig.** 4.3.

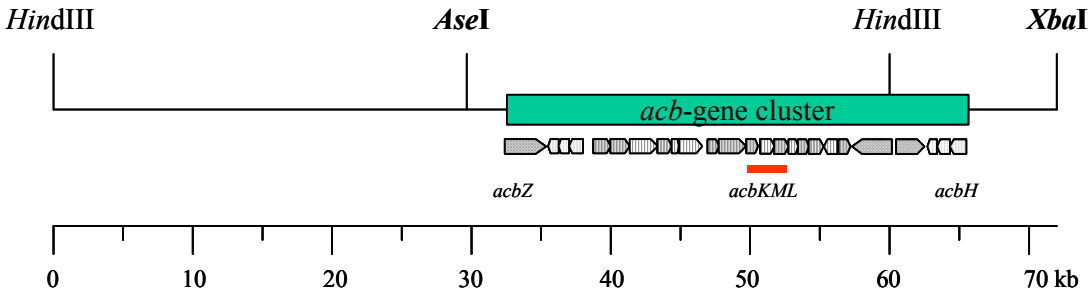


Fig. 4.3: Physical map of the location of the *acb*-cluster in the chromosome of *Actinoplanes* sp. SE50/110. The *acb*-gene cluster was represented by a rectangle (for the corresponding *acb*-genes see **Fig.** 1.6). The location of the *acbKML* probe is indicated by a small line.

This 45 kb *Xba*I/*Ase*I fragment was a manageable DNA fragment which could be cloned into a suitable vector such as cosmid pOJ446 (Bierman *et al*., 1992) after elution from the pulsed field gel. In a parallel work, a cosmid clone pHTWCos6 obtained by shot gun cloning was shown to contain the whole *acb*-cluster (Thomas, 2001). Therefore, no further attempt was made to clone the 45 kb *Xba*I/*Ase*I fragment.

4.2: Heterologous expression of the AcbK, M, L, N, O and AcbU proteins

4.2.1: The AcbK, AcbM, AcbL, AcbN, AcbO and AcbU proteins

Some genes of the *acb*-cluster had already been characterized experimentally (**Sect.** 1.7). The *acbC* gene has been shown to encode a C7-cyclitol synthase which catalyzes the conversion of sedo-heptulose-7-phosphate to 2-*epi*-5-*epi*-valiolone (Stratmann *et al.*, 1999). The *acbV* gene encodes a 6-deoxysugar aminotransferase (Diaz Guardamino, 2000) and the *acbD* gene encodes an extracellular acarbose-modifying glycosyl-transferase (Hemker *et al.*, 2001). However, the functions of most of the other Acb-proteins were still unclear. Therefore, it should be further investigated what roles they play in the biosynthesis and metabolism of acarbose. In contrast to the pathway for the biosynthesis of the 4-amino-4,6-didesoxysugar moiety of acarbose

(Stratmann, 1997; Diaz-Guardamino, 2000), there was little additional knowledge on the pathway leading to the formation of the unsaturated cyclitol moiety of acarbose, valienol (or valienamine). Therefore, the next goal of this work was to further elucidate several steps in the conversion of 2-*epi*-5-*epi*-valiolone during the biosynthesis of the acarviosyl moiety of acarbose on the biochemical level. Based on the comparisons of the deduced protein sequences with the gene bank, the genes *acbKMLNO*, coupled with the cyclase gene *acbC* in a putative operon (cf. **Fig.** 1.6, for sequence see **App.** 7.1), were investigated in this work. These genes were the most likely candidates which could encode at least some of the enzymes involved in the biosynthesis of the cyclitol moiety.

Tab. 4.3: *acb*-genes putatively involved in the biosynthesis of the cyclitol moiety of acarbose

Gene	aa [1]	Closest similarity to protein (accession no.)	COG [2]	Postulated function
acbK	300	Urf2 *Streptomyces* sp. (U08602)	0524	acarbpse-7-kinase
acbM	359	GlcK *Bacillus subtilis* (P54495)	1940	polyol kinase
acbL	366	SCF91.05c *Streptomyces coelicolor* (Q9RJB9)	1062	dehydrogenase?
acbN	257	SC9A4.23c *Streptomyces coelicolor* (AL391072)	1028	oxidoreductase?
acbO	270	no significant similarity		unknown
acbU	491	Pep2 *Streptomyces coelicolor* (AJ001205)		unknown (kinase?)

[1] : aa = coding capacity in number of amino acid residues;
[2]: COG = cluster of orthologous proteins in genomes (Tatusov *et al.*, 2001)

Sequence analysis had shown that the *acbK* gene encoded a protein which had the identical first 20 amino acids as the acarbose 7-phosphotransferase discovered and purified from the crude extracts of *Actinoplanes* sp. (Goeke *et al.*, 1996; Drepper and Pape, 1996). Therefore, the AcbK protein was designated as the acarbose 7-kinase (**Tab.** 4.3). The deduced AcbK protein sequence shows highly similarity (57.9% identity in 297 aa overlap) to Urf2 protein, a putative kinase from *Streptomyces* sp. (Chen *et al.*, 1995). Also, AcbK exhibits significant similarity to members of the ribokinase family of phosphotransferase (COG0524, **Tab.** 4.3; **App.** 7.3). The AcbM protein sequence is distantly related to members of glucokinase family (COG1940, **Tab.** 4.3, **App.** 7.4). The protein sequence of AcbL is significantly similar to that of the protein SCF91.05c (30.1% identity in 316 aa overlap) from *Streptomyces coelicolor* (Redenbach *et al.*, 1996), which belongs members of group I long-chain zinc-dependent alcohol dehydrogenases (COG1062). Based on the protein sequence comparison, the AcbN is significantly related to members of the short-chain dehydrogenases /reductases family (SDR) (COG1028). The deduced AcbN sequence exhibits 35.3% identity in 258 aa overlap to SC9A4.23c, a putative oxidoreductase of the zinc-dependent

alcohol dehydrogenase family (COG1028), from *Streptomyces coelicolor* (Redenbach *et al.*, 1996). AcbO had no significant similarity to any known protein. The possible function of AcbU has been discussed by Thomas (2001) as a cyclitol kinase.

4.2.2: The general strategy for the expression of the selected Acb-proteins

In order to characterize the enzymes encoded by the proposed genes for the pathway of acarbose biosynthesis, the overexpression of the individual genes in suitable hosts was necessary. The genes were first amplified by PCR from the chromosomal DNA of *Actionoplanes* sp. SE50/110 (**Tab.** 2.8, **Sect.** 2.15). The forward primers were designed for introduction of an *Nde*I site, changing the sequence at the natural start codon for the ability to create start codon fusions of these genes into the promoter/ribosome-binding-site cassettes of the expression vectors. The reverse primers were designed for the introduction of a suitable restriction site located immediately downstream of the stop codon for suitably orientated cloning into the expression vectors. In case of expressing the proteins as C-terminal His-tag forms, the reverse primers were designed to replace the natural stop condon by a *Bgl*II site (cf. **Tab.** 2.5, **Sect.** 2.4). The amplified DNA fragments were cloned into (i) two expression vectors of *E. coli*, pET11aP and pET16bP (N-terminal His-tag fusion protein), under the control of the *T7* promoter; (ii) another pair of expression vectors of *E. coli*, pJOE2702 and pJOE2775 (C-terminal His-tag proteins), under the control of the *rha* promoter; (iii) three *E. coli-Streptomyces* shuttle vectors, pUWL201, pPWW49 and pPWW50, under the control of the *ermE* up promoter and (iv) two *Streptomyces* vector, pIJ6021 and pIJ4123 (N-terminal His-tag fusion protein), under the control of the *tipA* promoter.

4.2.3: Heterologous expression of AcbK, AcbM, AcbN, AcbO and AcbU in *E. coli* K12 and in *S. lividans* 66

A number of plasmids were constructed to express the genes *acbK*, *acbM*, *acbL*, *acbN*, *acbO* and *acbU* in *E. coli* and in *S. lividans*. The cloning of the corresponding genes and the construction of the expression plasmids are described in **Sect.** 2.3 (**Tabs.** 2.3 & 2.4). The results of the expression of the six proteins (AcbK, M, L, N, O and AcbU) are summarized in **Tab.** 4.4.

The AcbK protein was overproduced both (i) as a soluble native protein in *E. coli* BL21 (DE3) pLysS/pCWK11 and (ii) a soluble N-terminal His-tag protein in *E. coli* BL21 (DE3) pLysS/pCWK16. Also, AcbK was well produced in *E. coli* JM109/pCW2072K as a soluble

native protein (**Tab.** 4.4). The genes *acbKML* were translationally coupled (**Fig.** 1.6; **App.** 7.1). Therefore, plasmids pCW201KM6 and pCW201KL were constructed originally for the expression of AcbM and AcbL as C-terminal His-tag proteins. In these cases, the expression of AcbK served as an easily detectable indicator to be able to optimize the culture conditions for the expression of AcbM and AcbL. Soluble AcbK protein was overproduced in *S. lividans* TK23/pCW201KM6 (lanes 2 and 5 in **Fig.** 4.4 A). In case of *S. lividans* TK23/pCW201KL, AcbK protein was produced only in the form of insoluble aggregates (lanes 3 and 4 in **Fig.** 4.4 A).

When using *E. coli* BL21 (DE3) pLysS as a host for expression, His-tag AcbM (N- or C-terminal) was overproduced but always in insoluble forms, independently of the expression vectors that was used (**Tab.** 4.4). AcbM was successfully overproduced in *S. lividans* TK64/pCW4123M (lane 5 and lane 6 in **Fig.** 4.4) as partially soluble N-terminal His-tag protein (lane 3 and lane 4 in **Fig.** 4.4 B; lane 3 in **Fig.** 4.5). The AcbL protein could be expressed in various recombinant clones: (i) in *E. coli* BL21 (DE3) pLysS/pCWL16 (N-terminal His-tag), (ii) in *E. coli* BL21 (DE3) pLysS/pCW11aL (C-terminal His-tag), and (iii) in *S. lividans* 1326/pMJL6, but in all cases only the insoluble protein was obtained. In contrast, soluble C-terminal His-tag AcbL was weakly produced in *S. lividans* TK23 /pCW201KL, which was only detectable by Western blot assays using anti-His-tag antibodies (lane 9 in **Fig.** 4.5). The N-terminal His-tag AcbL protein was successfully overproduced in *S. lividans* TK64/pCW4123L (lane 7 in **Fig.** 4.4 B) and partially soluble (lane 2 in **Fig.** 4.4 B; lanes 4-6 in **Fig.** 4.5). The AcbN protein was expressed in *E. coli* BL21 (DE3) pLysS harboring the plasmids (i) pMJN2 (native), (ii) pCWN16 (N-terminal His-tag) and (iii) pCW11aN (C-terminal His-tag), as well as in *S. lividans* TK23 containing the plasmids pMJN4 (native, lane 2 in **Fig.** 4.4 C) and pMJN5 (N-terminal His-tag, lane 1 in **Fig.** 4.4 C; lane 7 in **Fig.** 4.5). But all these expression systems yielded only partially soluble AcbN proteins. The AcbO protein was expressed in *E. coli* BL21 (DE3) harboring the plasmids (i) pCWO16 as N-terminal His-tag protein and (ii) pCW11aO as C-terminal His-tag protein and also (iii) in *S. livdans* TK64/pMJO7 as partially soluble N-terminal His-tag protein (lanes 2 & 3 in **Fig.** 4.4 D; lane 2 in **Fig.** 4.5). The AcbU protein was well expressed as N-terminal His-tag protein in *S. livdans* TK64/pCW4123U and was partially soluble (lane 1 in **Fig.** 4.4 E). The expressed His-tag proteins were also analyzed by Western blot assays using anti-His-tag antibodies (**Fig.** 4.5).

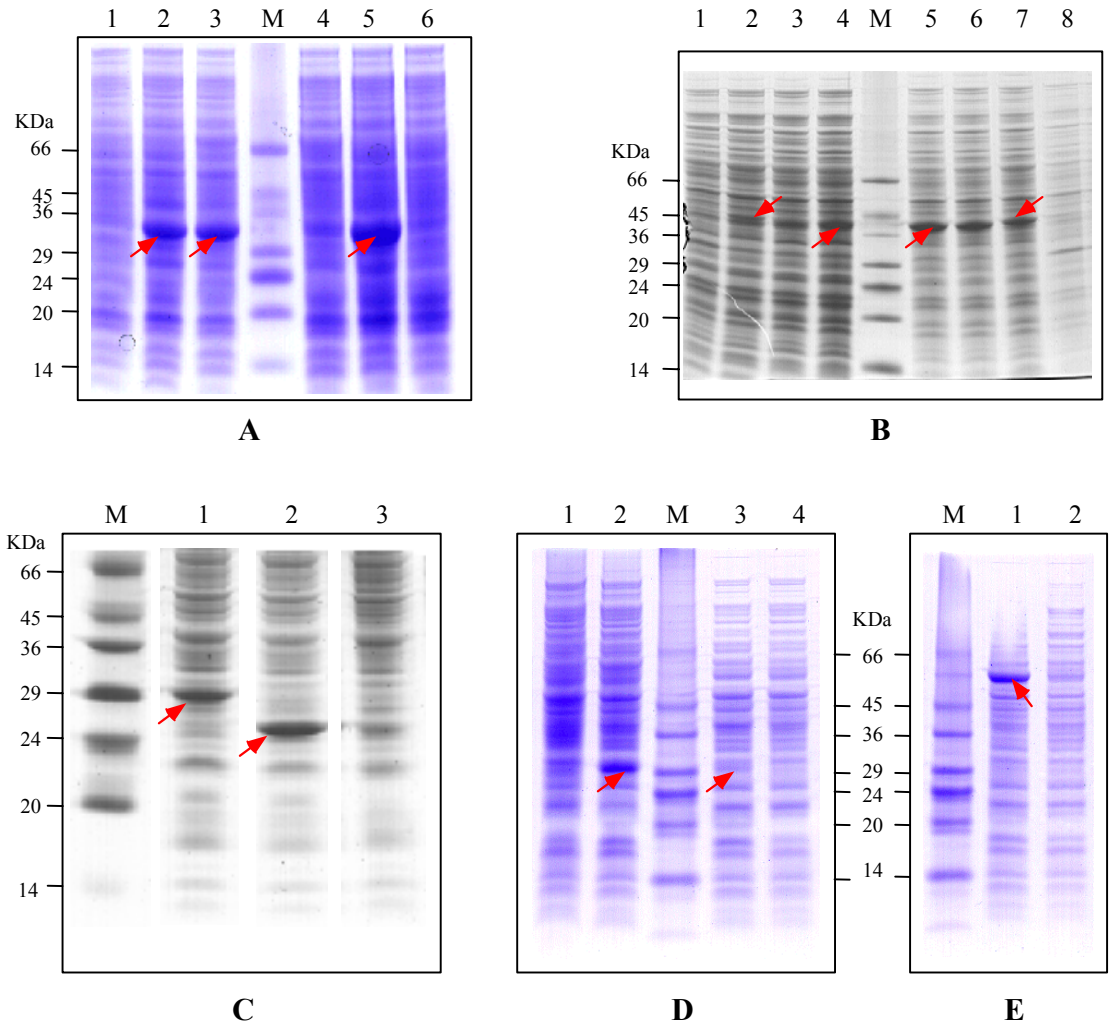


Fig. 4.4: SDS-PAGE analysis of the production of the proteins AcbK, AcbM, AcbL, AcbN, AcbO and AcbU in *S. lividans*. Electrophoresis was run in a 10% protein gel. **A: The production of AcbK in *S. lividans* TK23.** Cell extracts were obtained from *S. lividans* TK23 harboring the following plasmids: pUWL201 (control, *lanes* 1 and 6), pCW201KM6 (**AcbK**, *lanes* 2 and 5), pCW201KL (**AcbK**, *lanes* 3 and 4). *Lanes* 1-3 contained the crude cell extracts and *lanes* 4-6 contained the supernatants (soluble proteins). **B: The production of AcbM and AcbL in *S. lividans* TK64.** Cell extracts were from strains harboring the following plasmids: pIJ4123 (control, *lanes* 1 and 8), pCW4123L (N-terminal His-tag **AcbL**, *lanes* 2 and 7), pCW4123M (N-terminal His-tag **AcbM**, *lanes* 3-6). *Lanes* 1-4, the supernatants (soluble proteins); *lanes* 5-8, crude cell extracts. **C: The production of AcbN in *S. lividans* TK23.** Cell extracts were from strains harboring the following plasmids: pMJN5 (N-terminal His-tag **AcbN**, *lane* 1), pMJN4 (**AcbN**, *lane* 2), pUWL201 (control, *lane* 3). **D**: **The production of AcbO in *S. lividans* TK64.** Cell extracts were from strains harboring the following plasmids: pIJ4123 (control, *lanes* 1 and 4), pMJO7 (N-terminal His-tag **AcbO**, *lanes* 2 and 3). *Lanes* 1 and 2, crude cell extracts; *lanes* 3 and 4, supernatants (soluble proteins). **E: The production of AcbU in *S. lividans* TK64.** Soluble cell-free extracts were from strains harboring the following plasmids: pCW4123U (N-terminal His-tag **AcbU**, *lane* 1), pIJ4123 (control, *lane* 2). The molecular masses of the marker proteins (*lanes* M) were indicated. The respective proteins are depicted by arrows.

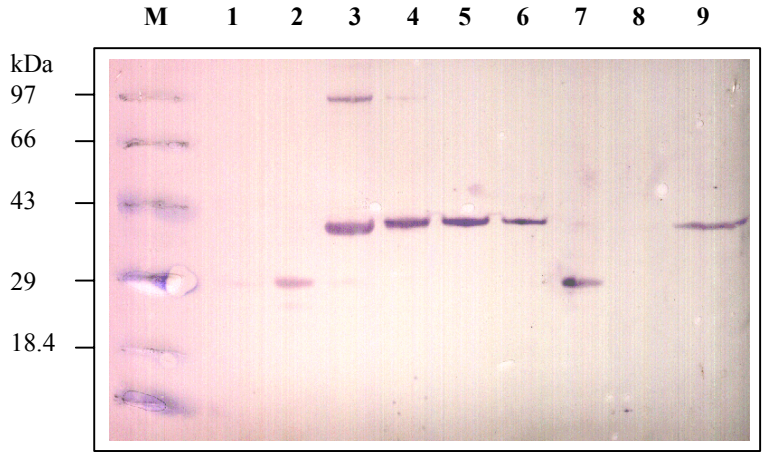


Fig. 4.5: Detection of the His-tag proteins AcbM, AcbL, AcbN and AcbO by Western blotting. Electrophoresis was run in a 10% protein gel. The following Western blotting was performed as described in **Sect.** 2.22. Soluble cell-free extracts were from *S. lividans* harboring the following plasmids: pIJ4123 (*lane* 1, control), pMJO7 (N-terminal His-tag **AcbO**, *lane* 2), pCW4123M (N-terminal His-tag **AcbM**, *lane* 3), pCW4123L (N-terminal His-tag **AcbL**, *lanes* 4 - 6), pMJN5 (N-terminal His-tag **AcbN**, *lane* 7), pCW201KM6 (C-terminal His-tag **AcbM**, *lane* 8), pCW201KL (C-terminal His-tag **AcbL**, *lane* 9)**.** The molecular masses of the marker proteins (*lane* M) were indicated.

Tab. 4.4: Summary of the expression of AcbK, M, L, N, O and AcbU

Protein	Plasmid	Vector (Promoter)	Host Strain	Culture Condition	MW (Da)	Solubility [a]
AcbK	pCWK11	pET11aP (*T7*)	*E. coli* BL21 (DE3) pLysS	LB, 28°C	31,709	+
	pCWK16 [b]	pET16bP (*T7*)	*E. coli* BL21 (DE3) pLysS	LB, 28°C	34,228	+
	pCW2072K	PJOE2702 (*rha*)	*E. coli* JM109	LB, 28°C	31,709	+
	pCW201KM6	pUWL201 (*ermE*)	*S. lividans* TK23	YEME, 28°C	31,709	+
	pCW201KL	pUWL201 (*ermE*)	*S. lividans* TK23	YEME, 28°C	31,709	-
AcbM	pCWM16 [b]	pET16bP (*T7*)	*E. coli* BL21 (DE3) pLysS	LB, 28°C	39,510	-
	pCW11aKM [c]	pET11a (*T7*)	*E. coli* BL21 (DE3) pLysS	LB, 28°C	37,708	-(WB)
	pCW4123M [b]	pIJ4123 (*tipA*)	*S. lividans* TK64	YEME, 28°C	39,143	±
AcbL	pCWL16 [b]	pET16bP (*T7*)	*E. coli* BL21 (DE3) pLysS	LB, 28°C	40,187	-
	pCW11aL [c]	pET11a (*T7*)	*E. coli* BL21 (DE3) pLysS	LB, 28°C	38,634	-
	pCW201KL [c]	pUWL201 (*ermE*)	*S. lividans* TK23	YEME, 28°C	38,634	+ (WB)
	pMJL6	pIJ6021 (*tipA*)	*S. lividans* 1326	YEME, 28°C	37,668	-
	pCW4123L [b]	pIJ4123 (*tipA*)	*S. lividans* TK64	YEME, 28°C	39,830	±
AcbN	pMJN2	pET11aP (*T7*)	*E. coli* BL21 (DE3)	LB, 28°C	26,518	±
	pCWN16 [b]	pET16bP (*T7*)	*E. coli* BL21 (DE3) pLysS	LB, 28°C	29,037	±
	pCW11aN [c]	pET11a (*T7*)	*E. coli* BL21 (DE3) pLysS	LB, 28°C	27,484	±
	pMJN4	pPWW49 (*ermE*)	*S. lividans* TK23	YEME, 28°C	26,518	±
	pMJN5 [b]	pPWW50 (*ermE*)	*S. lividans* TK23	YEME, 28°C	29,037	±
AcbO	pCWO16 [b]	pET16bP (*T7*)	*E. coli* BL21 (DE3) pLysS	LB, 28°C	30,973	-
	pCW11aO	pET11a (*T7*)	*E. coli* BL21 (DE3) pLysS	LB, 28°C	29,388	-(WB)
	pMJO7 [b]	pIJ4123 (*tipA*)	*S. lividans* TK64	YEME, 28°C	30,616	±
AcbU	pCW4123U [b]	pIJ4123 (*tipA*)	*S. lividans* TK64	YEME, 28°C	55,971	±

[a]: +, soluble; -, insoluble; ±, partially soluble; (WB), detection in Western blot assays. [b]: N-terminal His-tag protein. [c]: C-terminal His-tag protein. Extracts from the strains shown in blue were used for enzyme assays (**Sect.** 4.3 - 4.6).

4.3: Characterization of the AcbK protein and the applications thereof

A phosphotransferase (kinase) which modifies the α-glucosidase inhibitor acarbose by phosphorylation at its C7-position has been isolated from the acarbose producer *Actinoplanes* sp. SN223/29 (Drepper and Pape, 1996). The first 20 amino acids of AcbK were exactly identical to this acarbose-7-phosphotransferase (Stratmann, 1997). This fact led to the conclusion that AcbK was the acarbose 7-phosphotransferase (kinase) and the phosphorylated product was identified as acarbose-7-phosphate (Goeke *et al.*, 1996). The function of AcbK was further characterized herein, using the heterologously overproduced AcbK proteins.

4.3.1: Phosphorylation of acarbose by heterologously expressed AcbK

When cell-free extracts of *E. coli* BL21 (DE3) pLysS harboring plasmids pCWK11 (native AcbK) or pCWK16 (N-terminal His-tag AcbK) were incubated with acarbose and ATP, a new product was detected on TLC (lane 3 and lane 5 in **Fig.** 4.6). The new product had a significantly lower Rf-value (0.57) relative to acarbose in solvent I (**Sect.** 2.23), which was consistent with the Rf-value of acarbose-7-phosphate described by Goeke *et al.* (1996). The new product was not formed in absence of ATP (lane 6 in **Fig.** 4.6) or using extracts of the control strain (lane 4 in **Fig.** 4.6). Therefore, the formation of the new product was dependent both on ATP and AcbK. When the assay was done with the cell-free extracts of *S. lividans* TK23/pCW201KM6 (AcbK), the same result was obtained (data not shown). When acarbose was incubated with [γ-^{32}P] ATP and cell-free extracts of *S. lividans* TK23/pCW201KM6, the formed product was radioactively labeled (lane 2 in **Fig.** 4.7 A). This labeled product was not produced in the control assay (lane 3 in **Fig.** 4.7 A). These data clearly indicated that the new product was due to the phosphorylation of acarbose by AcbK. The AcbK-specific activity was also detected in the cell-free extracts of *Actinoplanes* sp. SE50/110 (lane 1 in **Fig.** 4.7 A).

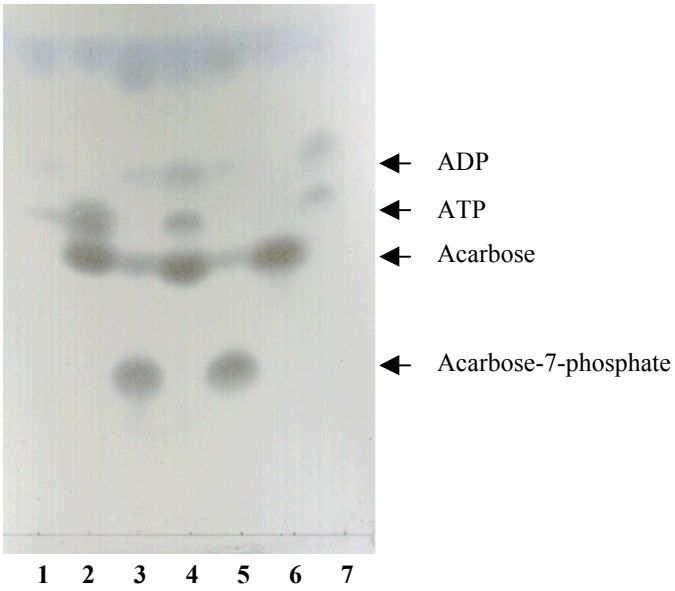


Fig. 4.6: TLC analysis of the phosphorylation of acarbose by the heterologously expressed AcbK. The AcbK reaction was performed as described in **Sect.** 2.25. TLC was developed in solvent I (**Sect.** 2.23). The cell-free extracts used in the reactions were from *E. coli* BL21 (DE3) pLysS harboring the following plasmids: pCWK11 (AcbK), *lanes* 1 - 3; pET11aP (control), *lane* 4; pCWK16 (His-tag AcbK), *lanes* 5 - 7. In *lane*1 and *lane* 7, no acarbose was added; in *lane* 2, the AcbK was inactivated by heating at 95°C for 3 min; in *lane* 6, no ATP was present. The spots were visualized by the Cer-reagent (**Sect.** 2.5.4) and heating. The positions of ATP, ADP, acarbose and acarbose-7-phosphate are indicated by *arrows*.

4.3.2: Phophorylation assays using AcbK and cyclitols

Several chemically synthesized cyclitols were available and used to study their roles in the biosynthesis of the valienamine moiety of acarbose, including valienol, 1-*epi*-valienol, 1-*epi*-5-*epi*-valiolol, 5-*epi*-valiolol and 2-*epi*-5-*epi*-valiolone (cf. **Fig.** 4.8). They all were putative precursors of the valienamine moiety of acarbose and they all share the common structural feature of a C7-hydroxylmethyl group which is the target for the AcbK in acarbose. AcbK assays were carried out using these cyclitols as substrates in both assay systems, using either cold or radioactive ATP. However, none of these cylitols was phosphorylated by AcbK (cf. **Tab.** 5.1).

4.3.3: Heterologous production of acarbose in *S. lividans* 1326/pHTWCos6

A cosmid bank of genomic DNA from *Actinoplanes* sp. SE50/110 has been constructed (Thomas, 2001). A recombinant cosmid, named pHTWCos6, was found to contain the entire *acb*-gene cluster for the biosynthesis of acarbose. AcbD activity was detected in the supernatant of *S. lividans* 1326/pHTWCos6 cultured in MD50 medium (A. Stratmann, p.c.; Thomas, 2001). The

AcbK-dependent activity was also detected in the cell-free extracts of *S. lividans* 1326/ pHTWCos6 (lane 4 in **Fig.** 4.7 A) but the conversion was much less when compared to that of the overproduced AcbK proteins and of cell-free extracts of *Actinoplanes* sp. SE50/110 (**Fig.** 4.7 A). These results led to the working hypothesis that acarbose could be produced heterologously by *S. lividans* 1326/pHTWCos6, due to the expression of biosynthetic Acb-proteins.

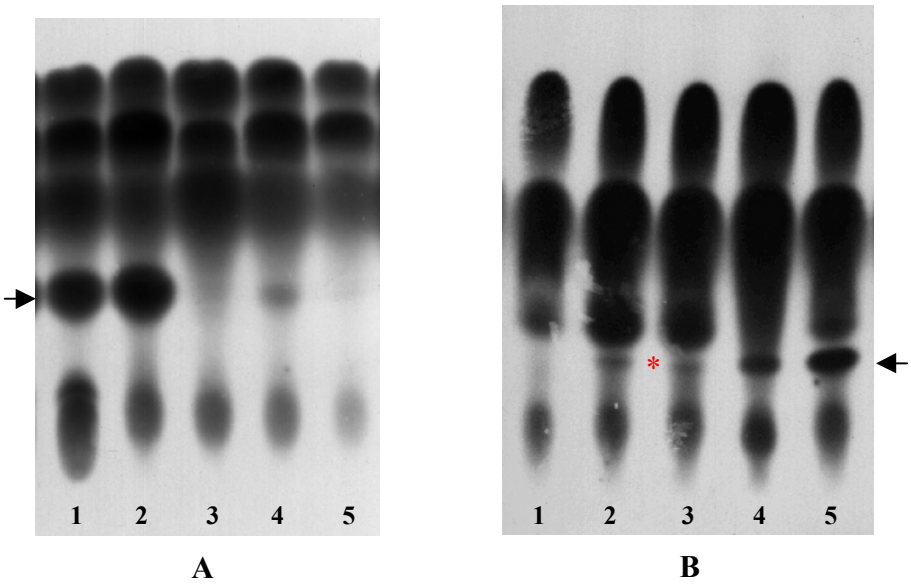


Fig. 4.7: Detection of the AcbK activity and the production of acarbose in *S. lividans*1326/pHTWCos6. The assays were performed with [γ-^{32}P] ATP as described in **Sect.** 2.25. TLCs were developed in solvent I (**Sect.** 2.23). TLC plates were autoradiographed at –70°C for 1 h (A) or 6 h (B). **A:** cell-free extracts were from *Actinoplanes* SE50/110 (*lane* 1), *S. lividans* TK23/pCW201KM6 (*lane* 2), *S. lividans* TK23/pUWL201 (*lane* 3), *S. lividans* 1326/pHTWCos6 (*lane* 4) and *S. lividans* 1326 (*lane* 5). **B:** supernatants of cell culture media from *S. lividans* 1326/pHTWCos6 (*lanes* 1 - 3) and *Actinoplanes* sp. SE50/110 (*lane* 5) served as the "substrate" for AcbK. In *lane* 3 the culture media were cooked at 100°C for 3 min. Cell-free extracts from *S. lividans* TK23/pUWL201 (*lane* 1, control) or from *S. lividans* TK23/pCW201KM6 (*lanes* 2 - 5, AcbK) were used in the assays. In *lane* 4 acarbose was added as the substrate. The positions of acarbose-7-phosphate are indicated by arrows. Phosphorylation of the new compound produced in *S. lividans* 1326/pHTWCos6 by AcbK is indicated with a star (*).

Based on the idea that the final product acarbose was secreted, the supernatant from the culture of *S. lividans* 1326/pHTWCos6 was used as the "substrate" for AcbK. A radioactively labeled product having the same mobility as acarbose-7-phosphate was detected when the culture medium of *S. lividans* 1326/pHTWCos6 was incubated with AcbK (cell-free extracts of *S. lividans* TK23 /pCW201KM6) and [γ-^{32}P] ATP (lane 2 and lane 3 in **Fig. 4.7** B). The formation of this labeled product required AcbK (lane 1 in **Fig. 4.7** B). When the culture medium of *S.*

lividans 1326 was used in the AcbK assay, this labeled product was not obtained (data not shown). These results led to the conclusion that: (i) a new product was produced by *S. lividans* 1326/pHTWCos6, (ii) this product could be phosphorylated by AcbK, (iii) the phosphorylated product had the same mobility as acarbose-7-phosphate in the TLC system used. These facts provide the first evidence that a new product, either acarbose or a derivative of acarbose, is heterologously produced in *S. lividans*.

4.4: Phosphorylation of 1-*epi*-valienol by cell-free extracts of *Actinoplanes* sp. SE50/110

To test for the hypothesis that the other enzymes responsible for the biosynthesis of acarbose should be also active, similar to AcbK, in the crude extract of *Actinoplanes* sp. and that they could catalyze the conversion of their corresponding substrates, various chemically synthesized cyclitols (**Fig.** 4.8; Block, 2000) were tested whether they could be converted by cell-free extracts of *Actinoplanes* sp. SE50/110 or SN223/29.

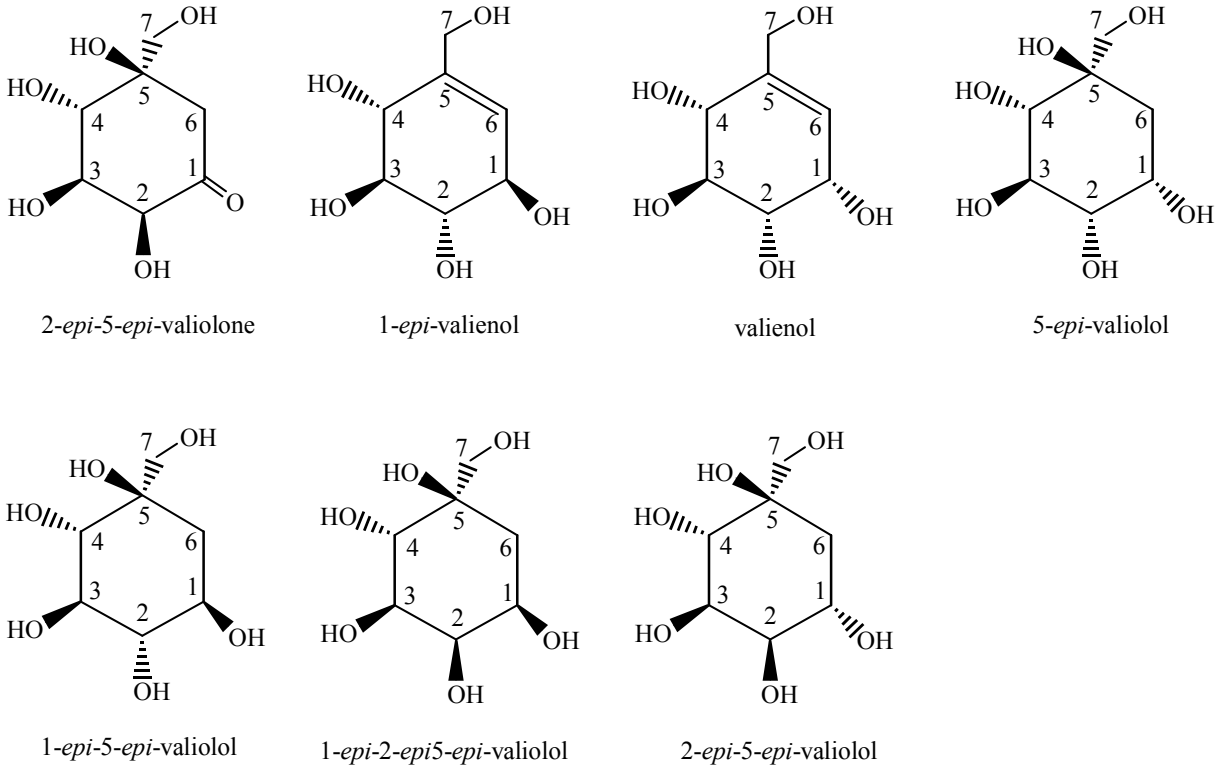


Fig. 4.8: Structure of the chemically synthesized cyclitols used in this work.

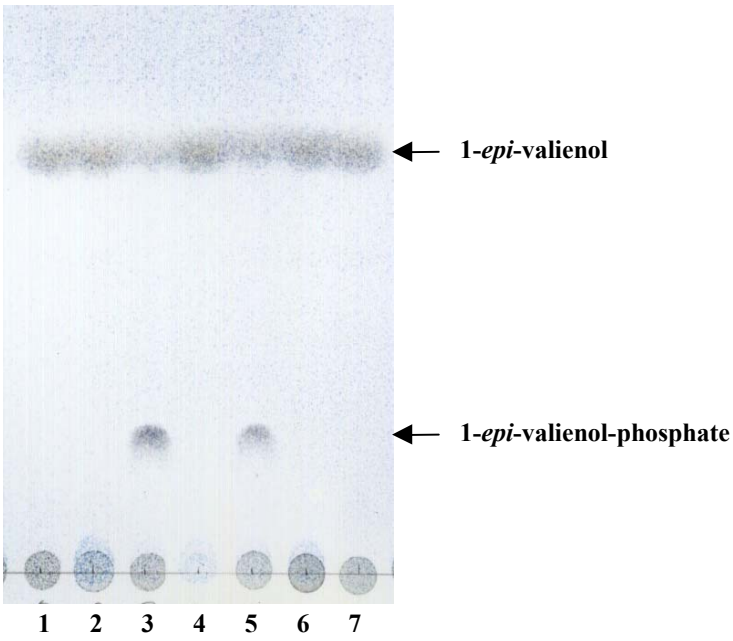


Fig. 4.9: TLC analysis of the conversion of 1-*epi*-valienol in the presence of ATP. The assays were performed as described in **Sect.** 2.26. TLC was developed in solvent II (**Sect.** 2.23). Cell-free extracts used in the assays were from *Actinoplanes* sp. SN223/29 (*lanes* 1 - 3) and SE50/110 (*lanes* 5 - 7). In *lane* 1 and *lane* 7 no ATP was present in the corresponding assays; in *lane* 2 and *lane* 6 the cell-free extracts were cooked at 100°C for 3 min; *lane* 3 and lane 5 represented the standard assay; *lane* 4, 1 µl 1-*epi*-valienol (100 mM). The positions of 1-*epi*-valienol and the conversion product are indicated.

When 1-*epi*-valienol was incubated with ATP and cell-free extract from *Actinoplanes* sp. SE50/110, a new product with a significantly lower mobility (Rf = 0.32, relative to 1-*epi*-valienol in solvent II) was detected on the TLC plate. As the chemically synthesized 1-*epi*-valienol was a racemic mixture, the conversion to the new product was only about 50%. The same result was obtained when the cell-free extract from *Actinoplanes* sp. SN223/29 was used. Further evidences showed that the formation of the new product was dependent on ATP (**Fig.** 4.9), indicating a phosphorylation of 1-*epi*-valienol. Therefore, the radioactive assay system with [γ-^{32}P] ATP was also employed. The product of 1-*epi*-valienol was indeed radioactively labeled (**Fig.** 4.10). This clearly showed that 1-*epi*-valienol was phosphorylated under these assay conditions. Other cyclitols, 2-*epi*-5-*epi*-valiolone, 1-*epi*-5-*epi*-valiolol, 5-*epi*-valiolol, 2-*epi*-5-*epi*-valiolol, 1-*epi*-2-*epi*-5-*epi*-valiolol and valienol (cf. **Fig.** 4.8) were also tested in assays using [γ-^{32}P] ATP. Of these cyclitols, valienol was also weakly phosphorylated, but no phosphorylation of the other tested cyclitols was detected (**Fig.** 4.10). From these results, it was concluded that, besides the overall structure, the sterochemistry at the C1-position of 1-*epi*-valienol played a crucial role for

this phosphorylation. These data hinted at a phosphorylation at C-1 and, therefore, the newly formed product most likely was 1-*epi*-valienol-1-phosphate. Because no synthesized 1-*epi*-valienol-1-phosphate was available as a standard, the real structure of this new product remained unclear.

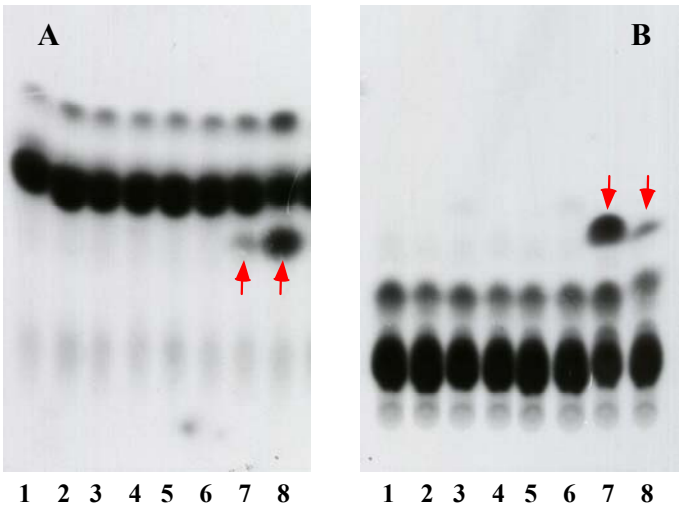


Fig. 4.10: Phosphorylation of 1-*epi*-valienol. Assays were done with [γ-^{32}P] ATP as described in **Sect.** 2.26. TLCs were run twice in solvent I (**A**) or solvent II (**B**). TLC plates were autoradiographed at room temperature for 2 h. Cell-free extracts were from *Actinoplanes* sp. SN223/29. The following substrates were used in the assays: *lane* 1, no substrate; *lane* 2, 1-*epi*-2-*epi*-5-*epi*-valiolol; *lane* 3, 2-*epi*-5-*epi*-valiolol; *lane* 4, 2-*epi*-5-*epi*-valiolone; *lane* 5, 5-*epi*-valiolol; *lane* 6, 1-*epi*-5-*epi*-valiolol; *lane* 7, valienol (**A**) or 1-*epi*-valienol (**B**); *lane* 8, 1-*epi*-valienol (**A**) or valienol (**B**). The conversion products of 1-*epi*-valienol and valienol are indicated by arrows.

A number of enzyme assays with various test conditions (dehydration, reduction, epimerization or phosphorylation) including chemically synthesized cyclitols (cf. **Fig.** 4.8) and combinations of the coenzymes ATP, NAD(P)$^+$, NAD(P)H, FAD$^+$, $FADH_2$, were performed using cell-free extracts of *Actinoplanes* sp. SE50/110 or SN223/29. However, no visible conversion of the cyclitols, 2-*epi*-5-*epi*-valiolone, 1-*epi*-5-*epi*-valiolol, 5-*epi*-valiolol, 2-*epi*-5-*epi*-valiolol, and 1-*epi*-2-*epi*-5-*epi*-valiolol was detected. Further experiments were also carried out to identify whether 1-*epi*-valienol was a real intermediate of the acarbose biosynthesis pathway. However, no conversion of 1-*epi*-valienol was detected using cell free extracts of *S. lividans* 1326/pHTWCos6 or the heterologously overexpressed Acb-proteins, AcbK, AcbM, AcbL, AcbN, AcbO and (data not shown). Therefore none of these proteins was responsible for the phosphorylation of 1-*epi*-valienol. Assays were also made with [^{14}C] ATP using cell-free extracts of *Actinoplanes* sp., but no further modification (adenylation) of 1-*epi*-valienol-phosphate was detected (data not shown). A number of attempts were also made in order to convert the known

precursor 2-*epi*-5-*epi*-valiolone to the assumed product 1-*epi*-valienol-1-phosphate, but no positive result could be achieved. Therefore, it remained unclear if 1-*epi*-valienol was a real intermediate for the biosynthesis of acarbose.

4.5: Enzyme-catalyzed conversion of 2-*epi*-5-*epi*-valiolone

2-*epi*-5-*epi*-valiolone has been shown to be the enzymatic product of the cyclase AcbC and was incorporated into acarbose in feeding experiments in *Actinoplanes* sp. (Stratmann *et al.*, 1999; Mahmud *et al.*, 1999; Dong *et al.*, 2001). Therefore, this work was focused on the enzymatical conversion of 2-*epi*-5-*epi*-valiolone using the selected Acb-proteins overproduced in *S. lividans* (**Tab.** 4.4). The conversion of 2-*epi*-5-*epi*-valiolone to the unsaturated cyclitol moiety of acarbose was assumed to require the following reaction steps in unknown order: (i) dehydration between C-5 and C-6, (ii) reduction of the C-1 keto group and (iii) epimerization at C-2. The deduced protein sequences of AcbL and AcbN exhibited similarities to dehydrogenase-related oxidoreductases (**Tab.** 4.3, **App.** 7.3 & 7.4). Hence they were the most likely candidates which might be responsible for the postulated steps (i) and (ii). Numerous enzyme assays were carried out with AcbL and/or AcbN in attempts to convert 2-*epi*-5-*epi*-valiolone in the presence of NAD^+, NADH, $NADP^+$, NADPH, FAD^+ or $FADH_2$ as coenzymes. However, no conversion was detected in TLC or in photometric analyzes (data not shown). As only small amounts of chemically synthesized 2-*epi*-5-*epi*-valiolone were available for these tests, it was necessary to synthesize this precursor cyclitol first. For this purpose, an enzymatical approach was employed.

4.5.1: Enzymatical synthesis and purification of 2-*epi*-5-*epi*-valiolone

The C7-cyclitol precursor 2-*epi*-5-*epi*-valiolone was biochemically synthesized using a coupled reaction with transketolase (EC: 2.2.1.1) and AcbC as enzymes (**Sect.** 2.27; **Fig.** 4.11). As *sedo*-heptulose-7-phosphate was not commercially available, it was first generated from hydroxypyruvate and ribose-5-phosphate by use of the transketolase reaction (Schärken, 1997) and then was further converted to 2-*epi*-5-*epi*-valiolone by AcbC (cell-free extracts of *S. lividans* 1326/pAS8/7, Stratmann *et al.*, 1999). Originally Tris-buffers were used for the transketolase reaction (Schärken, 1997) but these had negative effects on the AcbC activities (data not shown). Therefore, the conditions for this coupled reaction were optimized using phosphate buffer A

(**Sect.** 2.5.4). As shown in **Fig.** 4.12, 2-*epi*-5-*epi*-valiolone was efficiently produced by this method. The two substrates hydroxypyruvate and ribose-5-phosphate were nearly 100% converted to the desired product. The product synthesized enzymatically (lane 5) and chemically (lane 4) had the same mobility in the TLC system (**Fig.** 4.12).

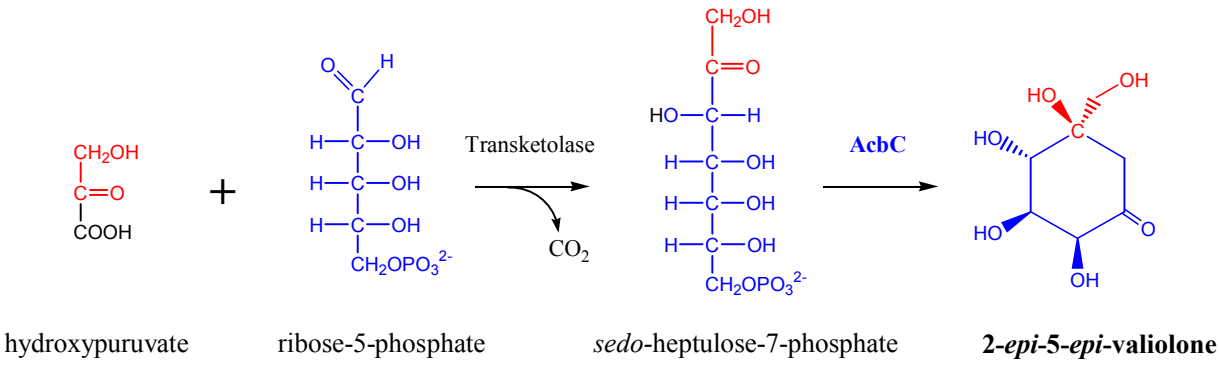


Fig. 4.11: Scheme for the enzymatical synthesis of 2-*epi*-5-*epi*-valiolone.

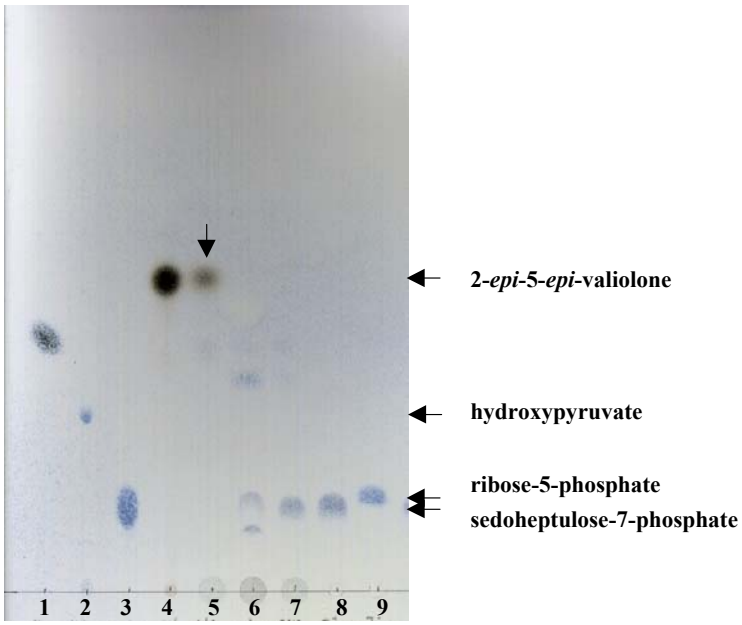


Fig. 4.12: Enzymatical synthesis of 2-*epi*-5-*epi*-valiolone by a coupled reaction with transketolase and AcbC. The reaction was performed in an analytical scale as described in **Sect.** 2.27. TLC was developed in solvent II and visualized using the Cer-reagent (**Sect.** 2.23). *Lane* 1: *sedo*-heptulose; *lane* 2, hydroxypyruvate; *lane* 3: ribose-5-phosphate; *lane* 4: chemically synthesized 2-*epi*-5-*epi*-valiolne (control); *lane* 5: assay with transketolase and cell-free extract of *S. lividans* 1326/pAS8/7 (AcbC); *lane* 6: assay with transketolase and cell-free extract of *S. lividans* 1326/pIJ6021 (control); *lane* 7: assay with transketolase and cooked cell-free extract of *S. lividans* 1326/pAS8/7 (heat-inactivated AcbC); *lane* 8: transketolase assay (without AcbC); *lane* 9: ribose-5-phosphate. Enzymatically produced 2-*epi*-5-*epi*-valiolone is indicated by an arrow.

The coupled reaction was performed preparatively in a total volume of 30 ml containing 10 mM hydroxypyruvate (31.2 mg) and 10 mM ribose-5-phosphate (82.2 mg) as described in **Sect.** 2.27. The resulting product was purified with a Dowex anion-exchange column according to **Sect.** 2.30. After purification, 20 mg of 2-*epi*-5-*epi*-valiolone were obtained as light yellow powder. NMR analysis showed it had identical ^{1}H and ^{13}C spectra as a sample of chemically synthesized 2-*epi*-5-*epi*-valiolone (**Tab.** 4.5, **Sect.** 4.5.4.2). During the purification process, an unknown byproduct with a lower Rf-value (= 0.38 in solvent II) compared to 2-*epi*-5-*epi*-valiolone (Rf = 0.50 in solvent II) was also detected (cf. **Fig.** 4.18 B). Part of 2-*epi*-5-*epi*-valiolone was converted to this unknown compound during freeze-drying. This conversion could be explained by (i) the spontaneous dehydration between C-5 and C-6 of 2-*epi*-5-*epi*-valiolone to give 2-*epi*-valienone due to the acidic condition in Dowex column or by (ii) the hydrtation of C-1 ketone function, which led to a lower recovery (35%, 20 mg relative to 57 mg in theory) of the desired product.

4.5.2: Phosphorylation of 2-*epi*-5-*epi*-valiolone by AcbM

Extracts of *S. lividans* TK23 with pMJN5 (AcbN) and of *S. lividans* TK64 harboring pCW4123M (AcbM), pCW4123L (AcbL) or pMJO7 (AcbO), respectively, were prepared and used in a number of various enzyme assays (dehydration-, reduction-, or epimerization-assays) including chemically synthesized cylitols (cf. **Fig.** 4.8) and combinations of the coenzymes ATP, $NAD(P)^{+}$, NAD(P)H, FAD^{+}, $FADH_2$. In these tests extracts containing a single overproduced Acb-protein and also combinations of the various extracts were analyzed in order to complement for potentially precedent reactions. The enzyme tests were monitored on TLC and also photometrically. In none of the assays a dehydrogenase, epimerase or dehydratase like activity was detected (data not shown). However, when chemically synthesized 2-*epi*-5-*epi*-valiolone was incubated with cell-free extracts including AcbM and ATP as a co-substrate, a new product (Rf = 0.40 relative to 2-*epi*-5-*epi*-valiolone in solvent II) was observed on the TLC and this product was missing especially in assays lacking ATP (lanes 8 and 9, **Fig.** 4.13). Therefore, it was assumed that the new product was 2-*epi*-5-*epi*-valiolone-phosphate. In subsequent assays using [γ-^{32}P] ATP it was shown that indeed the new product was radioactively labeled (lane 3, **Fig.** 4.14). The labeled product was missing in the control assay without AcbM (lane 1, **Fig.** 4.14). This clearly showed that 2-*epi*-5-*epi*-valiolone was phosphorylated. The other cyclitols (2-*epi*-5-*epi*-valiolol, 5-*epi*-valiolol, 1-*epi*-2-*epi*-5-*epi*-valiolol, 1-*epi*-5-*epi*-valiolol, 1-*epi*-valienol and

valienol; **Fig.** 4.8) were also tested for conversion in assays with AcbM-containing extracts. In the presence of [γ-^{32}P] ATP only in assays with 2-*epi*-5-*epi*-valiolol and 1-*epi*-2-*epi*-5-*epi*-valiolol weak radioactively labeled spots were detected on the autoradiogram of the TLC separation (lane 3 and 4, **Fig.** 4.14). In nonradioactive assays these spots were not visible, indicating very low and less specific phosphorylation of these compounds. As the chemically synthesized 2-*epi*-5-*epi*-valiolone consisted of a racemic mixture, only about 50% of the substrate used in the assays was converted (lane 8, **Fig.** 4.13). In order to overcome this problem the enzymatically synthesized 2-*epi*-5-*epi*-valiolone (**Sect.** 4.5.1) was incubated with AcbM-containing extracts and ATP. In these assays the conversion of 2-*epi*-5-*epi*-valiolone to the new phosphorylated spot on the TLC was nearly 100% (lane 5, **Fig.** 4.13). This result clearly demonstrated that the enzymatically produced 2-*epi*-5-*epi*-valiolone was the substrate for this phosphorylation step and AcbM was the 2-*epi*-5-*epi*-valiolone kinase.

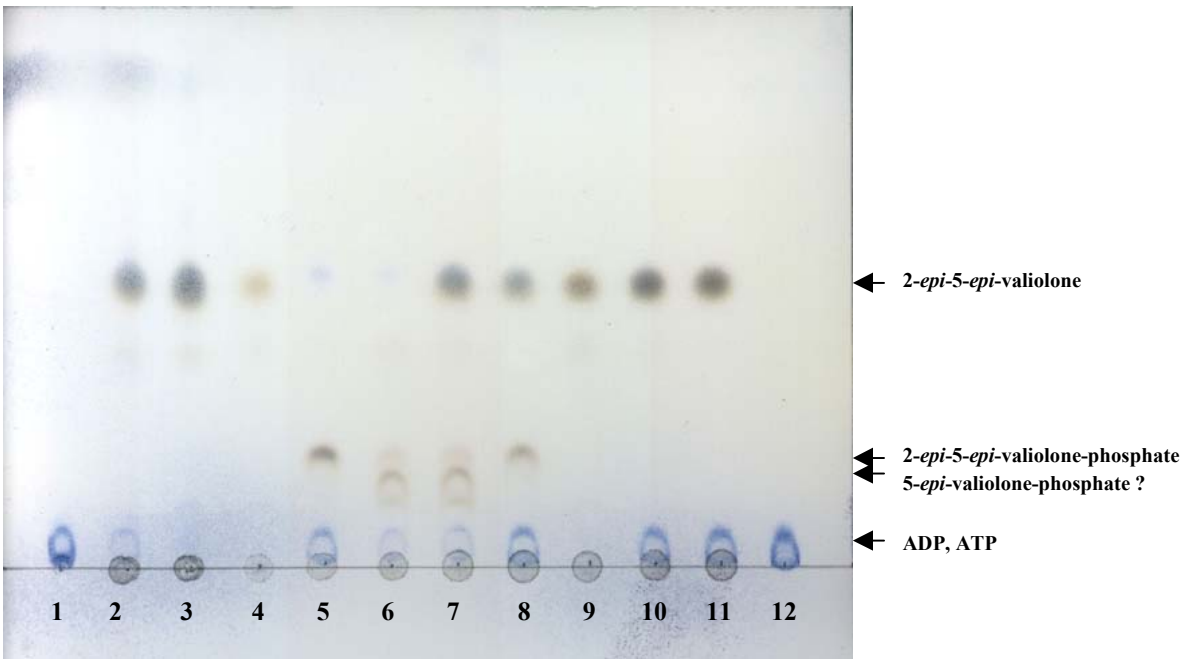


Fig. 4.13: TLC analysis of the conversion of 2-*epi*-5-*epi*-valiolone by AcbM and AcbO. Details for AcbO assays are described in **Sect.** 4.6. Extracts of *S.lividans* TK64 with pIJ4123 (control), pCW4123M (AcbM), or pMJO7 (AcbO) were used for these assays. Samples were prepared as described in **Sect.** 2.28. Samples were applied to the TLC and separated using the solvent II (**Sect.** 2.23); spots of cyclitol components were visualized by the use of a cer-/molybdate-containing reagent (**Sect.** 2.23). The respective spots are marked by arrows. For assays shown in *lanes* 2 - 6 enzymatically synthesized 2-*epi*-5-*epi*-valiolone was used, for assays in *lanes* 7 - 11 chemically synthesized 2-*epi*-5-*epi*-valiolone was used. *Lane* 1, 4 µl ADP (10mM); *lanes* 2 and 11, pIJ4123 (control); *lanes* 3 and 10, AcbO; *lanes* 4 and 9, AcbM (no ATP present in the assays); *lanes* 5 and 8, AcbM; *lanes* 6 and 7, AcbM and AcbO; *lane* 12, 4 µl ATP (10mM).

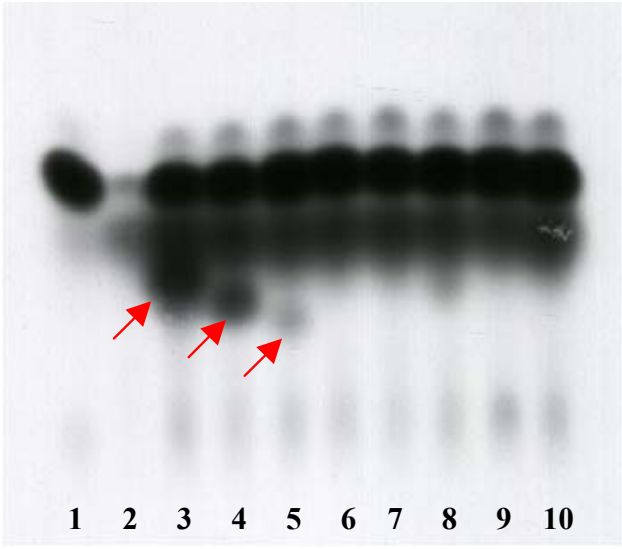


Fig. 4.14: Phosphorylation of cyclitols by AcbM. Assays were carried out in presence of [γ-^{32}P] ATP as described in **Sect.** 2.28. TLC was developed in solvent I. TLC plate was autoradiographed at room temperature for 2 h. Cell-free extracts were from *S. lividans* TK64 with pIJ4123 (control, *lane* 1) or with pCW4123M (AcbM, *lanes* 2 - 10). *Lane* 1: 2-*epi*-5-*epi*-valiolone; *lane* 2: no substrate; *lane* 3: 2-*epi*-5-*epi*-valiolone; *lane* 4; 2-*epi*-5-*epi*-valiolol; *lane* 5: 1-*epi*-2-*epi*-5-*epi*-valiolol; *lane* 6: 1-*epi*-5-*epi*-valiolol; *lane* 7: 5-*epi*-valiolol; *lane* 8: 1-*epi*-valienol; *lane* 9: valienol; *lane* 10: acarbose. The conversion products of cyclitols are indicated by arrows.

4.5.3: Purification of the His-tag AcbM protein and 2-*epi*-5-*epi*-valiolone-phosphate

From the experiments described in **Sect.** 4.5.2, it was concluded that AcbM acted as a 2-*epi*-5-*epi*-valiolone kinase. However, it was unclear in which position the cyclitol was phosphorylated. To solve this problem, NMR analysis of the newly formed product was necessary to elucidate its structure. Therefore, the product (2-*epi*-5-*epi*-valiolone-phosphate) had to be purified.

4.5.3.1: Partial purification of His-tag AcbM protein

In order to purify 2-*epi*-5-*epi*-valiolone-phosphate, the reaction conditions for AcbM were first optimized. The AcbM protein was overproduced in *S. lividans* TK64/pCW4123M in the form of a soluble N-terminal His-tag fusion protein (**Tab.** 4.4). This made it possible to purify the His-tag AcbM protein by an affinity chromatography using the Ni^{2+} HiTrap chelating column (Amersham Bioscience, Freiburg).

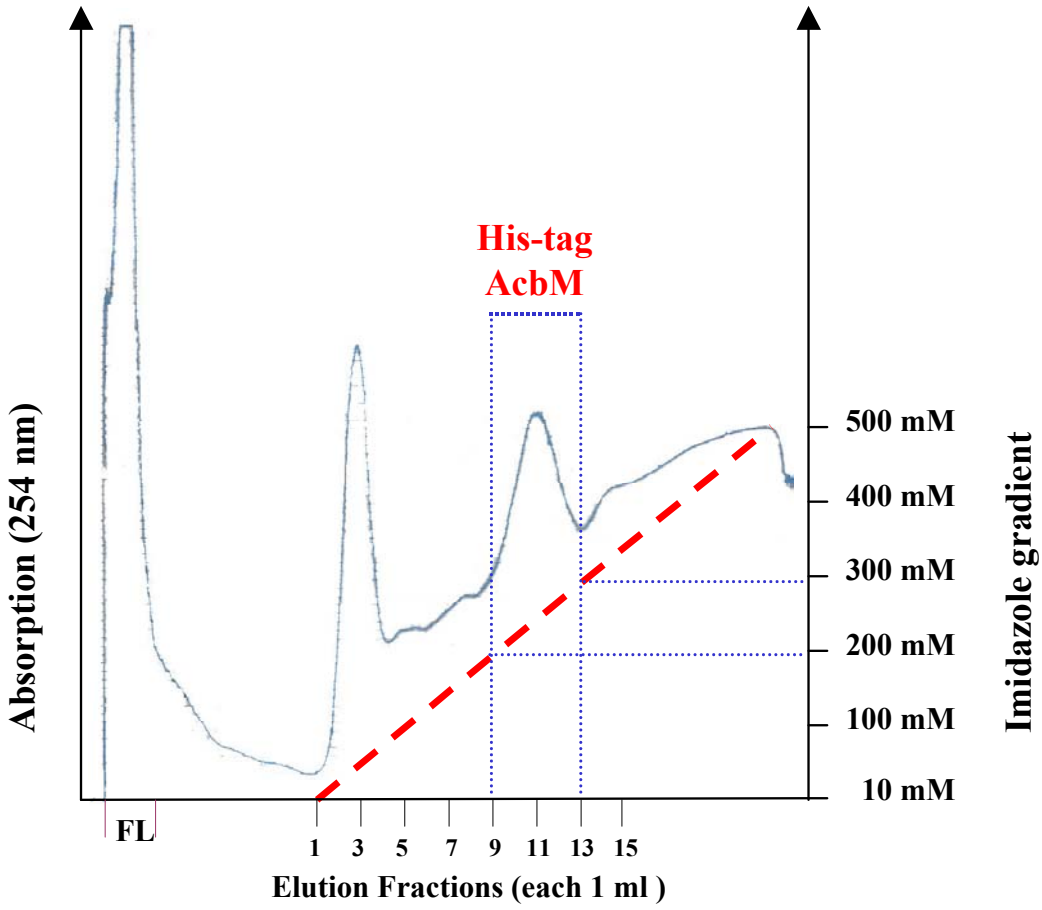


Fig. 4.15: Schematic diagram of the affinity chromatography. The purification was performed as described in **Sect.** 2.24. An elution chromatogram of His-tag AcbM from a Ni^{2+} HiTrap chelating column is shown (FL, flow through). The linear gradient of 10 – 500 mM imidazole is indicated by a broken line. The fractions containing His-tag AcbM are indicated with dotted lines. The fractions were collected as each of 1 ml.

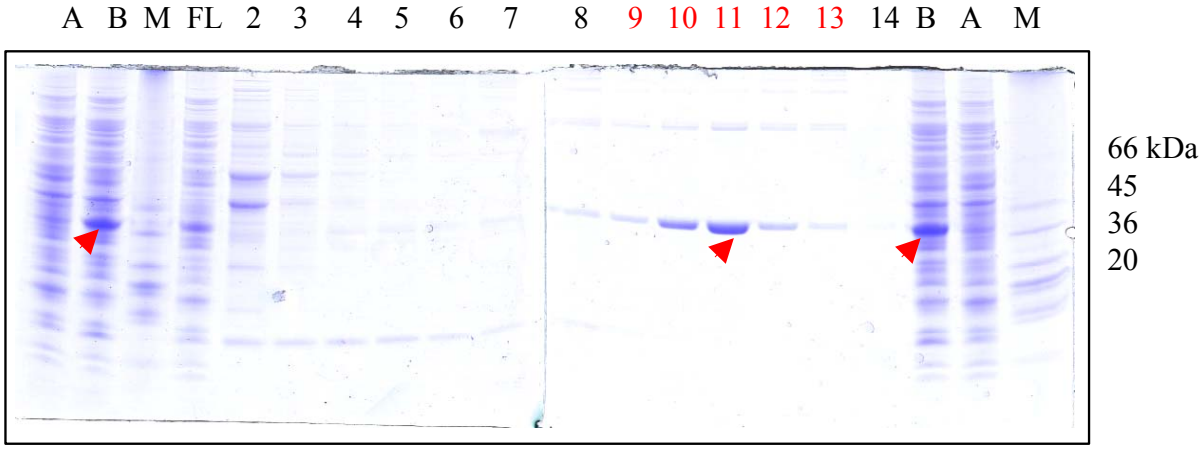


Fig. 4.16: SDS-PAGE analysis of the different elution fractions. Electrophoresis was run in a 10 % protein gel. *Lanes* A, cell-free extracts from *S. lividans* TK64/pIJ4123; *lanes* B, cell-free extracts from *S. lividans* TK64 /pCW4123M; *lanes* M, protein weight marker; *lane* FL, flow through; *lanes* 2 – 14, the elution fractions from 2 – 14 corresponding to those shown in **Fig.** 4.15. The band of the His-tag AcbM proteins is indicated with arrows.

As shown in **Fig.** 4.15 and **Fig.** 4.16, the His-tag AcbM was eluted from the Ni^{2+} HiTrap chelating column at a range of 200 – 300 mM imidazole. Different elution fractions (each of 1 ml) were analyzed by SDS-PAGE. The His-tag AcbM was nearly completely included in the fractions 9 – 13 (**Fig.** 4.16) which represented exactly the corresponding peak on the affinity chromatography (**Fig.** 4.15). Further enzyme assays showed that the His-tag AcbM was active in all these fractions (9 – 13) even in presence of high concentrations of imidazole (data not shown). Therefore these fractions were collected and dialyzed against 5 l of buffer 2 (**Sect.** 2.5.4) to remove imidazole. Finally a total volume of 5 ml of partially purified active His-tag AcbM protein was recollected.

4.5.3.2: Purification of 2-*epi*-5-*epi*-valiolone-phosphate

Preparative production of 2-*epi*-5-*epi*-valiolone-phosphate and the following purification steps were performed according to the general strategy shown in **Fig.** 4.17. The 20 mg purified 2-*epi*-5-*epi*-valiolone (**Sect.** 4.5.2) was incubated together with the whole volume of the 5 ml partially purified His-tag AcbM protein (**Sect.** 4.5.3.1) and 1 ml of ATP solution (100 mM in 100 mM Tris) in an end volume of 10 ml. The reaction was monitored by use of the TLC method. After 6 h incubation at 30°C, nearly all 2-*epi*-5-*epi*-valiolone was converted to the desired product. The byproduct generated during the purification of 2-*epi*-5-*epi*-valiolone for some unknown reasons (**Sect.** 4.5.2) was not a substrate for AcbM (lanes 1 and 15, **Fig.** 4.18 B). The proteins were removed by ultrafiltration (**Sect.** 2.31) from the reaction solution. The collected flow-through was concentrated to 3 ml by freeze-drying and then was applied to the Dowex anion-exchange column (**Sect.** 2.31). The chromatography profile was shown in **Fig.** 4.18 A. The unphosphorylated compounds, e.g., the unknown compound (peak I) and 2-*epi*-5-*epi*-valiolone (peak II), were eluted from the column with water (**Fig.** 4.18 A). The phosphorylated compounds were eluted with a linear gradient of 0 – 600 mM NaCl and fractionated (each of 2 ml). The following TLC analysis showed that most of the desired product, 2-*epi*-5-*epi*-valiolone-phosphate, was included in the fractions E33 - E54 (**Fig.** 4.18 B). It was quantitatively separated from ATP, ADP and AMP (peak III, **Fig. 4.18** A). Fractions E33 – E56 were then pooled (total volume of 48 ml) and concentrated to about 5 ml by freeze-drying. The sample was desalted on a Sephadex G-10 gel filtration column (**Sect.** 2.31). Finally, a sample of 12 mg of purified 2-*epi*-5-*epi*-valiolone-phosphate were obtained as a white powder.

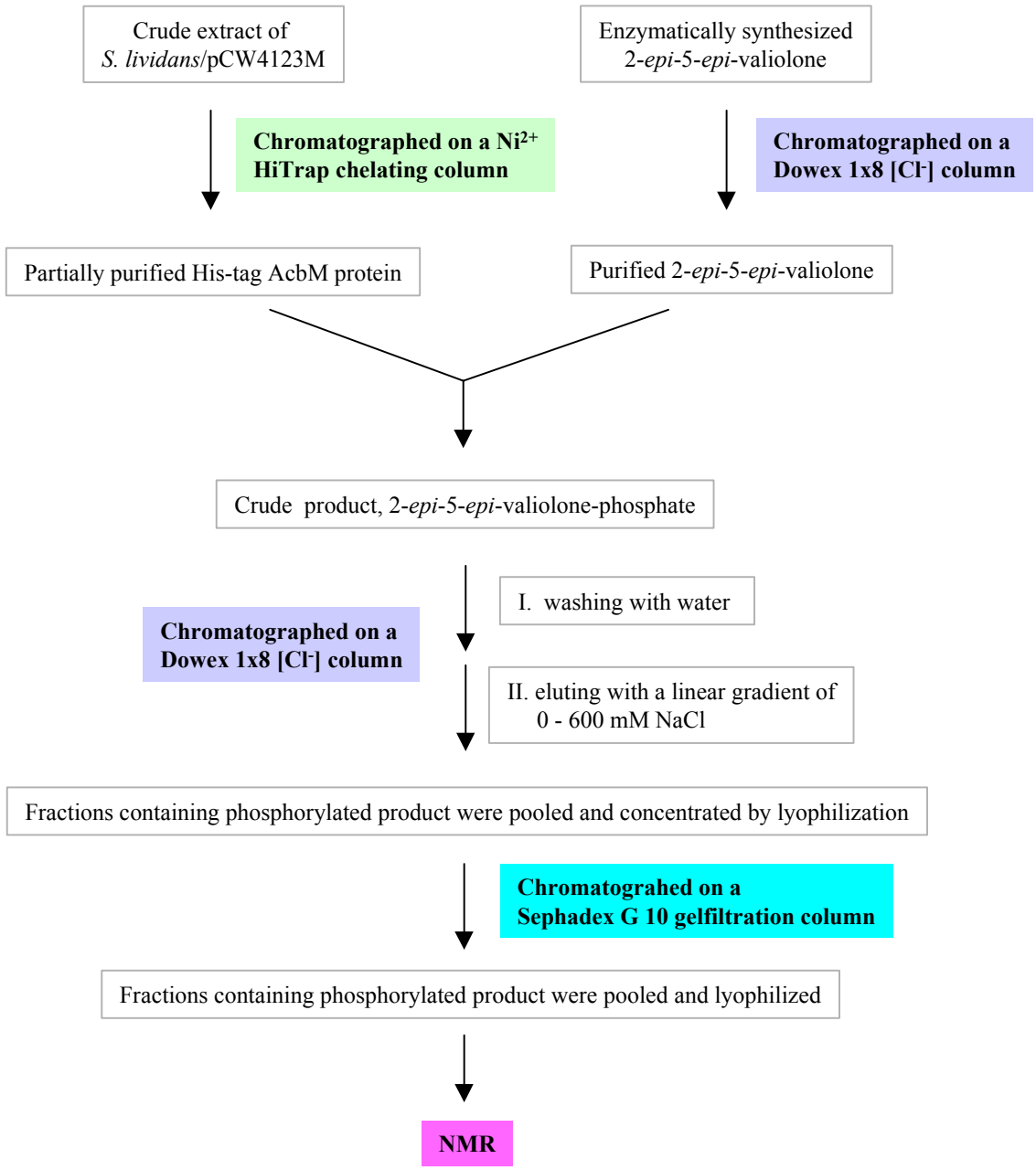


Fig. 4.17: General strategy for the purification of 2-*epi*-5-*epi*-valiolone-phosphate.

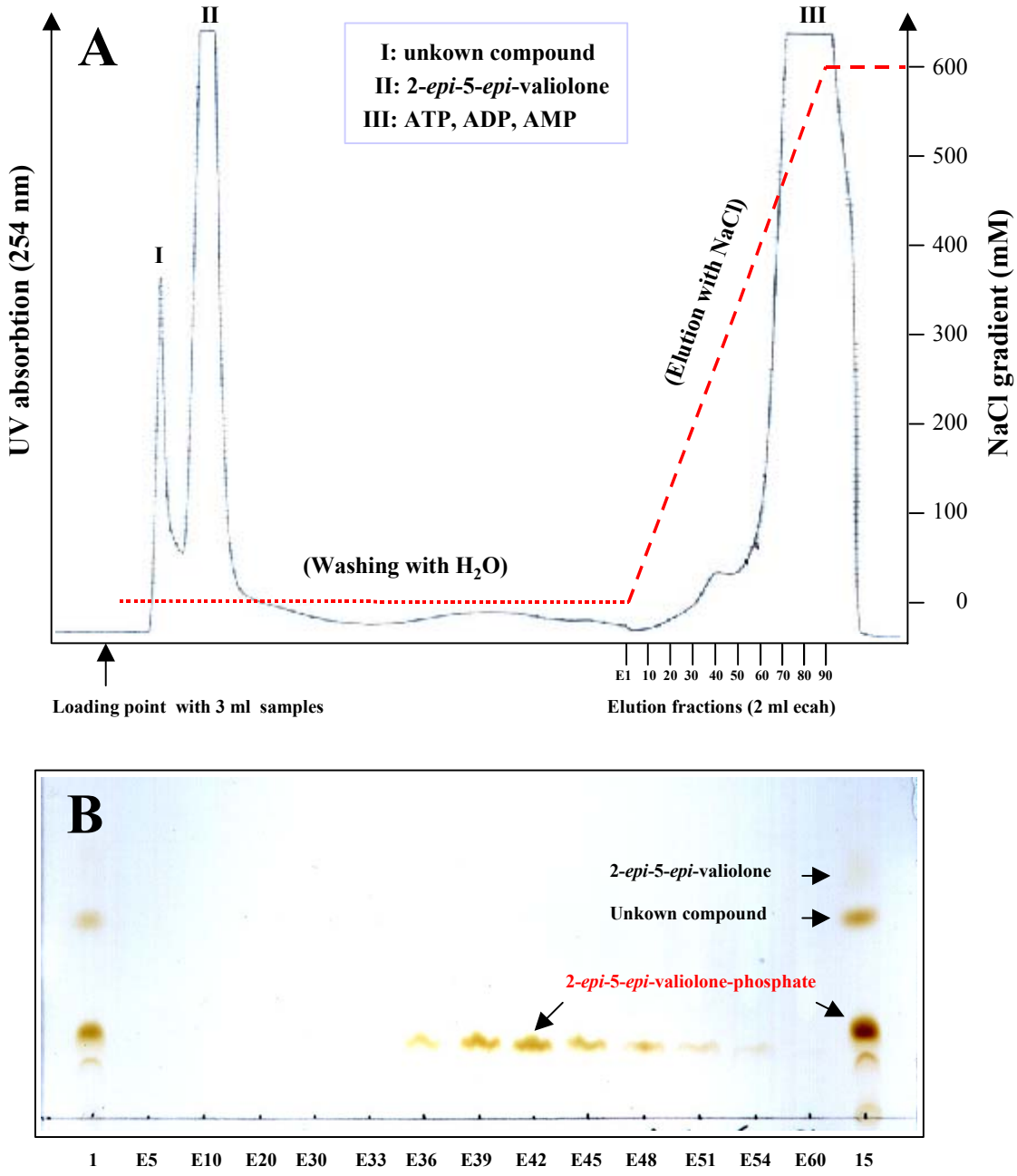


Fig. 4.18: Purification of 2-*epi*-5-*epi*-valiolone-phosphate with a Dowex anion-exchange column. A: Schematic diagram of the anion-exchange chromatography. The loading point of the 3 ml of concentrated AcbM reaction samples is indicated by an arrow. The column was first washed with H_2O (the dotted line) and eluted with a linear gradient of 0 - 600 mM NaCl (the broken line). The elution fractions were collected as each of 2 ml**. B: TLC analysis of the elution fractions.** TLC was developed in solvent II (section 2.23) and visualized by heating. *Lane* 1: AcbM reaction solution (3 µl out of 10 ml); *lane*s E5 - E60: the corresponding elution fractions (E5 – E60) as shown in the chromatography **A** (10 µl out of 2 ml); *lane* 15: the concentrated reaction solution (2 µl out of 3 ml). Positions of the unknown compound, 2-*epi*-5-*epi*-valiolone and 2-*epi*-5-*epi*-valiolone-phosphate are indicated with arrows.

4.5.4: Elucidation of the structure of 2-*epi*-5-*epi*-valiolone-phosphate

4.5.4.1: Determination of the molecular weight by IC-MS

In order to get more information about the product of the AcbM reaction, IC-MS (Ion chromatography coupled to mass spectrometry) was performed as described in **Sect.** 2.32 to determine the molecular mass of the product. The analysis revealed that there is a dominant new peak (12.77 min in IC) with a mass of 272 (*m/z* 271) in the AcbM reaction solutions, in comparison with the control, as shown in **Fig.** 4.21 and **Tab.** 4.6 (see **Sect.** 4.6). This value corresponds exactly to that of the calculated mass of 2-*epi*-5-*epi*-valiolone-phosphate. Furthermore, the purified AcbM product (**Sect.** 4.5.3.2) was analyzed by IC-SRM (Ion chromatography coupled with selected reaction monitoring). The result showed that the product (*m/z* 271) indeed contained a phosphate group (data not shown). These data provided strong evidence that the new product generated by AcbM is 2-*epi*-5-*epi*-valiolone-phosphate.

4.5.4.2: NMR-based investigations of the AcbM reaction product

In order to find out in which position 2-*epi*-5-*epi*-valiolone was phosphorylated by AcbM, the purified product (12 mg, **Sect.** 4.5.3.2) was dissolved in D_2O and subjected to NMR analysis carried out as described in **Sect.** 2.34. Then ^{1}H, ^{13}C and ^{31}P-NMR data of the purified substance were determined (**Fig.** 4.19, **Tab.** 4.5). In the ^{1}H-NMR-spectrum (**Fig.** 4.19 A) the coupling pattern of the former hydroxymethyl-group had changed significantly indicating that it had been functionalized. A signal in the ^{31}P-NMR at 5.55 ppm was indicative of a phosphate group and a ^{1}H-^{31}P-COSY experiment proved that the phosphate group was sitting at the primary position (**Fig.** 4.19). The methylene-group occurred in the ^{1}H-NMR as an ABX-spinsystem at 3.58 ppm and 3.99 ppm with a coupling constant $^{3}J_{H\text{-}7a,P}$ = 6.6 Hz and $^{3}J_{H\text{-}7b,P}$ = 9.4 Hz. Interestingly, while dissolved in D_2O, the AB pattern of the ring methylene group was replaced by two pseudo singulets at 2.40 ppm and 2.78 ppm within thirty minutes. This phenomenon could be explained by a fast exchange of the methylene protons by deuterium from the solvent and had also been observed for the 2-*epi*-5-*epi*-valiolone system itself (Dong *et al.*, 2001). In the ^{13}C-spectrum the C-7 appeared as a dublet at 70.2 ppm with a coupling constant $^{2}J_{C\text{-}7,P}$ = 5.1 Hz. Instead of the expected carbonyl group C-1 showed a resonance for a quaterneric carbon at 101.23 ppm, indicating a hydratization of the ketone function (**Tab.** 4.5). From the described result the

purified product of the AcbM reaction can be unequivocally identified as 2-*epi*-5-*epi*-valiolone-7-phosphate, being interpreted here to represent the second intermediate in the cyclitol branch of the acarbose pathway.

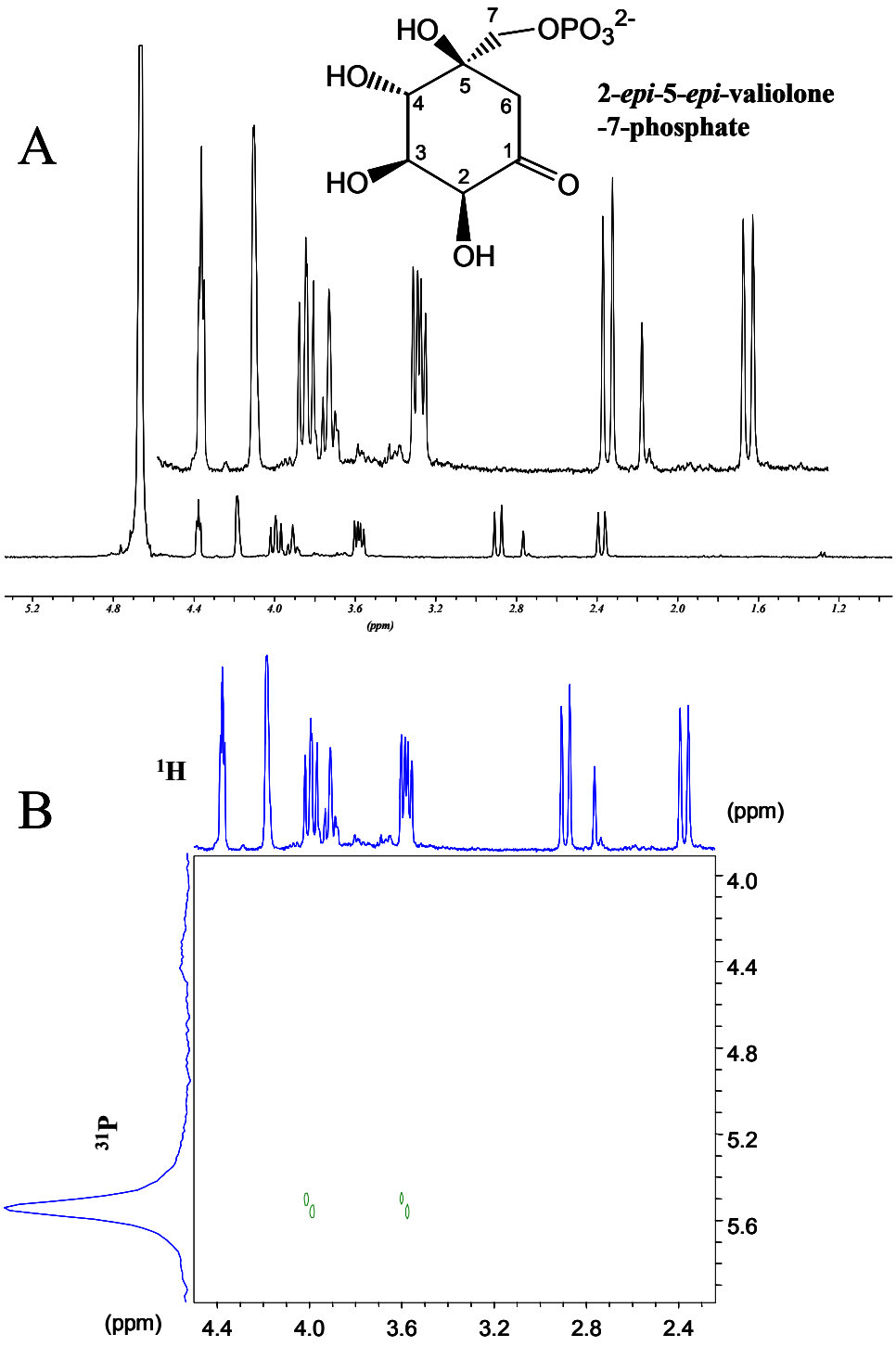


Fig.4.19: NMR spectra of 2-*epi*-5-*epi*-valiolone-7-phosphate. A. ^{1}H-NMR spectra; B. ^{1}H-^{31}P-COSY spectra.

Tab. 4.5: Chemical shift values (ppm) of 2-*epi*-5-*epi*-valiolone (I) and 2-*epi*-5-*epi*-valiolone-7-phosphate (II) in the ^{1}H and ^{13}C NMR spectra

^{13}C NMR (400MHz)	I (*d4*-MeOH)	II (D_2O)	^{1}H NMR (100 MHz)	I (*d4*-MeOH)	II (D_2O)
C-1	209.8	101.23	H-2	4.59 (d, *J* = 4.0 Hz)	4.71 (d, HDO, *J* = 4.0 Hz)
C-2	76.1	76.74	H-3	4.27 (Ψt, *J* = 4.0 Hz)	4.38 (Ψt, *J* = 3.8 Hz,)
C-3	79.7	79.62	H-4	4.03 (m)	4.18 (m)
C-4	70.9	71.19	H-6_{ax}	2.33 (dd, *J* = 13.7 Hz, *J* = 1.7 Hz)	2.38 (d, *J* = 13.8 Hz,)
C-5	81.5	82.86	H-6_{eq}	2.84 (d, *J* = 13.7 Hz,)	2.89 (d, *J* = 14.2 Hz,)
C-6	46.0	46.38	H-7a	3.43 (d, *J* = 11.3 Hz)	3.58 (dd, *J* = 6.6 Hz, *J* = 11.7 Hz)
C-7	67.7	70.17 (d, *J* = 5.1 Hz)	H-7b	3.64 (d, *J* = 11.3 Hz,)	3.99 (dd, *J* = 9.4 Hz, *J* = 11.5 Hz)

d, dublet; t, triplet; m, multiplet; Ψt, pseudotriplet; the coupling constant *J* is given in Hz.

4.6: Conversion of 2-*epi*-5-*epi*-valiolone-7-phosphate by AcbO

4.6.1: 2-*epi*-5-*epi*-valiolone-7-phosphate is the substrate of AcbO

To get further insight into the biosynthetic pathway for valienol moiety, cell extracts including AcbM were combined with extracts containing AcbO, AcbL or AcbN. In all tests 2-*epi*-5-*epi*-valiolone and ATP were used as substrates without or in combination with various dinucleotides as redox or epimerase coenzymes (NAD, NADH, NADP, NADPH). Only when extracts with AcbM/ATP in combination with AcbO were used, a new spot was observed on the TLC plates (lane 5 and lanes 7 – 9, **Fig.** 4.20). The conversion of 2-*epi*-5-*epi*-valiolone-7-phosphate to the new product by AcbO was nearly 100% as judged from the TLC analysis. When AcbM extracts were combined with other extracts (AcbN and/or AcbL), the substrate 2-*epi*-5-*epi*-valiolone was only phosphorylated by AcbM, no further conversion was observed (lanes 3, 4 and 6, **Fig.** 4.20). Without AcbM and in presence of ATP alone no conversion of 2-*epi*-5-*epi*-valiolone by AcbO was monitored (lanes 3 and 10, **Fig.** 4.13). These data gave an important hint for AcbO being the next enzyme in cyclitol conversion. In these assays no dinucleotide co-enzyme was required, though extensively dialyzed cell-free extracts were used. This finding indicated that the reaction catalyzed by AcbO was either co-enzyme independent or, if a co-factor would be involved, that it must have been tightly bound to the enzyme. Therefore, it was further investigated whether AcbO could be either (i) the epimerase that catalyzes the epimerization at C-2 to give 5-*epi*-

valiolone-phosphate or (ii) a dehydratase that catalyzes the formation of 2-*epi*-valienone-7-phosphate.

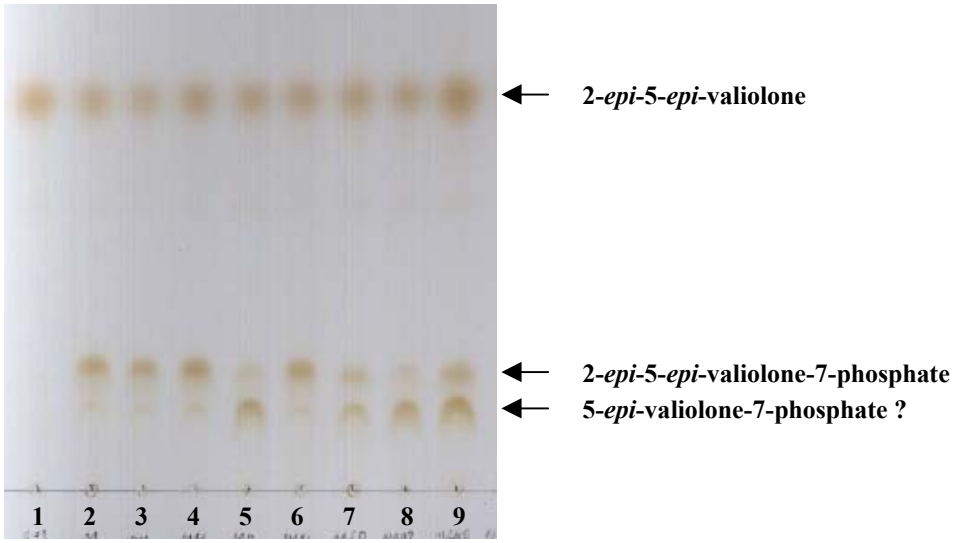


Fig. 4.20: TLC analysis of the enzyme assays for conversion of 2-*epi*-5-*epi*-valiolone-7-phosphate by other Acb-proteins. Cells were from *S. lividans* TK64 harboring the plasmids pIJ4123 (control), pCW4123M (AcbM), pCW4123L (AcbL), pMJO7 (AcbO) and *S. lividans* TK23/pMJN5 (AcbN). For combined assays, cells were first mixed at a ratio of 1:1 and then disrupted by sonication. The corresponding cell-free extracts were used in the assays (as described in **Sect.** 2.29). In all these assays, chemically synthesized 2-*epi*-5-*epi*-valiolone was used as a substrate. Extracts or combined extracts were from: *lane* 1, control; *lane* 2, AcbM; *lane* 3, AcbML; *lane* 4, AcbMN; *lane* 5, AcbMO; *lane* 6, AcbMLN; *lane* 7, AcbMLO; *lane* 8, AcbMNO; *lane* 9, AcbMLNO. TLC was developed once in solvent II (**Sect.** 2.23) and visualized by heating.

4.6.2: Characterization of the AcbO product

In order to identify the conversion product from 2-*epi*-5-*epi*-valiolone-7-phosphate that was obtained in the reaction catalyzed by AcbO, IC-MS data of the reaction product were analyzed. In assays containing 2-*epi*-5-*epi*-valiolone/ATP and the overproduced enzymes AcbM alone or AcbM and AcbO together, respectively, three new mass peaks of phosphorylated compounds were detected: *m/z* 253 (13.27 min), 271 (12.77 min; ratio 2:1) and 287 (12.97 min). These peaks were missing in assays without AcbM (**Fig.** 4.21). The dominant mass peak corresponded exactly to the expected mass for 2-*epi*-5-*epi*-valiolone-7-phosphate. As the same dominant mass peak (*m/z* 271) was detected in assays with AcbO (**Tab.** 4.6), it was concluded that AcbO most likely catalyzed the epimerization at C-2 yielding 5-*epi*-valiolone-7-phosphate. The peak (*m/z* 287) could be explained by the hydration of C-1 ketone function which was also observed in the NMR

analysis of purified 2-*epi*-5-*epi*-valiolone-7-phosphate (cf. **Sect.** 4.5.4), whereas the peak (*m/z* 253) indicated a natural dehydration between C-5 and C-6 to give 2-*epi*-valienone-7-phosphate due to the basic condition used in IC-MS assays.

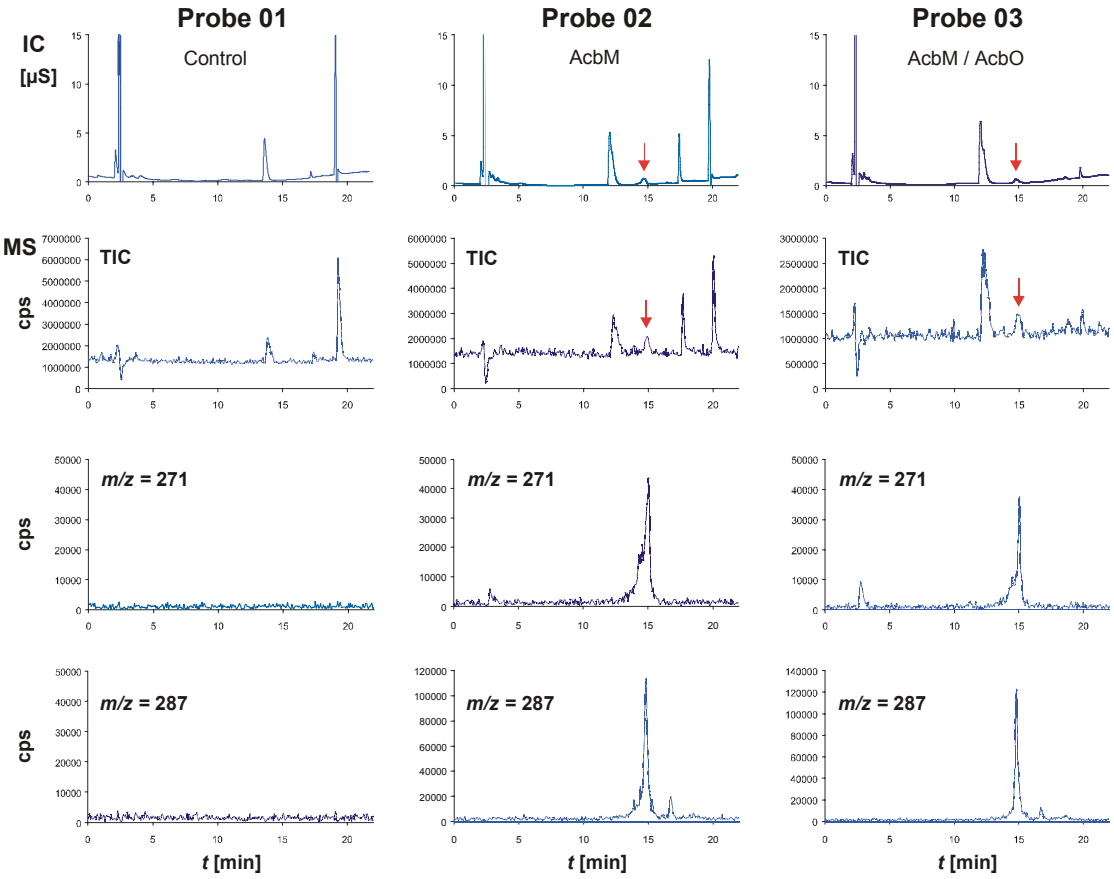


Fig. 4.22: Analysis of the conversion products by ion chromatography and mass spectrometry (IC-MS). cps, counts per second; TIC, total ion current. Probes are from the enzyme assays including ATP, 2-*epi*-5-*epi*-valiolone and the cell-free extracts from *S. lividans* TK64 harboring the following plasmids: pIJ4123 (control, probe 01), pCW4123M (AcbM, probe 02) and pCW4123M/pMJO7 (AcbM/AcbO, probe 03).

Tab. 4.6: IC-MS data of the new products in the AcbM and AcbMO assays

Enzyme assays	retention time	m/z (-1)
Control	---	---
AcbM	12.77min	271
	12.97min	287
	13.27min	253
AcbMO	12.77min	271
	12.97min	287
	13.27min	253

Details for enzyme assays see **Fig.** 4.22.

5: Discussion

The thesis includes two distantly related parts – "gene sponge" and the biosynthesis of acarbose. Though both topics were not in any direct relationship there was a common general goal in their motivation: the expansion of the genetic/biochemical potential of microorganisms for the production of bioactive natural products. Towards their aim for both parts the basic knowledge is still largely lacking, especially concerning the genetic and biochemical ecology of organisms and their products. Thus, the knowledge of the conditions and roles of, e.g. acarbose production could help to design microcosms in which gene clusters for similar products could be tranferred to the type of gene sponges used in this study (cf. Piepersberg, 2001).

5.1: Preliminary experiments for testing a "gene sponge" approach towards direct isolation of new eco-genetic traits from natural gene pools

For the biotechnical exploitation of the diverse and non-cultivatable genetic material in natural microhabitats (e.g. in soil), the possibility and feasibility of the *in vivo* "gene sponge" approach was tested in this work. Some preliminary experiments were carried out to test the natural gene transfer to the selected "gene sponge" strains in regular mating crosses, as well as in sterile and nonsterile soil microcosms.

5.1.1: *S. lividans* 66 strains with large deletions as potential "gene sponges"

The four derivatives of the strain *S. lividans* TK64, namely Jni13C1, Jni14C1, WP and AJ100, were chosen to serve as the potential recipients ("gene sponges"). These strains, such as their wild type origin, were expected to have little barriers in taking-up foreign DNA. Furthermore, the four strains have lost large parts of their variable genome fraction via large deletions in the chromosomal end regions and they had circular chromosomes (Volff *et al.*, 1996; Volff *et al.*, 1997a; cf. **Fig.** 3.1). These genetic features were expected to give them more pressure to cope with the natural microflora when seeded into nonsterile soil. In turn, they should be prone to soak up genetic material, encoding environmental traits such as secondary metabolites, from the shared vast gene pool in the residential microflora, for their survival (cf. **Sect.** 1.4).

The preliminary investigations on this matter, presented in this work, prove that the four deletion strains had the potential to serve as "gene sponges". First, the transformation experiments showed that these four deletion strains have no restriction to take up naked plasmid DNA, whether methylated or not. This feature provided an increasing chance (with little bias) for their uptake of free DNA molecules which seem to ubiquitously exist in the environment, e.g. soil, by natural transformation (Lorenz and Wackernagel, 1994). Secondly, with mutational antibiotic resistances/sensitivities these four strains had positively selectable markers for their reisolation from the soil. They all showed high resistance to streptomycin (up to at least 100 µg/ml) and were highly sensitive to chloramphenicol and kanamycin (**Tab.** 3.2). Therefore, firstly, they could be easily reisolated from the nonsterile soil using high concentrations of streptomycin and, secondly, they were identifiable via these markers.

5.1.2: Transfer and mobilization of large chromosomal fragments by the conjugative plasmid pIJ903

An ideal "gene sponge" should have the potential to take up large DNA fragments which encode an entire pathway for bioactive compounds. The strains like WP and Jni13C1 were tested for the ability to integrate large chromosomal fragments in mating crosses. Matings between *Streptomyces* sp. were employed early in building up a chromosomal linkage map (Hopwood *et al*., 1983; 1993). Conjugative plasmids are common in the genus *Streptomyces* and are shown to be highly self-transmissible and nearly all have chromosome-mobilizing ability (Hopwood and Kieser, 1993). In this work, the conjugative plasmid pIJ903 was employed to study the mobilization and transfer of chromosomal markers into the "gene sponges". The experiments resulted in some very interesting genetically modified exconjugants, such as M1-48, M1-107 and M3-1, which were different from both parent strains. These exconjugants had identical phenotypes as their potential parental strains like WP and Jni13C1 except for the additional presence of *cmlR* and chromosomal ends. Interestingly, they had duplicated left chromosomal ends together with the *cmlR* gene (cf. **Sect.** 3.7.4). These three strains (M1-48, M1-107 and M3-1) were unlikely to be derived solely from the strain TK23/pIJ903 without interactions with the other parent strain, namely Jini13C1 or WP. This conclusion was deduced from the fact that no similar strains were recovered in screening mutants resistant to both streptomycin and chloramphenicol from spore suspensions of strain TK23 prepared from nonselective plates. Some

models are presented here based on the idea that the derivation of these strains probably was a multiple step process (deletion-recombination) which was highly related to the genetic instability of *Streptomyces*.

5.1.2.1: General models for the generation of mutant strains with large end-deletions and with duplicated chromosomal ends

Genetic instablility is apparently ubiquitous in the genus *Streptomyces* (**Sect.** 1.1). The discovery of the linearity of the chromosome and the high instability of its telomeres (Lin *et al.*, 1993; Redenbach *et al.*, 1993) was an important step towards understanding genetic instability. In most cases, deletion of one or both telomers is found in the mutants (Lin *et al.*, 1993; Rendenbach *et al.*, 1993; Fischer *et al.*, 1997; Lezhava *et al.*, 1997). In experiments studying DNA amplifications in *S. lividans*, Rauland *et al.* (1995) reported a series of deletion mutants which retained only the left chromosome end. These strains are very similar to strains recovered in this work, like M1-107 and M3-1. Therefore, the question arises how these strains are created naturally.

A model (cf. **Fig.** 5.1) for interpreting the generation of these strains is derived from that proposed for the repair of collapsed replication forks in *E. coli* (Kuzminov, 1995; Volff *et al.*, 1997a; Volff and Altenbuchner, 1998). A replication fork moving from the center of the chromosome will collapse when it reaches a single-strand break. This collapsed fork may be repaired by homologous recombination allowing replication to continue. Lack of such a repair event would lead to the occurrence of the hypothetical mutant Mu1, in which one chromosomal end was deleted, but the other end was retained. However, it is unlikely that the strain Mu1 would survive with an unprotected chromosomal end (newly created on the right side), since no structure was available for the attachment of the terminal protein. Therefore, as a further step it is speculated that some sequences in the chromosomal left end region frequently undergo illegitimate recombination with the free end from the collapsed fork. When this assumed event occurred in the chromosome of Mu1 itself, it would result in the deletion of both telomeres and the subsequent circularization of the chromosome. Such an illegitimate recombination process was reported in the formation of *S. griseus* deletion mutants with a circular chromosome (Kameoka *et al.*, 1999). Therefore, the generation of the deletion strains like WP and Jni13C1 might resemble a similar process occurring in *S. lividans* TK64. Alternatively, the illegitimate recombination event could also happen between the free chromosomal end of Mu1 and a sequence, somewhere in the left

chromosomal end region of TK23, in an inverted orientation. This recombination event between two chromosomes would give rise to a new chromosome of the hypothetical strain Mu2 with identical ends on each side (cf. **Fig.** 5.1).

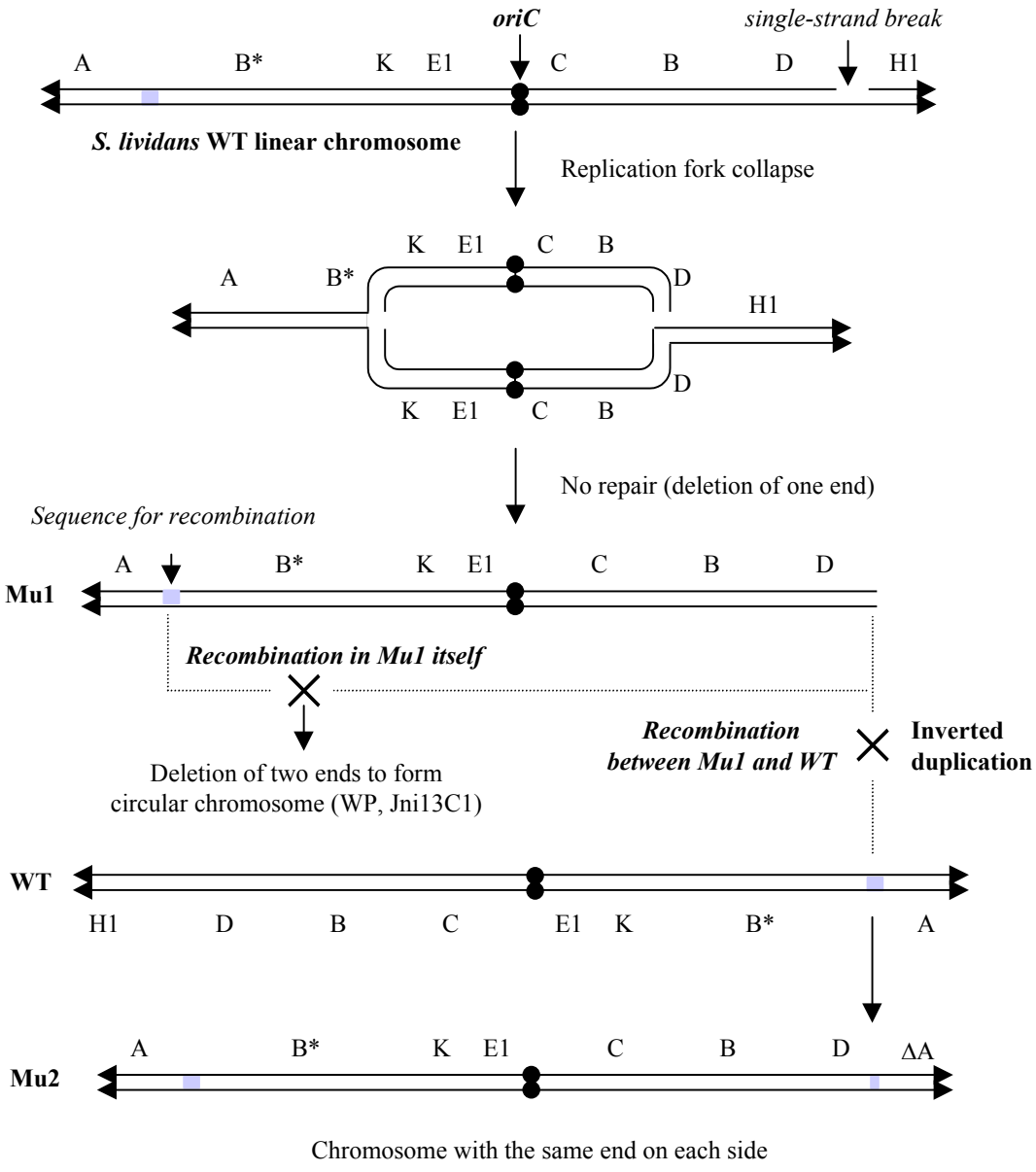


Fig. 5.1: Models for generation of deletion strains and mutants with duplicated chromosomal ends in *Streptomyces* sp. The map is not drawn to scale. The chromosomes are symbolized by lines. The corresponding *Ase*I fragments of wild type (WT) *S. lividans* (A, B*, K, E1, C, B and D) are given; ΔA means reduced A fragment. The arrows indicated the TIR. The *oriC* was symbolized by dots. The small rectangle indicates a sequence for recombination. WT refers to the wild type strain. Mu1 and Mu2 designate two types of hypothetical mutants generated in this "deletion-recombination" process. (modified after Volff and Altenbuchner, 1998)

Another model for explaining strains with duplicated chromosome ends is shown in **Fig.** 5.2. An example for this model had been reported in mutants of *S. ambofaciens*, which had also two identical chromosomal ends (Fischer *et al.*, 1998). In this case, duplication of one chromosomal end was interpreted to be due to an event of homologous recombination between two copies of a duplicated DNA sequence (*hasL* and *hasR*) located on each chromosomal arm of the wild type *S. ambofaciens* strain. This event would replace part of one of the chromosomal arms and generate derivatives with identical chromosomal ends on both sides (**Fig.** 5.2).

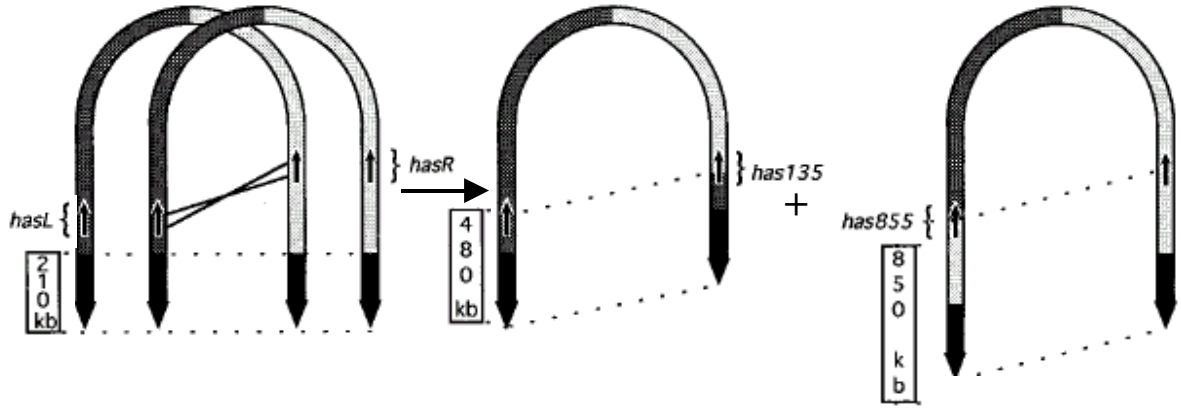


Fig. 5.2: Sister chromosome exchange via homologous recombination in *Streptomyces ambofaciens*. The left and right chromosomal arms are shown in dark and light gray, respectively; the TIRs of the wt strain are shown as thick, black arrows. The transcriptional orientation is defined by the thin arrow. The new TIRs are delimited by dotted lines. *hasL* and *hasR* are two copies of a duplicated DNA sequence. For details see Fischer *et al.* (1998).

5.1.2.2: Models for the generation of strain M3-1

The models shown in figures 5.1 and 5.2 could explain the generation of the deletion strains with circular chromosomes and also of the strains with duplicated chromosomal ends. However, neither could explain the generation of strain M3-1. As a result of either of the two models, the newly-born chromosome would keep one arm unaltered. For example, the chromosome of hypothetical strain Mu2 (cf. **Fig.** 5.1) would have the same left arm as TK23, namely the entire *Ase*I-A fragment. In contrast, the chromosome of strain M3-1 had a smaller *Ase*I-A fragment, as compared to that of TK23 (**Fig.** 3.11 I.A). Furthermore, neither of the two models could explain the absence of the AUD1 and *argG* sequences and the presence of phenotypes like *str-6*, *spc-1* and *pro-2* in strain M3-1, which were different from the parental strain TK23. These facts led to the proposal of the third model (**Fig.** 5.3).

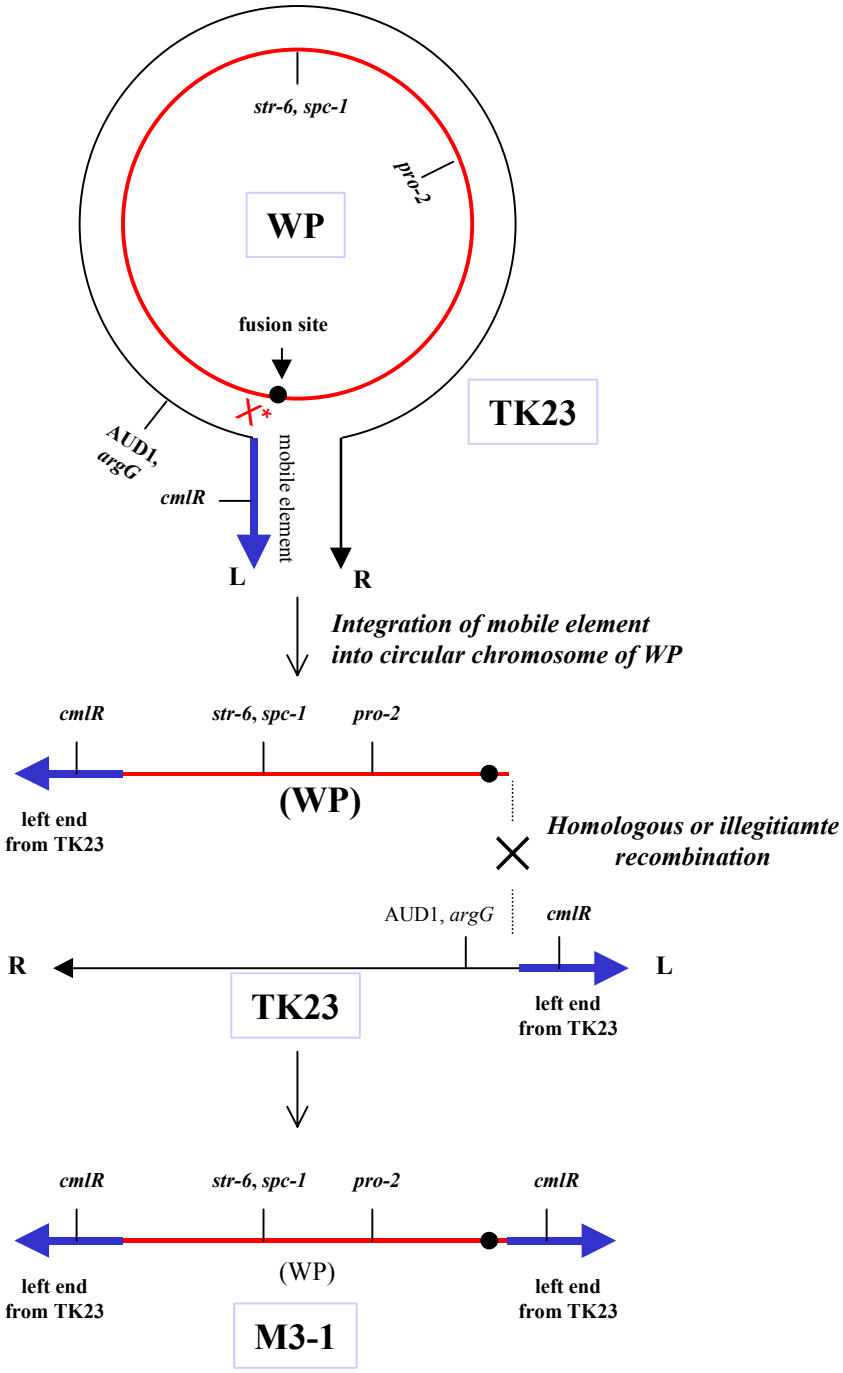


Fig. 5.3: A model for the generation of strain M3-1. The map is not drawn to scale. The chromosome of strain TK23 is depicted by a thin line (black) and of strain WP by a bold line or circle (red). Left chromosomal end (mobile element, cf. **Fig.** 3.14) is represented by bold arrows (blue) and the right end by a thin arrow (black). L and R mean the left and right TIRs. X or X* indicate probable homologous or illegitimate recombination. The positions of the genetic markers (*cmlR*, *argG*, AUD1, *str-6*, *spc-1* and *pro-2*) are indicated (cf. **Fig.** 3.13). Interpretations see text. The black dot indicates the fusion site at which the circular chromosome of WP was formed via illegitimate recombination (cf. **Fig.** 5.1).

The strain M3-1 was derived from the mating pairs WP x TK23/pIJ903 and recovered in the subsequent screening of mutants resistant to three antibiotics (chloramphenicol, streptomycin and thiostrepton). According to the model shown in **Fig.** 5.3, it was assumed that the first event was the integration of one copy of the left end of TK23 ("500 kb mobile element") into the circular chromosome of WP. This event could be driven by the selective pressure (chloramphenicol) and by help of the conjugative plasmid pIJ903. The strain WP had to take up this transfer-prone "500 kb mobile element" including *cmlR* from TK23 for survival. This large fragment could be integrated into WP by illegitimate or a repeated sequence-mediated homologous recombination, leading to the generation of a linear chromosome with only one end (left TIR and *cmlR* but no AUD1) protected by covalently-bound proteins, and one end free. This chromosome structure resembled the one of Mu1 shown in **Fig.** 5.1. Similarly, the second event was assumed to be the illegitimate or repeated sequence-mediated homologous recombination between this free end (cf. **Fig.** 5.3) and the left chromosome end of TK23 in an inverted orientation according to the general model shown in **Fig.** 5.1. This second event would generate the chromosome of M3-1. As a result of this model, it was concluded that M3-1 was derived from parental strain WP by insertion of two copies of the left chromosome end derived from the chromosome of TK23.

Clearly, this model is just one likely explanation for strain M3-1. There are many other possibilities for the formation of this strain. However, it is also clear that the generation of strain M3-1 must be a multiple step process in which exchanges between the chromosomes of WP and TK23 had occurred. The discovery of such recombinant strains like M3-1 provided evidence that strains like WP (or Jni13C1) could be appropriate candidates as "gene sponges".

5.1.2: Gene exchange in sterile soil

Soil microcosms are a valuable tool for the study of genetic interactions between microorganisms in natural soil environments. Sterile soil microcosms had been first tested in this work for screening of the "gene sponge" approach in a feasiblility study. Sterile soil "matings" were carried out for testing the viability of the "gene sponge" in presence of a number of streptomycetes (e.g. *Streptomyces lincolnensis*). The possibility was tested, whether they could acquire known traits, e.g., plasmid DNA (pIJ702), pigment-production (melanin), antibiotic resistance (the *aacC4* gene cassette encoding apramycin resistance), and antibiotic production

(lincomycin). However, no such event of interspecific transfer could be clearly demonstrated under the conditions used.

Earlier research demonstrated that *Streptomyces* sp. can survive for a long time in sterile and nonsterile soil samples as spores after a short mycelial growth phase of 2 to 3 days (Wellington *et al.*, 1990). Data from this work showed that the two tested recipient strains Jni13C1 and WP could survive for at least one month in sterile Warwick soil (**Sect.** 3.3). However their protoplasts seemed not to be able to regenerate in the sterile soil, since there were no survivors recovered from soil.

From some earlier experiments it was concluded that gene transfer between streptomycetes in soil was a relatively rare event (Cresswell *et al.*, 1992) and most recent studies focused on the intra- or interspecific transfer of conjugative plasmids (Wellington *et al.*, 1992; Vionis *et al.*, 1998; Ravel *et al.*, 2000). As expected, besides the size and design of the soil microcosms, a number of other factors were determined to have influence on the conjugative transfer of plasmids between streptomycetes in soil, such as soil moisture and nutrient levels (Bleakley and Crawford, 1989; Karagouni et al., 1993), donor/recipient ratios (Rafii and Crawford, 1989; Wellington *et al.*, 1990), and the presence of antibiotics (Herron *et al.*, 1998). Furthermore, it was found that a conjugative plasmid could mobilize nontransferable plasmids in soil (Rafii and Crawford, 1988; 1989). But there are no data available on the transfer of a non-conjugative plasmid by itself in soil. In this work, it has been demonstrated for the first time the transfer of a non-conjugative plasmid, pIJ702, intraspecifically in *S. lividans* strains in sterile soil in absence of other mobilizing plasmids (cf. **Sect.** 3.4). The time scale of the transfer of the plasmid pIJ702 was also different from the transfer of a conjugative plasmid. Plasmid transfer via conjugation in batch soil microcosms occurred only during the first two days. Since conjugative gene transfer requires an active mycelial growth phase, such transfer events are interpreted to occur in a mycelial state lasting only about 48 h (Clewlow *et al.*, 1990; Vionis *et al.*, 1998). In contrast, the establishment of pIJ702 in the recipient Jni13C1 was first observed after 9 days. This result indicated that the mechanism of the transfer of pIJ702 was not due to conjugation, but rather natural transformation. Probably, after seeded into sterile soil for 6 - 8 days, lysis of the cells of the donor strain TK23 released free pIJ702 DNA, which was taken up by the naturally developed competent recipient strain Jni13C1 to form the new recombinants. The fact that no transfer of

pIJ702 into the alternative recipient strain WP was detected under the identical conditions, probably meant that strain WP did not become naturally transformable.

However, these interpretations could not explain why no interspecific transfer of pIJ702 from plasmid-containing *S. lincolnensis* NRRL 2936 to the strain Jni13C1 was detectable in sterile soil. Also, in these experiments, no exchange of chromosomal markers, such as the known traits of melanin-production, apramycin resistance (*aacC4*) and lincomycin-production, was observed. DNA exchange or efficient recombination might have not occurred because of several possible reasons:

(1) the donor strain did not lyze readily and set free enough DNA for the detection of natural transformation;
(2) the frequency of gene transfer per viable recipient cell was too low;
(3) the recipient cells were not competent for uptake of DNA from other species;
(4) the DNA from *S. lincolnensis* could not be established in *S. lividans* under these conditions, e.g. because of interferences with the recombination apparatus or of lacking sequence homology;
(5) the phenotypic changes due to new genetic material in strain Jni13C1 could not be detected because of lacking expression of the respective genes.

At present it is not possible to decide on which level the limiting factor(s) occur. More experiments would be necessary to answer these questions. Nevertheless, strains like Jni13C1 seem to be efficient and suitable recipients for the transfer of foreign DNA as exemplified by the uptake of non-conjugative pIJ702 in sterile soil.

5.1.3: Gene exchange in nonsterile soil (chracterization of soil isolates)

Series of infection tests in natural soil populations have been carried out in this work, varying soil types, incubation time and temperature, water content (soil moisture), and various kinds of selective pressures (the presence of chloramphenicol or kanamycin in soil microcosms, or treating the soil microcosms via sonication), in attempts to find out optimal conditions for potential gene transfer to the strains Jni13C1 and WP (**Sect.** 3.6). However, mostly negative and no interpretable results were achieved, which meant that more control experiments were necessary in order to stepwise answer the many open questions.

Data showed that the strain Jni13C1 could survive rather long time even in nonsterile soil (**Fig.** 3.6). This feature was a disadvantage for it to serve as an optimal "gene sponge". Ideal "gene sponges" are assumed to be strains which are badly survivable in a populated foreign habitat. Most cells of the seeded "gene sponges" should be eliminated by the natural process of selection and competition with the resident microflora. Rather, they should be forced to acquire new ecologically relevant traits via gene transfer. They should be enriched by this way and have inherited new features from the soil's gene pool, such as the traits encoded for secondary metabolism (Piepersberg, 1993; 1994; 2001). An optimized condition of this kind would reduce the later work for the screening of strains exhibiting production of biochemically active compounds.

It should be stated here that the results of this work do not disprove the feasibility of the general "gene sponge" approach. There is enough evidence from the currently available data, including whole genome sequences, that bacterial gene transfer rates in nature including the uptake of whole chromosomes of foreign organisms should be high enough to detect them in the time scale and under other conditions necessary for an experimental or even industrial exploitation. For instance, evidence of increasing resistance to antibiotics in isolates from soil and other natural sources highlights the importance and speed of horizontal transfer of the resistance genes in bacteria (Nwosu, 2001). Horizontal gene transfer in bacteria is favored by the presence of many different mobile genetic elements. Especially, the genome of the model streptomycete *S. coelicolor* A(3)2 is exceptionally full of those elements (transposons, integrated virus- or plasmid-related genes, resistance gene sets, and all other kinds of secondary metabolic traits; Piepersberg, 2001; http://www.sanger.ac.uk/Projects/S_coelicolor/).

Addition of antibiotics to the soil microcosm led to reduced numbers of recipients and the reduction in conjugation frequencies (Herron *et al.*, 1998). On the other hand, nutrient amended soil allowed introduced inoculants to colonize and grow in the microcosm (Wellington *et al.*, 1993). Obviously, this condition allowed higher production of vegetative cells which were fit in the competition for nutrients with the natural microbial population; which in turn facilitated conjugative gene transfer in soil. Therefore, in this work nutrient amendments (starch and chitin) and chloramphenicol were added together to nonsterile soils seeded with sonicated mycelia *S. lividans* Jni13C1 (**Fig.** 3.6). The results showed that the addition of chloramphenicol had no significant effect on the survival of Jni13C1 in the microcosm (data not shown). Possible

explanations are that chloramphenicol was inactivated through adsorption to soil particles, biological degradation or through deterioration due to its own intrinsic chemical instability (Williams and Vickers, 1986) or that surviving cells were dominant since the drug acts only on fully biochemically active, translating cells (Witmer et al., 1975).

During this experiment, some *Streptomyces*-like strains were isolated, most of which were resistant to chloramphenicol. Among them, four isolates (E30d-2. E30d-3, E50d-1 and E50d-3) showed strong similarities in their phenotypes to *S. lividans* (cf. **Sect.** 3.6.2). The question arose how to know whether they were natural soil isolates or the Cml^R derivatives of recipient Jni13C1. The effort to further characterize them would have required to analyze larger parts of their chromosomal DNA, e.g. by restriction mapping, hybridization, PCR, and sequencing, which was not possible within the scope and time scale of this work. However, experiments of this kind should be repeated in the future. The four Cml^R soil strains were characterized by the detection of special chromosomal regions via PCR, genomic fingerprinting assays including RAPD, RFLPs of 16S rRNA and PFGE. However, the combination of these methods still could not bring a clear conclusion. The four isolates showed similar RAPD and RFLPs patterns to the strain Jni13C1 but some differences in PFGE. The result of the PFGE rather led to the conclusion that the four strains were not likely to be derived from Jni13C1 (cf. **Sect.** 3.6). However, it was also possible that the differences were due to large scale rearrangements after the integration and recombination of the Cml^R determinants to the chromosome of Jni13C1. Similar effects of large scale genome rearrangement had been frequently observed in *Streptomyces* sp. (cf. **Sect.** 1.1). In conclusion, the feasibility of the "gene sponges" approach is still likely and should be further evaluated in the future.

5.2: Experiments on acarbose biosynthesis in *Actinoplanes* sp. SE50/110

The second main goal of this work was to elucidate several biosynthetic steps for acarbose in *Actinoplanes* sp. SE50/110. The *acb*-gene cluster for acarbose production had been identified and sequenced before, but several partial sequences had to be proven again throughout this work. Biosynthetic functions of several Acb-proteins were investigated in this work by expressing them heterologously in *E. coli* K12 and in *S. lividans* 66, and characterizing their catalytic activities.

5.2.1: Heterologous expression of Acb-proteins in *E. coli* K12 and in *S. lividans* 66

In order to achieve the soluble proteins of AcbK, AcbM, AcbL, AcbN, AcbO and AcbU for enzyme assays, different expression systems in *E. coli* and in *S. lividans* were tested. *E. coli* is the most frequently used prokarotic expression system for the high-level production of heterologous proteins (Hannig and Makrides, 1998). The dominance of *E. coli* in this field is a reflection of the depth of information on its genetic and biochemical systems generated over many decades of research, both academic and industrial (Binnie *et al.*, 1997). Several *E. coli* expression systems have been tested in this work, such as the expression under the control of the *T7* (pET-vectors) and *rha* (pJOE2702 and pJOE2775) promoters. *E. coli* is probably unsurpassed in its ability to generate products in high yield within the bacterial cytoplasm. The protein is usually produced in the form of inclusion bodies (Binnie *et al.*, 1997). For example, in this work, the AcbM, AcbL and AcbO proteins were overproduced in the insoluble form as N-terminal His-tag fusion proteins in *E. coli*. It seemed that addition of His-tag to the N- or C-terminus facilitated the expression of the *acb*-genes in this case because none of these proteins was overexpressed in the native form under the same conditions. Alternatively, strains of the actinomycete *S. lividans* were employed as hosts for heterologous production of Acb-proteins. Surprisingly, nearly in all cases (AcbM, AcbL, AcbN, AcbO and AcbU), the overproduced Acb-proteins with N-terminal His-tag were only partially soluble in *S. lividans*, too (cf. **Tab.** 4.4).

There are some possible explanations for the formation of inclusion bodies (Wall and Plückthun, 1995). Successful protein folding requires that the end product is a thermodynamically stable entity. Many reports of only insoluble material being produced upon expression of a particular protein in *E. coli* are not surprising, in that something thermodynamically impossible was attempted. Almost invariably, truncated domains are severely destabilized and often totally

unable to fold to monomeric protein. Similarly, dimeric complexes or multi-subunit assemblies may not tolerate the absence of a subunit, by virtue of a large hydrophobic subunit interface becoming exposed. In such cases, aggregation may be the only option for the protein to cover its hydrophobic surface. Several expression strategies could be developed to aid the formation of the native three-dimensional protein structure, including coexpressing molecular chaperones, using thioredoxin-deficient host strains to maintain a favorable redox potential, reducing the rate of protein synthesis, growing bacterial cultures at lower temperatures and using highly soluble polypeptides as fusion partners (Hannig and Makrides, 1998). However, none of these methods had been really adapted as yet to actinomycete expression systems.

Another interesting phenomenon appeared during the heterologous production of the AcbK protein. In all cases AcbK was overproduced. However, in case of *S. lividans* TK23/pCW201KL, AcbK was produced only in the form of inclusion bodies. And no AcbK activity was detectable in the corresponding cell-free extract, suggesting that soluble proteins are essential in determining their enzyme activity. In this case the insert of expressing plasmid pCW201KL (3.1 kb *acbKL* fragment) was not fully sequenced. Hence, some mutations could have occurred during PCR and might have changed some amino acid residues of the produced AcbK protein. By this way a correct folding of the AcbK protein could have been impaired.

5.2.2: Two kinases – AcbK and AcbM

The gene for the acarbose 7-kinase, *acbK*, was identified in a putative operon, *acbKLMNOC* (cf. **Fig.** 1.6), by the identity of the first 20 amino acids of the N-terminal peptide sequence obtained from the purified enzyme by others (Drepper and Pape, 1996), with that of the deduced AcbK protein sequence. Also, AcbK exhibited significant similarity to members of the ribokinase family of phosphotransferases (COG0524, **App.** 7.3) (Tatusov *et al.*, 2001). The gene *acbM* was also included in this putative operon. The deduced AcbM protein is distantly related to members of one of the bacterial glucokinase families (represented by the GlcK proteins from *Bacillus subtilis* and *S. coelicolor* A3(2); COG1940, **App.** 7.4). In this work, both proteins were heterologously overproduced in *S. lividans* 66 strains and characterized *in vitro* as acarbose 7-kinase (AcbK) and 2-*epi*-5-*epi*-valiolone 7-kinase (AcbM), respectively.

The C-7 position of the cyclitol moiety of free acarbose molecules is phosphorylated inside cells by the acarbose 7-kinase, encoded by the *acbK* gene (Goeke *et al.*, 1996; Drepper and Pape,

1996). During tests for the substrate specificity of AcbK, a weak activity had also been measured with two C7-cyclitols not directly related to the valienol metabolism (Drepper and Pape, 1996). These assays had been carried out with an indirect measurement of ATP consumption and no direct determination of the phosphorylated products was achieved. However, in all experiments carried out in this work, the heterologously overproduced AcbK acted only on the oligomeric end product, acarbose, and not on any of the monomeric C7-cyclitols being likely precursors of valienol or valienol itself (cf. **Fig.** 4.9, **Tab.** 5.1).

Tab. 5.1: Substrate spectrum of AcbK and AcbM.

Substrate	Phosphorylation by	
	AcbK	AcbM
2-*epi*-5-*epi*-valiolone	-	+
2-*epi*-5-*epi*-valiolol	-	(±)
1-*epi*-2-*epi*-5-*epi*-valiolol	-	(±)
1-*epi*-5-*epi*-valiolol	-	-
5-*epi*-valiolol	-	-
1-*epi*-valienol	-	-
Valienol	-	-
Acarbose	+	-

All substrates were used in phosphorylation assays using nonlabelled ATP and [γ-^{32}P] ATP as described in **Sect.** 2.25. +, phosphorylation; (±) weak phosphorylation, detectable only with [γ-^{32}P] ATP; -, no phosphorylation

Very interestingly, it was demonstrated in this work that 7-*O*-phosphorylation of 2-*epi*-5-*epi*-valiolone should be the next step after cyclization of *sedo*-heptulose-7-phosphate catalyzed by the AcbC protein during the biosynthesis of acarbose. This unexpected step is catalyzed by the novel kinase AcbM (**Sect.** 4.5). The similarity of AcbM with glucokinases is intriguing since both these enzymes, D-glucose 6-kinase (GlcK) and 2-*epi*-5-*epi*-valiolone 7-kinase (AcbM) phosphorylate the primary hydroxyl groups on a cyclic polyol with a six-member ring system. This might indicate that not only valienol, but also its precursor 2-*epi*-5-*epi*-valiolone, had a distinct structural resemblance to hexoses and therefore the C7-cyclitol unit of acarbose and its precursors functionally behaved as sugar-related metabolites. However, the 7-*O*-phosphorylation by AcbM suggests that most of the cyclitol precursors were metabolized inside cells mainly in their phosphorylated or nuleotidylated forms. Furthermore, AcbM specifically phosphorylated only the monomeric cyclitol, 2-*epi*-5-*epi*-valiolone, the first cyclic intermediate of valienol biosynthesis, but none of the other monomeric C7-cyclitols tested (cf. **Tab.** 5.1). Therefore, it is

concluded that AcbM is a biosynthetic enzyme, whereas AcbK has another, possibly protective and/or transport-prone function(s) (see below).

Interestingly, both AcbM and AcbK phosphorylated the C7-cyclitol moiety at the 7-hydroxyl group. The phosphorylation could be necessary in order to prevent an inhibitory effect of the C7-cyclitol- and acarviosyl-containing intracellular metabolites on cytoplasmic enzymes, such as α-glucosidases or glucomaltases of the producer, during the *de novo* biosynthesis of acarbose. For instance, the cytoplasmic and acarbose-sensitive maltase activity of *Actinoplanes* sp. SE50 is no longer strongly inhibited by the modified inhibitor acarbose-7-phosphate (Drepper and Pape, 1996). Therefore, this AcbK-catalyzed phosphorylation can be regarded somehow as a resistance like self-protection mechanism, similar to those in other aminoglycoside producers, which frequently use phosphorylation for inactivation of their self-toxic products (Piepersberg and Distler, 1997; Cundliffe, 1989). Similarly, it was shown previously that two 6-phosphorylation steps were also involved in the *de novo* biosynthesis of streptomycin in *S. griseus* strains, which were catalyzed by a biosynthetic 6-kinase (probably StrN) acting on a monomeric cyclitol intermediate, N-amidino-streptamine, and a resistance-conferring streptomycin 6-kinase (AphD), respectively (reviewed in Piepersberg and Distler, 1997; Walker, 1975). These phosphorylations always keep the bioactive metabolites in an inactivated state intracellularly. However, the 6-phosphorylated streptomycin also seems to be the export form of this antibiotic and becomes only dephosphorylated to the bioactive form by a specific phosphatase (StrK) outside the cell (Mansouri and Piepersberg, 1991). This seems to be a wide-spread strategy used in producers of self-toxic secondary metabolites since similar modification and export strategies in biosynthetic routes, resistance mechanisms, and active export are used also in the producers of macrolides (by glucosylation; Mendez and Salas, 2001), puromycin and phosphinothricin (by acetylation; Thompson and Seto, 1995; Tercero *et al.*, 1996). Therefore, it was speculated that the 7-phosphorylation of acarbose-related metabolites (the intracellular end product of the underlying pathway has not yet been identified) in addition has a role in their export to the environment.

5.2.3: A probable epimerase - AcbO

The *acbO* gene lies also in the putative transcription unit *acbKMLNOC* (**Fig.** 1.6). The deduced AcbO protein sequence showed no significant similarity to those in data banks. The AcbO protein is only distantly related to SGBU (28.9% identity in 159 aa overlap; **Fig.** 5.4), a putative

hexulose-6-phosphate isomerase from *E. coli*, which probably catalyses the isomerization of D-arabino-6-hexulose-3-phosphate to D-fructose-6-phosphate (Reizer *et al.*, 1996).

In this work, it was demonstrated that the enzyme AcbO probably represented a novel type of isomerase. In the presence of AcbO, 2-*epi*-5-*epi*-valiolone-7-phosphate was converted to a new product with the same molecular mass, but the non-phosphorylated 2-*epi*-5-*epi*-valiolone was not a substrate for AcbO. Furthermore, coenzymes such as $NAD(P)^+$, NAD(P)H were not required for this conversion. These data indicated that AcbO catalyzed an epimerization of 2-*epi*-5-*epi*-valiolone-7-phosphate, probably at C-2, to give 5-*epi*-valiolone-7-phosphate, which is postulated to be the second conversion step in modifying the cyclitol precursor 2-*epi*-5-*epi*-valiolone after phosphorylation by AcbM during the biosynthesis of acarbose. Evidentially, a similar epimerization step was also assumed to be involved in the biosynthesis of validamycin (Dong *et al.*, 2001; see below). However, to get a clear conclusion, the product of the AcbO-catalyzed reaction should be also preparatively purified and subjected to NMR analysis for elucidation of its structure in the future.

```
AcbOAsp         -VTCRVGLTEWRLAP--SGAAAIRLAAAVGADGIQLDFGGPGR--GVLVDGPGRAGQLRA   55
SGBUEcoli       MRNHQLGIYEKALAKDLSWPERLVLAKSCGFDFVEMSVDETDERLSRLDWSAAQRTSLVA   60
                  . ::*: *  **   * .  : ** : * * :::... ...  . *  ...:  .* *

AcbOAsp         VADEAGVDLLALAGNLLNDIGLTSQPAVVQP----VLARLADTATELGVPLLIVPSFRR-   110
SGBUEcoli       AMIETGVGIPSMCLSAHRRFPFGSRDEAVRERAREIMSKAIRLARDLGIRTIQLAGYDVY   120
                .  *:**.: ::. .  . : : *:  .*:     ::::    * :**:  : :..:

AcbOAsp         SAITDAMSFTRTAAALRWAVSLAEARGIVLASENVLPP-----ARARQLVEEVGSPAFRL   165
SGBUEcoli       YEDHDEGTRQRFAEGLAWAVEQAAASQVMLAVEIMDTAFMNSISKWKKWDEMLASPWFTV   180
                    *  :  * * .* ***. * *  ::** * : ..     :: ::  * :.** * :

AcbOAsp         LLDTFNPVRYGLEPAWLATELRPWWADQIHLKDGPPDTGPSP----LLGAGQGGVRRTLT   221
SGBUEcoli       YPDVGNLSAWGND-VPAELKLGIDRIAAIHLKDTQPVTGQSPGQFRDVPFGEGCVDFVGI   239
                  *. *   :* : .    :*       *****  * ** **     :  *:* *  .

AcbOAsp         ALRGSPAPVRALVLENDYRDGHGARLRADLEWARRAAVNARESEKGKLT 270
SGBUEcoli       FKTLHKLNYRGSFLIEMWTEKAKEPVLEIIQARR--WIEARMQEAGFIC 286
                         *. .* : : :     :   ::  *   ::** .* * :
```

Fig. 5.4: Similarity between AcbO and a putative isomerase from *E. coli*. AcbOAsp = AcbO protein from *Actinoplanes* sp. SE50/110, SGBUEcoli = a putative hexulose-6-phosphate isomerase from *E. coli* (U00039).

5.2.4: Two putative dehydrogenases – AcbL and AcbN

The genes, *acbL* and *acbN*, are also identified in the putative operon *acbKMLNOC* (cf. **Fig.** 1.6). The deduced protein sequences of AcbL and AcbN are distantly related to the alcohol dehydrogenase (ADH, EC: 1.1.1.1), which catalyzes the reversible oxidation of ethanol to

acetaldehyde with the concomitant reduction of NAD(P). Currently three structurally and catalytically different types of alcohol dehydrogenases are known: (i) zinc-containing 'long-chain' alcohol dehydrogenases (COG1062), (ii) insect-type, or 'short-chain' alcohol dehydrogenases (COG1028), (iii) iron-containing alcohol dehydrogenases (COG1979) (Reid and Fewson, 1994). AcbL is clearly a member of the zinc-containing family of ADHs, whereas AcbN shows similarity to members of the family of short-chain dehydrogenases/reductases (SDR). Zinc-containing ADHs are dimeric or tetrameric enzymes that bind two atoms of zinc per subunit and have a highly conserved signature in their protein sequence: G-H-E-x(2)-G-x(5)-[GA]-x(2)-[IVSAC], which is also present in the deduced AcbL protein sequence (cf. **App.** 7.5). The family of the short-chain dehydrogenases/reductases (SDR) is a very large family of enzymes, most of which are known to be NAD- or NADP-dependent oxidoreductases. Most members of this family are proteins of about 250 to 300 amino acid residues. Most dehydrogenases possess at least 2 domains, the first binding the coenzyme, often NAD(P), and the second binding the substrate. A conserved Gly-rich pattern (GXXXGXG) is observed in members of this family (Reid and Fewson, 1994), which is also found in the AcbN protein sequence (cf. **App.** 7.6). These indicated that both AcbL and AcbN proteins may act as dehydrogenases or related enzymes, using a catalytically NAD(P)-dependent oxidoreductase mechanism (such as dehydratases). Therefore, AcbL and AcbN were the most likely candidates which could catalyze the dehydration between C5 and C6 and the reduction of the C-1 keto group, respectively.

The postulated functions of AcbL and AcbN were tested in this work. Neither could catalyze the conversion of 2-*epi*-5-*epi*-valiolone and other tested cyclitols (cf. **Fig.** 4.9). The discovery of AcbM activity suggests that both proteins AcbN and AcbL require phosphorylated cyclitols as substrates. However, in the assays combining all four heterologously overproduced proteins (AcbMLNO), no visible further conversion of the AcbO product (5-*epi*-valiolone-7-phosphate) was observed (cf. **Fig.** 4.20). This negative result could be due to the following reasons: (i) in these assays, coenzymes, $NAD(P)^+$ and NAD(P)H were not included, which could be essential for activity of these oxidoreductases; (ii) the tested proteins (AcbL and AcbN) were in the form of N-terminal His-tag versions, in which the N-terminal extender peptide in direct neighborhood to the dinucleotide binding site (which is also at the N-terminus of both proteins) might have had a negative effect on the enzyme activity; (iii) the conditions of the combined assays were not optimal for the activity of these distinct oxidoreductases, e.g. pH or the presence of ATP, salts

and their concentrations and Tris buffer. Therefore, the activity of these two proteins should be further investigated, also in their native or C-terminal His-tag forms. Further assays should include the coenzymes such as $NAD(P)^+$, NAD(P)H, some other cofactors (e.g. Zn^{2+} for AcbL), and use the purified 5-*epi*-valiolone-7-phosphate.

5.2.5: 1-*epi*-valienol, an intermediate of the acarbose biosynthesis pathway?

In an earlier report, two cyclitols, valienol and 1-*epi*-valienol, were identified in the fermentation broth and cell-free extracts of *Actinoplanes* sp. SN223/29, a derivative of strain SE50 (Mahmud *et al.*, 1999). Analyses of the time course of the concentrations of these two compounds in the cultures indicated that valienol appeared rather late in the fermentation, after substantial amounts of acarbose had accumulated, suggesting that it might be a degradation product of acarbose. Whereas 1-*epi*-valienol appeared early in the fermentation, consistent with a possible role as a precursor of acarbose. Furthermore, 1-*epi*-valienol was found to be phosphorylated by the cell-free extract of *Actinoplanes* sp. SE50/110 in this work (see **Sect.** 4.4). Interestingly, the presence of NH_4Cl in the cell disruption buffer turned out to be essential for the 1-*epi*-valienol kinase activity in the cell-free extract. Absence of this salt gave no phosphorylation of 1-*epi*-valienol and the activity could not be restored by the addition of NH_4Cl to the reaction buffer (data not shown). This indicated that the presence of NH_4Cl during protein release from intact cells was crucial for maintaining this enzyme activity.

However, none of the currently available overproduced Acb-proteins, including AcbK, M, L, N, O and AcbU, could be identified to be responsible for the phosphorylation of 1-*epi*-valienol. Especially, this kinase activity was not detected in cell-free extracts of *S. lividans* 1326 /pHTWCos6, although acarbose or acarobose-like compounds were produced by this strain (cf. **Sect.** 4.3.3). Furthermore, feeding experiments gave no incorporation of 1-*epi*-valienol into acarbose (Mahmud *et al.*, 1999). These facts excluded the possibility that 1-*ep*i-valienol was a direct intermediate in the formation of the cyclitol moiety of acarbose. However, the discovery of the 2-*epi*-5-*epi*-valiolone 7-kinase, AcbM, suggested that 1-*epi*-valienol-7-phosphate but not 1-*epi*-valienol was the right precursor. Furthermore, no experimental evidence was available so far to show that, in which position (e.g. C1 or C7) the substrate 1-*epi*-valienol was phosphorylated. Therefore, structure elucidation of this phosphorylated product via NMR is necessary in further understanding the role of 1-*epi*-valienol (phosphate) in acarbose biosynthesis.

5.2.6: A new model proposed for the biosynthesis of acarbose in *Actinoplanes* sp. SE50/110

Previously, labeling and feeding studies with possible cyclitol precursors of both acarbose and validamycin A had been performed in the producers *Actinoplanes* sp. SE50 and *S. hygroscopicus* var. *limoneus*, respectively, in attempts to elucidate the biosynthetic pathway leading to the formation of the common valienol moiety shared by both drugs. However, only the product of the cyclase AcbC, 2-*epi*-5-*epi*-valiolone, was identified as a common precursor for both acarbose and validamycin A biosyntheses by this way (Stratmann *et al.*, 1999; Mahmud *et al.*, 1999). In contrast to acarbose, besides 2-*epi*-5-*epi*-valiolone, some more intermediates (5-*epi*-valiolone, valienone, validone) were also efficiently incorporated into the trehalase inhibitor validamycin A (Dong *et al.*, 2001). These data indicated that there must be fundamental differences between the two pathways for acarbose and validamycin A. Based on the currently available information from the feeding experiments, here a pathway for validamycin A is presented (**Fig.** 5.5).

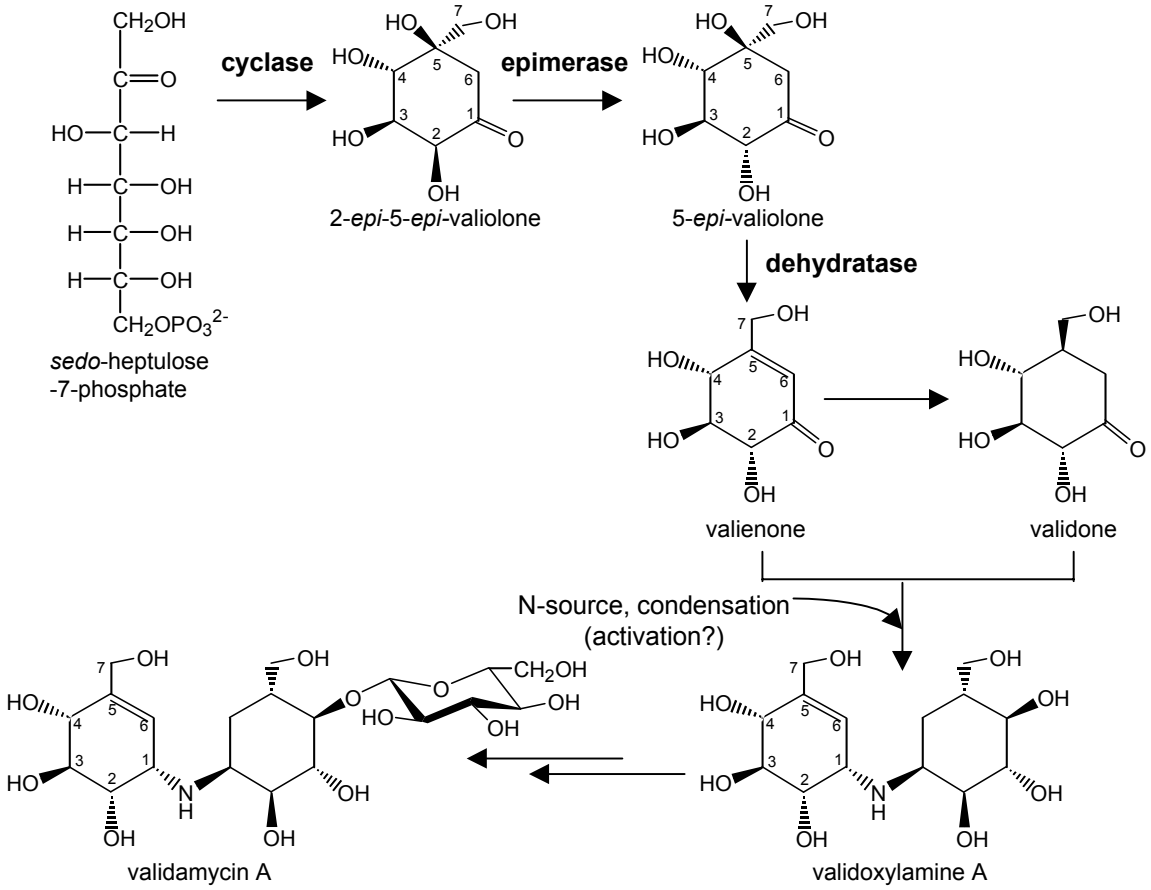


Fig. 5.5: Proposed pathway for the formation of the cyclitol moiety of validamyin A in *Streptomyces hygroscopicus* var. *limoneus*. For details see Mahmud *et al.* (2001b).

The biosynthesis of the valienamine moiety of validamycin also starts with a C-C cyclization of *sedo*-heptulose-7-phosphate to 2-*epi*-5-*epi*-valiolone, which then becomes further epimerized to 5-*epi*-valiolone and dehydrated to give valienone. The incorporation of the nitrogen into validamycin is still unclear. Validone, the second cyclitol moiety of validamycin is postulated to be synthesized from valienone in a reduction step (cf. Fig. 5.5; Mahmud *et al.*, 2001a; 2001b). So far, no needs for phosphorylated intermediates nor their presence were detected in the validamycin biosynthetic pathway. Thus, it can be concluded that *S. hygroscopicus* var. *limoneus* does not use phosphorylated intermediates throughout the pathway forming validamycins. However, it can not be excluded that a phosphorylation of either valienamine or validone is necessary, at least for the activation before the condensation step. Furthermore, the genetics and biochemistry of validamycin biosynthesis has not yet been studied in the producing strain so far.

Investigations on acarbose biosynthesis were carried out in parallel by the isolation of the *acb*-gene cluster from the producer *Actinoplanes* sp. SE50/110 (cf. **Sect.** 1.7). The identification and fully sequencing of the *acb*-gene cluster made it possible to study the biosynthesis of acarbose on the biochemical level by performing single enzyme assays. By this way, some Acb-proteins, e.g. AcbC, AcbD and AcbV had already been characterized experimentally before, which gave useful information in the elucidation of the pathway for acarbose (cf. **Sect.** 1.8). Another important step towards its further understanding has been made in this work by the discovery of the novel kinase, AcbM, catalyzed the 7-*O*-phosphorylation of 2-*epi*-5-*epi*-valiolone. This led to the proposal of a new and much more consistent biosynthetic pathway for the acarviosyl moiety of acarbose (**Fig.** 5.6). After the two initial steps catalyzed by AcbC (step 1) and AcbM (step 2), further conversion of the AcbM product, 2-*epi*-5-*epi*-valiolone-7-phosphate, is catalyzed by the probable 2-epimerase AcbO (step 3). These facts suggest that phosphorylated intermediates are required for acarbose biosynthesis also in the downstream pathway and explain why in feeding experiments no free cyclitol intermediates other than 2-*epi*-5-*epi*-valiolone could be incorporated into acarbose. Further modifications are assumed to be followed by 5,6-dehydration (step 4), 1-reduction (step 5) in unknown order, resulting in 1-*epi*-valienol(-7-phosphate) and by a second phosphorylation (step 6). A subsequent nucleotidylation (step 7) at C-1 then results in NDP-1-*epi*-valienol(-7-phosphate). The incorporation of the amino nitrogen occurs by transamination of the dTDP-4-keto-6-deoxyglucose of the sugar moiety but not the cyclitol moiety catalyzed by AcbV (Diaz-Guardamino, 2000).

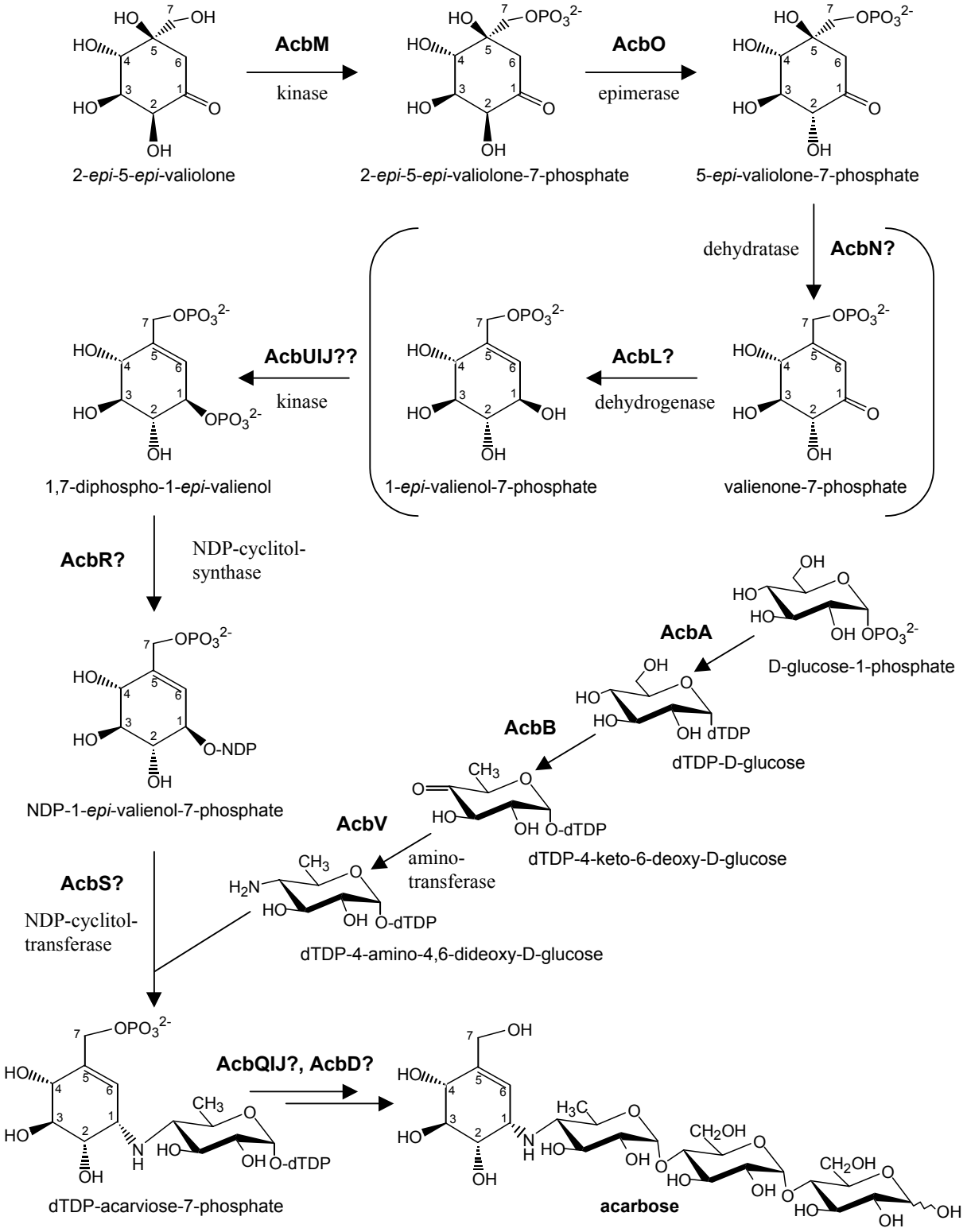


Fig. 5.6: Newly proposed pathway for the biosynthesis of acarbose in *Actinoplanes* sp. SE50/110. The order of steps in the bracketed part is unknown. For detailed explanations see text.

The other still hypothetical steps of valienol formation (steps 4 - 7) are postulated on the basis of the genetic record deduced from the *acb*-gene cluster (cf. **Fig.** 1.6, **Tab.** 1.4). The functions of the AcbN and AcbL proteins have been discussed in **Sect.** 5.2.4. They are the most likely candidates which catalyze the 5,6-dehydration and 1-reduction, respectively. The AcbU protein putatively is a member of the antibiotic protein kinase superfamily and therefore could catalyze the 1-phosphorylation of 1-*epi*-valienol(-7-phosphate). The *acbR* gene encodes a GlgC (ADP-glucose synthase, COG0448)-like protein, which could catalyze the probable nucleotidyl activation of 1-*epi*-valienol-1-phosphate (or more likely 1-*epi*-valienol-1,7-diphosphate). The identification of the *acbS* gene, which encodes a glycosyltransferase like enzyme (COG0297), leads to the proposal that the acarviose is the condensation product of NDP-(1-*epi*-)valienol-7-phosphate and dTDP-4-amino-4,6-dideoxy-D-glucose. This condensation could be fulfilled by the AcbS protein. It is unclear whether a pathway-specific regulation system exists for the biosynthesis of acarbose in *Actinoplanes* sp. SE50/110. The *acbP* gene possibly encodes a NUDIX-hydrolase, which has been discussed to be likely involved in the regulation of acarbose biosynthesis on the level of substrate pools (Thomas, 2001). If, e.g. the AcbV product, dTDP-4-amino-4,6-didesoxy-D-glucose, is limiting for the condensation with NDP-1-*epi*-valienol-7-phosphate to form dTDP-acarviose-7-phosphate during acarbose biosynthesis (cf. **Fig.** 5.6), the AcbP protein will hydrolyze the toxic NDP-1-*epi*-valienol-7-phosphate to prevent its intracellular accumulation. The hydrolyzed product 1-*epi*-valienol-7-phosphate might be further lysed by a phosphatase (e.g. encoded by *acbI* or *acbJ*), resulting in the intracellular accumulation of 1-*epi*-valienol, as observed by Mahmud *et al.* (1999). In this view, 1-*epi*-valienol is postulated to be a product of a regulation process rather than an intermediate for the biosynthesis of acarbose.

Furthermore, it is still unclear, whether acarviose (or activated avarviose, or acarviose-7-phosphate) or acarbose(7-phosphate) itself is the final product that is synthesized inside the cell. So far it is also unknown whether the glucose or maltose moieties of acarbose are introduced inside the cell or after the export of the activated acarviose. If acarviosylglucose(-7-phosphate) is the exported product, the acarviosyltransferase AcbD described by Hemker *et al.* (2001) could be a candidate that adds, via transglycosylation, the maltose or maltotriose residues to the acarviosyl unit outside the cell to form acarbose and the higher order oligomeric components extended at the reducing end of the molecule found in the fermentation broths of *Actinoplanes* sp. SE50.

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7: Appendices

7.1: The nucleotide and encoded amino acid sequences of the *acbKMLNO* genes (under accession number: Y18523)

```
1/1                                     31/11
acbK  --->
M   S   E   H   T   D   V   L   V   L   G   G   A   G   V   D   T   I   A   Y
ATG TCG GAG CAC ACC GAC GTC CTC GTC CTC GGC GGC GCC GGC GTC GAC ACC ATC GCT TAC

61/21                                   91/31
V   P   E   L   P   L   P   F   Q   D   S   Y   V   V   A   A   I   E   P   R
GTG CCC GAA CTG CCG CTG CCG TTC CAG GAC AGC TAC GTG GTC GCG GCC ATC GAA CCA CGA

121/41                                  151/51
A   G   Q   T   G   D   N   V   A   L   G   L   H   T   L   G   L   R   T   M
GCC GGG CAG ACC GGC GAC AAC GTC GCT CTC GGC CTG CAC ACG CTG GGC CTG CGC ACG ATG

181/61                                  211/71
H   V   D   V   L   G   D   D   P   E   G   D   L   V   R   A   F   H   T   R
CAC GTC GAC GTG CTC GGC GAC GAC CCG GAA GGT GAC CTG GTC CGG GCC TTC CAC ACC CGG

241/81                                  271/91
H   G   L   P   F   A   A   L   P   T   A   A   G   T   K   R   A   V   N   L
CAC GGT CTG CCG TTC GCC GCC CTG CCC ACG GCC GCC GGC ACC AAG CGG GCG GTG AAC CTG

301/101                                 331/111
V   G   P   D   G   R   R   L   S   L   W   D   G   S   R   E   A   E   E   D
GTC GGC CCG GAC GGC CGC CGG CTG TCG CTG TGG GAC GGC AGC CGG GAA GCC GAG GAG GAC

361/121                                 391/131
R   Y   P   A   A   L   I   A   A   H   T   A   H   A   R   H   V   H   V   C
CGC TAC CCG GCC GCC CTG ATC GCC GCG CAC ACC GCC CAC GCC CGG CAC GTG CAC GTC TGC

421/141                                 451/151
I   T   P   P   G   Q   H   V   F   G   Q   L   N   D   L   P   V   T   V   S
ATC ACC CCG CCC GGG CAG CAC GTG TTC GGC CAG CTC AAC GAC CTG CCG GTC ACC GTC TCC

481/161                                 511/171
T   D   L   H   N   W   D   G   A   Y   E   G   F   E   V   Y   A   F   N   A
ACC GAC CTG CAC AAT TGG GAC GGC GCC TAC GAG GGC TTC GAG GTC TAC GCC TTC AAC GCC

541/181                                 571/191
D   L   V   F   L   S   A   T   A   L   T   D   V   A   A   T   M   R   R   V
GAC CTG GTG TTC CTG TCG GCG ACC GCG CTG ACC GAC GTG GCC GCG ACG ATG CGC CGC GTC

601/201                                 631/211
I   D   R   G   R   A   R   L   V   V   A   T   G   R   A   H   G   G   S   V
ATC GAC CGG GGC CGG GCC CGG CTG GTG GTG GCC ACC GGA CGG GCC CAC GGC GGA TCG GTG

661/221                                 691/231
L   V   R   R   R   K   A   E   V   R   R   Y   A   A   V   A   P   E   A   P
CTG GTG CGC CGG CGG AAA GCG GAG GTC CGG CGG TAC GCG GCG GTG GCC CCC GAG GCG CCG

721/241                                 751/251
V   V   D   S   N   G   A   G   D   A   F   V   S   G   F   L   F   G   H   L
GTG GTG GAC TCC AAC GGC GCC GGT GAC GCG TTC GTG TCC GGC TTC CTC TTC GGG CAC CTG
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781/261                                 811/271
A   G   E   P   L   E   T   C   P   R   Y   G   A   I   A   G   A   Y   A   C
GCC GGC GAA CCG CTG GAG ACC TGC CCT CGC TAC GGC GCG ATC GCC GGC GCC TAC GCG TGC

841/281                                 871/291
                                                          acbM  ---> V
T   I   P   A   T   R   A   G   A   I   D   R   A   A   L   L   R   P   A   A
ACC ATC CCC GCC ACC CGG GCC GGC GCC ATC GAC CGG GCC GCG CTG CTG CGG CCG GCG GCG

901/301                                 931/311
  K   R   P   P   H   H   P   V   T   V   A   D   V   G   G   T   H   L   R   W
*  --->  acbK
TGA AGC GGC CAC CGC ACC ACC CGG TCA CGG TGG CCG ACG TGG GCG GCA CCC ACC TGC GCT

961/321                                 991/331
  A   R   W   S   P   D   G   G   L   G   E   V   H   T   T   P   S   P   G   H
GGG CAC GGT GGT CAC CGG ACG GCG GAC TGG GCG AAG TGC ACA CCA CGC CGT CTC CGG GGC

1021/341                                1051/351
  A   R   R   P   G   A   G   A   A   D   L   Q   A   E   L   I   R   E   L   A
ACG CCC GGC GGC CCG GCG CCG GTG CCG CCG ACC TGC AGG CGG AAC TGA TCC GCG AGC TGG

1081/361                                1111/371
  S   R   V   E   P   G   A   R   A   G   V   S   L   G   A   A   M   D   H   H
CGT CGC GGG TCG AGC CGG GTG CGC GGG CCG GTG TCT CGC TCG GCG CGG CCA TGG ACC ACC

1141/381                                1171/391
  S   G   Y   A   Y   A   S   A   P   L   W   G   P   Q   V   S   P   F   D   V
ACA GTG GTT ACG CGT ACG CCT CCG CGC CGT TGT GGG GTC CGC AGG TCA GCC CGT TCG ACG

1201/401                                1231/411
  P   A   A   L   R   A   A   R   P   D   V   H   W   T   V   V   N   D   V   T
TGC CGG CCG CCC TGC GCG CCG CCC GGC CGG ACG TGC ACT GGA CCG TGG TCA ACG ACG TCA

1261/421                                1291/431
  A   G   L   L   H   L   A   E   M   V   R   D   A   G   V   R   K   A   C   L
CGG CGG GGC TGC TGC ACC TGG CCG AGA TGG TTC GCG ACG CCG GCG TCC GCA AGG CGT GCC

1321/441                                1351/451
  V   T   I   S   T   G   I   A   C   R   T   M   D   L   R   T   G   G   I   P
TGG TCA CGA TCA GCA CCG GCA TCG CCT GCC GGA CGA TGG ACC TGC GCA CCG GCG GTA TCC

1381/461                                1411/471
  V   D   A   A   G   L   Q   G   E   I   G   H   L   P   A   T   V   L   A   D
CGG TCG ACG CGG CCG GCC TCC AGG GCG AGA TCG GTC ACC TGC CGG CGA CCG TGC TGG CCG

1441/481                                1471/491
  G   V   P   V   V   T   R   C   D   C   G   E   P   G   H   V   A   A   S   S
ACG GGG TGC CGG TGG TGA CCC GCT GCG ACT GCG GTG AGC CGG GGC ACG TGG CGG CGT CCT

1501/501                                1531/511
  S   G   P   G   I   R   R   V   A   A   V   L   A   R   R   D   P   A   T   W
CGT CCG GGC CGG GCA TTC GAC GGG TCG CGG CCG TCC TGG CCC GGC GTG ACC CGG CCA CCT

1561/521                                1591/531
  A   G   S   G   P   T   T   R   M   M   A   G   S   G   F   E   D   A   F   R
GGG CGG GCT CCG GGC CGA CCA CCC GGA TGA TGG CGG GCT CGG GCT TCG AGG ACG CGT TCC

1621/541                                1651/551
  A   A   L   D   D   G   D   P   V   A   A   D   L   L   T   A   V   T   A   P
GGG CCG CTC TGG ACG ACG GCG ATC CGG TCG CGG CGG ACC TGC TGA CGG CGG TGA CCG CCC

1681/561                                1711/571
  I   A   D   L   L   R   T   A   L   C   L   D   P   E   L   D   L   I   A   L
CGA TCG CGG ACC TGC TGC GGA CCG CCC TCT GCC TGG ACC CGG AAC TCG ACC TGA TCG CGC
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1741/581                                    1771/591
  T   G   G   V   A   H   G   L   E   P   H   Y   S   A   A   V   H   D   H   L
TGA CCG GTG GAG TCG CCC ACG GGC TGG AAC CGC ACT ACT CGG CGG CGG TGC ACG ACC ACC

1801/601                                    1831/611
  R   R   R   G   L   Y   L   T   S   E   R   E   P   D   W   L   T   G   R   I
TGC GGC GCC GGG GGC TCT ACC TGA CCT CCG AGC GGG AGC CGG ACT GGT TGA CCG GGC GGA

1861/621                                    1891/631
  R   V   V   P   P   A   T   A   D   P   L   V   G   A   G   L   A   A   L   A
TCC GGG TCG TCC CGC CGG CGA CGG CCG ATC CGT TGG TCG GCG CCG GTC TGG CCG CAC TGG

1921/641                                    1951/651
  A   G   P   V   P   A   Y   S   G   G   G   R   E   A   L   V   G   R   * ---> acbM
                                                          acbL --->   M   S
CGG CGG GGC CGG TCC CGG CGT ACT CCG GTG GTG GCC GGG AAG CGT TGG TCG GGC GAT GAG

1981/661                                    2011/671
 R   H   R   A   I   V   R   T   G   T   G   V   V   V   A   D   V   P   T   P
CCG GCA CCG CGC GAT CGT GCG CAC CGG CAC CGG CGT GGT GGT CGC CGA CGT CCC GAC CCC

2041/681                                    2071/691
 V   P   G   P   G   E   L   L   V   G   T   E   W   A   G   L   C   G   T   D
GGT GCC GGG GCC GGG AGA GCT GTT GGT CGG CAC CGA GTG GGC GGG ACT GTG CGG CAC CGA

2101/701                                    2131/711
 I   Q   M   L   R   G   L   R   D   D   P   E   P   I   I   G   H   E   G   I
CAT CCA GAT GCT GCG CGG CCT GCG CGA CGA TCC CGA ACC GAT CAT CGG CCA CGA GGG GAT

2161/721                                    2191/731
 A   R   V   I   A   A   G   D   G   V   P   G   T   L   R   P   G   T   L   V
CGC CCG GGT GAT CGC GGC CGG CGA CGG CGT GCC CGG CAC GCT GCG GCC GGG GAC CCT GGT

2221/741                                    2251/751
 A   V   N   P   T   H   R   D   D   P   S   F   L   L   G   H   N   V   P   G
GGC GGT CAA CCC GAC CCA CCG GGA CGA TCC GTC GTT CCT GCT CGG GCA CAA CGT TCC CGG

2281/761                                    2311/771
 L   L   Q   E   R   T   L   L   P   A   T   A   V   S   G   G   L   V   L   P
CCT GCT GCA GGA ACG GAC GCT GCT GCC GGC CAC CGC GGT CTC CGG TGG CTT GGT GCT CCC

2341/781                                    2371/791
 L   P   D   G   T   D   V   T   L   G   P   L   L   E   P   L   A   V   V   S
GCT GCC GGA CGG CAC CGA CGT CAC GCT GGG CCC GCT GCT GGA ACC GCT GGC CGT GGT CAG

2401/801                                    2431/811
 Y   A   L   S   Q   L   G   A   V   R   P   A   T   L   V   I   V   G   D   G
CTA TGC GCT GAG CCA GCT CGG CGC CGT CCG CCC GGC CAC CCT GGT GAT CGT CGG CGA CGG

2461/821                                    2491/831
 T   V   G   H   L   A   A   R   A   A   V   T   W   L   A   E   R   P   R   V
AAC GGT GGG TCA CCT GGC CGC CCG GGC TGC GGT GAC CTG GCT GGC GGA GCG GCC CCG GGT

2521/841                                    2551/851
 V   L   V   H   H   T   A   A   G   R   D   F   S   A   A   G   P   H   P   A
GGT GCT GGT GCA CCA CAC GGC AGC GGG GCG GGA CTT CAG CGC GGC CGG GCC GCA TCC GGC

2581/861                                    2611/871
 D   V   L   L   T   T   G   E   L   A   G   R   P   L   P   G   P   V   A   A
GGA CGT TCT GCT CAC CAC CGG CGA GCT GGC GGG CAG ACC GCT GCC GGG ACC GGT GGC CGC

2641/881                                    2671/891
 L   L   A   T   P   R   D   A   T   V   E   A   L   E   A   V   L   A   A   A
CCT GCT GGC GAC CCC ACG GGA CGC GAC TGT CGA GGC CCT GGA GGC GGT CCT GGC CGC GGC
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2701/901                                    2731/911
 G   A   D   V   L   I   D   V   L   G   G   L   P   P   G   A   R   S   A   M
CGG CGC CGA TGT GCT GAT CGA CGT GCT GGG CGG CCT GCC CCC GGG CGC GCG ATC GGC GAT

2761/921                                    2791/931
 L   P   G   V   D   L   T   A   V   R   A   A   N   C   G   G   F   P   E   P
GCT GCC CGG TGT CGA TCT CAC CGC CGT GCG GGC GGC CAA CTG CGG CGG CTT CCC GGA ACC

2821/941                                    2851/951
 A   L   V   T   T   T   S   G   G   T   R   L   F   G   H   R   G   V   G   N
GGC TCT GGT CAC CAC CAC CTC CGG CGG AAC CCG GCT CTT CGG GCA TCG CGG GGT GGG CAA

2881/961                                    2911/971
 A   H   L   R   A   A   A   G   E   L   A   R   D   P   D   R   Y   R   D   L
CGC GCA CCT GCG GGC GGC GGC CGG CGA GCT TGC CCG CGA CCC GGA CCG GTA CCG GGA TCT

2941/981                                    2971/991
 V   T   H   R   N   D   L   A   G   A   A   R   I   M   T   A   L   R   S   G
CGT CAC CCA TCG CAA CGA TCT GGC CGG CGC GGC CCG GAT CAT GAC CGC GCT CCG GAG CGG

3001/1001                                   3031/1011
 R   D   R   I   I   D   G   R   R   L   V   K   L   A   I   Q   V   N   V   R
CCG GGA CCG GAT CAT CGA CGG CCG CCG ACT GGT GAA GCT CGC GAT CCA GGT CAA TGT CAG

3061/1021                                   3091/1031
 E   D   H   R   * ---> acbL
    acbN --->   M   S   G   T   L   V   G   R   R   A   L   V   V   G   G   T
AGA GGA TCA TCG ATG AGC GGG ACT CTG GTG GGC CGC CGC GCC CTG GTG GTG GGC GGA ACC

3121/1041                                   3151/1051
T   G   I   G   R   G   I   A   D   A   W   A   A   A   G   A   E   V   T   V
ACG GGC ATC GGC CGG GGC ATC GCC GAC GCG TGG GCC GCG GCC GGC GCC GAG GTG ACC GTC

3181/1061                                   3211/1071
C   G   R   S   E   P   A   G   D   G   A   A   G   L   H   W   E   P   L   D
TGT GGC CGC AGC GAG CCG GCC GGC GAT GGC GCC GCC GGG TTG CAC TGG GAG CCG CTC GAC

3241/1081                                   3271/1091
L   T   R   T   G   E   A   G   A   R   L   E   R   I   A   G   D   R   V   D
CTG ACC CGG ACC GGG GAG GCC GGT GCG CGC CTG GAA CGG ATC GCC GGT GAC CGG GTC GAT

3301/1101                                   3331/1111
L   A   C   F   A   A   V   S   Y   G   A   R   R   A   V   F   E   D   V   A
CTG GCC TGC TTC GCC GCG GTC TCC TAC GGG GCC CGG CGC GCC GTC TTC GAG GAC GTG GCG

3361/1121                                   3391/1131
V   D   E   W   R   T   Q   L   D   I   N   V   H   G   L   W   L   T   L   R
GTG GAC GAA TGG CGT ACC CAG CTG GAC ATC AAC GTG CAC GGC CTG TGG CTC ACC CTG CGG

3421/1141                                   3451/1151
A   A   L   P   G   L   R   A   A   R   P   G   L   F   V   G   V   S   S   E
GCG GCA CTG CCG GGG CTG CGT GCC GCC CGG CCC GGC CTG TTC GTG GGC GTC TCG TCC GAG

3481/1161                                   3511/1171
V   V   Y   N   A   G   P   G   R   S   G   Y   T   A   S   K   A   A   A   K
GTG GTC TAC AAC GCC GGG CCC GGC CGC TCC GGG TAC ACC GCC TCC AAG GCG GCC GCG AAG

3541/1181                                   3571/1191
A   L   L   E   S   V   A   Q   E   E   D   E   S   R   V   R   I   V   Q   V
GCG CTG CTG GAG TCG GTG GCC CAG GAG GAG GAC GAG AGC CGG GTC CGG ATC GTG CAG GTG

3601/1201                                   3631/1211
L   P   A   G   M   V   D   T   P   S   I   R   R   R   R   P   A   G   F   D
CTG CCG GCC GGG ATG GTC GAT ACT CCG AGC ATC CGG CGC CGT CGC CCG GCC GGC TTC GAC
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3661/1221                                   3691/1231
Y   S   D   Y   M   V   P   A   D   F   A   R   N   A   L   E   L   A   A   T
TAC AGC GAC TAC ATG GTG CCC GCC GAT TTC GCG CGC AAC GCG CTG GAA CTC GCC GCC ACG

3721/1241                                   3751/1251
A   G   A   G   H   H   G   D   S   L   V   V   D   R   G   G   D   W   W   S
GCC GGC GCC GGC CAC CAC GGG GAC AGC CTG GTG GTC GAC AGG GGC GGC GAC TGG TGG TCG

3781/1261                                   3811/1271
                                                  acbO ---> V   T   C   R   V   G
V   R   E   R   M   P   V   S   Q   S   R   P   V   R   T   *  ---> acbN
GTG CGC GAG CGG ATG CCG GTG TCG CAG AGC CGG CCG GTG CGG ACG TGA CCT GCC GGG TGG

3841/1281                                   3871/1291
  L   T   E   W   R   L   A   P   S   G   A   A   A   I   R   L   A   A   A   V
GGC TGA CCG AGT GGC GGC TGG CGC CCT CGG GTG CGG CGG CCA TCC GGC TGG CCG CGG CGG

3901/1301                                   3931/1311
  G   A   D   G   I   Q   L   D   F   G   G   P   G   R   G   V   L   V   D   G
TCG GTG CCG ACG GGA TCC AGC TCG ACT TCG GCG GCC CGG GCC GTG GCG TGC TGG TGG ACG

3961/1321                                   3991/1331
  P   G   R   A   G   Q   L   R   A   V   A   D   E   A   G   V   D   L   L   A
GCC CGG GCC GTG CCG GGC AGC TGC GGG CGG TCG CCG ACG AGG CGG GAG TGG ACC TGC TCG

4021/1341                                   4051/1351
  L   A   G   N   L   L   N   D   I   G   L   T   S   Q   P   A   V   V   Q   P
CGC TGG CCG GCA ACC TGC TCA ACG ACA TCG GGC TCA CGT CGC AGC CGG CGG TGG TCC AGC

4081/1361                                   4111/1371
  V   L   A   R   L   A   D   T   A   T   E   L   G   V   P   L   L   I   V   P
CGG TGC TGG CCC GGC TGG CCG ACA CGG CGA CCG AGC TCG GGG TGC CGC TGC TCA TCG TGC

4141/1381                                   4171/1391
  S   F   R   R   S   A   I   T   D   A   M   S   F   T   R   T   A   A   A   L
CCA GCT TCC GGC GCA GCG CGA TCA CCG ACG CGA TGT CCT TCA CCC GCA CAG CAG CCG CCC

4201/1401                                   4231/1411
  R   W   A   V   S   L   A   E   A   R   G   I   V   L   A   S   E   N   V   L
TGC GCT GGG CCG TGT CGC TGG CCG AGG CCC GGG GAA TCG TGC TGG CCA GCG AGA ACG TCC

4261/1421                                   4291/1431
  P   P   A   R   A   R   Q   L   V   E   E   V   G   S   P   A   F   R   L   L
TGC CCC CGG CGC GGG CCC GGC AGC TGG TCG AGG AGG TCG GCT CAC CGG CTT TCC GGC TGC

4321/1441                                   4351/1451
  L   D   T   F   N   P   V   R   Y   G   L   E   P   A   W   L   A   T   E   L
TGC TGG ACA CCT TCA ACC CGG TCC GGT ACG GAC TCG AAC CGG CCT GGC TGG CCA CCG AGC

4381/1461                                   4411/1471
  R   P   W   W   A   D   Q   I   H   L   K   D   G   P   P   D   T   G   P   S
TCC GGC CGT GGT GGG CCG ACC AGA TCC ACC TCA AGG ACG GCC CGC CGG ACA CCG GGC CGT

4441/1481                                   4471/1491
  P   L   L   G   A   G   Q   G   G   V   R   R   T   L   T   A   L   R   G   S
CGC CGC TGC TCG GTG CGG GAC AGG GCG GGG TGC GGC GCA CCC TGA CCG CGC TCA GGG GGA

4501/1501                                   4531/1511
  P   A   P   V   R   A   L   V   L   E   N   D   Y   R   D   G   H   G   A   R
GCC CCG CAC CGG TAC GGG CCC TGG TGC TGG AGA ACG ACT ACC GCG ACG GCC ACG GTG CGC

4561/1521                                   4591/1531
  L   R   A   D   L   E   W   A   R   R   A   A   V   N   A   R   E   S   E   K
GGC TGC GAG CGG ACC TGG AGT GGG CCC GCC GGG CCG CGG TGA ACG CGA GAG AAT CCG AGA
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4621/1541                                    4651/1551
  G   K   L   T   *  ---> acbO
     acbC --->   V   S   G   V   E   T   V   G   V   H   A   D   A   H   R   D
AAG GGA AGC TGA CGT GAG TGG TGT CGA GAC GGT AGG GGT GCA CGC GGA TGC GCA CCG CGA

4681/1561                                    4711/1571
 S   W   Q   V   R   A   Q   K   Q   I   T   Y   E   V   R   F   R   D   D   V
CTC GTG GCA GGT GCG GGC CCA GAA GCA GAT CAC CTA CGA GGT GCG CTT CCG GGA CGA CGT

4741/1581                                    4771/1591
 F   G   L   D   S   T   D   L   L   E   A   G   A   D   G   A   G   S   R   R
GTT CGG GCT GGA CTC CAC CGA CCT GCT GGA GGC CGG GGC GGA CGG GGC CGG TTC ACG GCG

4801/1601                                    4831/1611
 R   F   V   V   V   D   S   A   V   D   A   L   Y   G   S   R   I   R   E   Y
GCG GTT CGT GGT GGT GGA CAG CGC CGT CGA CGC CTT GTA CGG GTC CCG GAT CCG GGA GTA

4861/1621                                    4891/1631
 F   T   H   H   G   I   D   H   S   I   L   V   M   R   V   G   E   T   V   K
CTT CAC CCA TCA CGG CAT CGA TCA TTC GAT CCT GGT GAT GCG GGT GGG CGA GAC GGT CAA

4921/1641                                    4951/1651
 D   F   D   T   A   G   R   I   V   A   A   M   D   A   F   G   L   A   R   R
GGA CTT CGA CAC GGC GGG CCG CAT CGT CGC CGC GAT GGA CGC CTT CGG ACT GGC CCG CCG

4981/1661                                    5011/1671
 R   E   P   M   I   V   V   G   G   G   V   L   M   D   V   A   G   L   V   A
CCG GGA GCC GAT GAT CGT CGT CGG TGG TGG GGT GCT GAT GGA CGT GGC CGG TCT GGT GGC

5041/1681                                    5071/1691
 S   L   Y   R   A   R   H   A   V   L   R   V   P   T   T   L   V   G   L   I
CAG CCT CTA CCG GGC GCG GCA CGC CGT TCT GCG GGT GCC GAC GAC ACT GGT CGG ACT GAT

5101/1701                                    5131/1711
 D   A   V   S   R   E   D   R   V   N   F   N   G   H   K   E   P   A   G   Y
CGA CGC GGT GTC GCG CGA AGA CCG GGT CAA CTT CAA CGG CCA CAA GGA ACC GGC TGG GTA

5161/1721                                    5191/1731
 V   R   P   A   D   L   T   L   L   D   R   R   F   L   A   T   L   D   R   R
CGT ACG CCC GGC TGA TCT GAC CCT GCT GGA CCG CCG CTT CCT GGC CAC CCT GGA CCG GCG

5221/1741                                    5251/1751
 H   L   S   N   G   L   A   E   M   L   K   I   A   L   I   K   D   A   E   L
CCA CCT CAG CAA CGG GCT CGC CGA GAT GCT CAA GAT CGC GCT GAT CAA GGA TGC CGA GCT

5281/1761                                    5311/1771
 F   Q   L   L   E   R   H   G   R   V   L   I   E   E   R   F   Q   G   V   P
GTT CCA GCT GCT GGA GCG GCA CGG GCG GGT CCT GAT CGA GGA ACG GTT CCA GGG CGT ACC

5341/1781                                    5371/1791
 E   P   V   T   G   P   P   S   G   P   C   A   R   H   P   W   H   A   G   G
GGA ACC GGT GAC CGG GCC GCC GTC CGG GCC CTG CGC GCG CCA CCC ATG GCA TGC TGG AGG

5401/1801                                    5431/1811
 T   R   P   N   L   W   E   S   R   L   E   R   S   V   D   Y   G   H   T   F
AAC TCG GCC CAA TCT GTG GGA GAG CCG GCT GGA ACG CAG TGT CGA CTA CGG GCA CAC GTT

5461/1821                                    5491/1831
 S   P   T   I   E   M   R   A   L   P   A   L   L   H   G   E   A   V   C   V
CAG CCC GAC CAT CGA GAT GCG CGC GCT GCC GGC TCT GCT GCA CGG CGA GGC CGT GTG TGT

5521/1841                                    5551/1851
 D   M   A   L   T   T   V   L   A   Y   R   R   G   L   L   D   V   A   Q   R
GGA CAT GGC GCT GAC CAC GGT GCT GGC GTA CCG GCG GGG TCT GCT CGA CGT CGC GCA GCG
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5581/1861                                   5611/1871
 D   R   I   F   A   V   M   T   A   L   G   L   P   T   W   H   P   L   L   T
GGA CCG GAT CTT CGC GGT GAT GAC CGC CCT GGG CCT GCC GAC CTG GCA TCC GCT GCT CAC

5641/1881                                   5671/1891
 P   E   V   L   E   A   A   L   Q   D   T   V   R   H   R   D   G   W   Q   R
GCC GGA GGT GCT GGA GGC GGC GTT GCA GGA CAC CGT CCG GCA CCG GGA CGG GTG GCA GCG

5701/1901                                   5731/1911
 L   P   L   P   V   G   I   G   G   V   T   F   V   N   D   V   T   A   G   R
GCT GCC ACT GCC GGT GGG GAT CGG GGG TGT CAC GTT CGT CAA CGA CGT GAC GGC CGG CCG

5761/1921
 A   A   G   R   P   R   *  ---> acbC
AGC TGC AGG CCG CCC GCG CTG A
```

The start codon (ATG or GTG) of each gene is indicated in bold letters, whereas the stop condon (TGA) is underlined and indicated by a star. The first number shows the position of the nucleotide and the second for the amino acid in the first frame of translation.

7.2: Oligonucleotides used for the inserts in the pMJ-series of plasmids [1]

Primer	Nucleotide sequence [2]	Target
pMJM.2S	5'- GGCCGGCCATATGAAGCGGCCAC -3'	1.07 kb *acbM*, *Nde*I
pMJM.1E	5'- CGGTGCCGGTACCCACGATCGCGC -3'	1.07 kb *acbM*, *Kpn*I
pMJL.1S	5'- TTGGTCGGCATATGAGCCGGCA -3'	1.42 kb *acbL*, *Nde*I
pMJL.1E	5'- GTACGGAATTCGTCCACCGCCAC -3'	1.42 kb *acbL*, EcoRI
pMJN.4S	5'- GAGGATCACATATGAGCGGGACTCTGGTG -3'	0.86 kb *acbN*, *Nde*I
pMJN.1E	5'- TCGAGCTGGATCCCGTC -3'	0.86 kb *acbN*, *Bam*HI
pMJO.1S	5'- CGGTGCGCATATGACCTGCCGGGTG -3'	0.87 kb *acbO*, *Nde*I
pMJO.1E	5'- TACCGTCTCGACAGATCTCACGTCAGCTTCCCT -3'	0.87 kb *acbO*, *Bgl*II

[1]: M. Jarling, p.c.
[2]: The sequences for restriction enzymes are underlined

7.3: Sequence alignment of the AcbK protein with other kinases

```
AcbKAsp         -------MSEHTDVLVLGGAGVDTIAYVPELPLPFQDSYVVAA-IEPRAGQTGDNVALGL  52
URF2            MSNTTAFMSRQTDVLVLGGAGVDTIVHVPELPLPFADSYMIRPGIETRAGQSGDFVSLGL  60
MTH1544         -------MSEDRDLLAVGHTAFDYIIHLDEFPEPNTSTAIKRM--RNLHGGAAANVALVG  51
MTH404          ---------MRFDAVGLGALNMDQLHMVERIAGPDEETFVRGL--VESCGGSAANTMIGL  49
PAB0280         ---------MRFDVICMGNLNYDIVFLMEKFPGIHEKVNAKGV--FTGLGGSAGNTATWL  49
                            * : :*    * :  : .:.    .            * :.  .

AcbKAsp         HTLGLRTMHVDVLGDDPEGDLVRAFHTRHGLP-FAALPTAAGTKRAVNLVGPDGRRLSLW  111
URF2            LALGLRTHHLDMIGDDHEGDLVRALHRDRGIP-LTAVPAPGGTKRAVNLVGPDGRRLSLY  119
MTH1544         SRLGLRTSLVSAVGGDFEGSEYRELLESSGID-IESMILVADESTPTAFVMTDSDHNQIS  110
MTH404          SRLGLRTAHIGKVADDREGGLLRSNLSSEGVTDFTVVADTGRSGRVMGFVDPDGNRALYV  109
PAB0280         AKLGLKVGFIGAVGNDDFGRLHLEFFREIGVDTSGIKVVNDATGIAVTMVKGEDKRIVKY  109
                  ***:.  :. :..*  *          *:                 :*  :. :

AcbKAsp         DGSREAEEDRYPAALIAAHTAHARHVHVCITPP------GQHVFGQLNDLPVTVSTDLHN  165
URF2            DATRSRETDRFDEPAVRQLAGAARHAHVVITQP------CALALPVLREAGVTISTDLHN  173
MTH1544         YFYWGAARYFKDAETPADAIKSARAVHLATGDPSFNCRCGEFARSLGKIISFDPGQDLHM  170
MTH404          DP---GVNDTLRVDEVADEALNTELLHLTSFAGDGINVQVEVIEALDESVTVSLDPGHIY  166
PAB0280         PG------ANRWKEVNTEYLKRARHLHLSSNPIELIREAVEKAKELGLTVSFDPGE----  159
                              :.  *:                       .  .

AcbKAsp         WDGAYEGFEVYAFNADLVFLSATALTDVAATMRRVID--RGRARLVVATGRAHGGSVLVR  223
URF2            WDGADPYHEAFALQADLVFLSAMDLDDPERILRNIAA--RGRARTVVATAGADGSYLLY-  230
MTH1544         YS--RSQLERAVGVCDILFGNHHEIDRICSKLSVDIHGLREMGPGVVVKTYGKEGSIIY-  227
MTH404          ASRGVSELSDILERTDILLTNQRELELMTGSADPEEAASLLGIGVVVVKMGARGVRAWD-  225
PAB0280         ----MEVPRDVEKELDILMMNEDEFKAKYGSLDRIKD---VKARIAIATLNGGGALVRD-  211
                         *::: .   :                   .:..  .

AcbKAsp         RRKAEVRRYAAVAPEAPVVDSNGAGDAFVSGFLFGHLAGEPLETCPRYGAIAGAYACTIP  283
URF2            --DDELTHTPAVTPPAPVVDSNGAGDAFAAAFLLGTLNGEHPRRCALYGTVAGAYACTVP  288
MTH1544         --SDDVIKIDAIPREA--VDPTGAGDSYRAGFMRAYLRGADLKTCGRFASAVASFIVEDE  283
MTH404          ---GESVMVDALSTEC--VDTTGAGDAFNAGFIYAWLEGFGLEVSCRFGNYIASRCIGGY  280
PAB0280         -EKGEVHEVRGLSAKA--IDTTGGGDAFNAGFLYGFLSGWDVVNSAKLGMLLAYLTVQEV  268
                    :     .:.  .  :*..*.**:: :.*: . * *     .   .   .

AcbKAsp         ATRAGAIDRAALLRPAA---------  300
URF2            STRTGAIGREELLARVAEVEKDRTP-  313
MTH1544         GTQTNIPDTGEAVKRFTAQWGYEPPI  309
MTH404          GATESLPGTGPGCPQEVR--------  298
PAB0280         GARSAVRPLDEIKKIAQELKLNLPI-  293
                .:
```

The AcbK sequence is significantly related to members of the ribokinase family (COG0524). Identical (*) and conserved (:) amino acids are indicated. Gaps are indicated by "-". The sequences aligned are from the following sources (accession codes in brackets): AcbKAsp = AcbK protein from *Actinoplanes* sp. SE50 (Y18523); URF2 = putative kinase from *Streptomyces* sp. (U08602); MTH1544 = putative ribokinase from *Methanobacterium thermoautotrophicum* (AAB86018); MTH404 = putative ribokinase from *Methanobacterium thermoautotrophicum* (AAB84910); PAB0280 = putative ribokinase from *Pyrococcus abyssi* (E75157).

7.4: Sequence alignment of the AcbM protein with other kinases

```
GlcKBsu         ---MDEIWFAGIDLGGTTIKLAFINQYGEIQHKWEVPTDKTGDTITVTIAKTIDSKLDEL  57
MLR7234         ---MSIEAALAIDLGGTELRAALVDRDGKILAFAAVPTQAQAGPDVVIGQIEALAATVHA  57
GlkASli         -----MGLTIGVDIGGTKIAAGVVDEEGNILSTHKVPTP-TTPEAIVDAIASAVEGARVG  54
GlkARsa         ---MPSTLAIGIDIGGTKVAAGVVDEHGVVLEERRCSTPGSDPRAVEETIVELVRDLGQR  57
AcbMAsp         VKRPPHHPVTVADVGGTHLRWARWSPDGGLGEVHTTPSPGHARRPGAGAADLQAELIREL  60
                          *:*** :  .  .  * :      .:

GlcKBsu         QKPKHIIKYIGMGAPGPVDMAAGVVYETVNLG-WKNYALKNHLETETGIP---AVIENDA  113
MLR7234         EAPGLAIVGVGVGAPGPLDPLAGIAVGPPTLAGWQDVPLADILERRLGLP---VRLENDA  114
GlkASli         ----HEIVAVGIGAAGYVNRQRSTVYFAPNID-WRQEPLKEKVEARVGLP---VVVENDA  106
GlkARsa         ----HEIASVGIGAAGWMDLSGGTVLFSPHLA-WRNEPLRENLERLLCRP---VRLTNDA  109
AcbMAsp         ASRVEPGARAGVSLGAAMDHHSGYAYASAPLWGPQVSPFDVPAALRAARPDVHWTVVNDV  120
                     *:.  . ::   . .  .  :   :  .:          *     : **.

GlcKBsu         NIAALGEMWKGAGDGAKDVILVTLGTGVGG--GIIANGEILHGINGAGGEIGHICSIPEG  171
MLR7234         NAAALGEWRFGAGHGARSLVFVTVSTGIGG--GVVADGRILHGRRGLAAEIGHMT-ITNE  171
GlkASli         NAAAWGEYKFGGGKGHRNVICITLGTGLGG--GIIIGNKLRRGHFGVAAEFGHIRMVPDG  164
GlkARsa         DAAAWAEWRFGSGRDESRLVCITLGTGIGG--AMVMDGRIERGRFGVAGEFGHQIIMPGG  167
AcbMAsp         TAGLLHLAEMVRDAGVRKACLVTISTGIACRTMDLRTGGIPVDAAGLQGEIGHLPATVLA  180
                  .         . .      :*:.**:.     :  . :  .  *  .*:**

GlcKBsu         G-----APCNCGKTGCIETIASATGIVRIAKGKIANAKKTT--RLKAT----EQLSARDV  220
MLR7234         G-----ERCVCGVVGCFEAIASGTALGRRANAATSAFDGSTLRRLSAN----AEVTGRHV  222
GlkASli         ------LLCGCGSQGCWEQYASGRALVRYAKQRANATPERAEVLLALGDGTPDGIEGKHI  218
GlkARsa         ------YRCECGNRGCWEQYASGNALGREARELARANSPVAQEILRAVDGDADLITGAII  221
AcbMAsp         DGVPVVTRCDCGEPGHVAASSSGPGIRRVAAVLARRDPATWAGSGPTTRMMAGSGFEDAF  240
                       * **  *     :*. .: * *                              .

GlcKBsu         FEAAGENDEIALEVVDYVAK-HLGLVLGNLASSLNPSKIVLGGGVSRAGE-LLRSKVEKT  278
MLR7234         VEAARLQDDLALALLEEEAR-WLGVGFTNLLHLYSPDVLVVGGGIANGLD-LMHPVIEAT  280
GlkASli         SVAARQGCPVAVDSYRELAR-WAGAGLADLASLFDPSAFIVGGGLSDEGD-LVLDPIRKS  276
GlkARsa         TDLARAGDAASIELIEDVGS-WLGLGMANLAAALDPGMFVIGGGLCEAGE-LLLEPARRA  279
AcbMAsp         RAALDDGDPVAADLLTAVTAPIADLLRTALCLDPELDLIALTGGVAHGLEPHYSAAVHDH  300
                         :           .     *    . . : : **:.   :       .

GlcKBsu         FRKCAFPRAAQAAD-ISIAALGNDAGVIGGAWIAKN----------------EWLKHQN  320
MLR7234         IRQRAM-RAYRDVP-VVQAQLGRHAGLVGAASLVLFDDGSLAARMPVGPSTFPEARRDFN  338
GlkASli         YKRWLVGGNWRPVADVIAAQLGNKAGLVGAADLAREPDPIM------------------  317
GlkARsa         FGRNLTGRGFRPAAAIALAELGPSAGLIGAADLSRSASGS--------------------  319
AcbMAsp         LRRRGLYLTSEREPDWLTGRIRVVPPATADPLVGAGLAALAAGPVPAYSGGGREALVGR-  359
                  :       .       . :   .   . . :

GlcKBsu         C  321
MLR7234         G  339
GlkASli         -
GlkARsa         -
AcbMAsp         -
```

The AcbM sequence is distantly related to members of the glucokinase family (COG1940). Identical (*) and conserved (:) amino acids are indicated. Gaps are indicated by "-". The sequences aligned are from the following sources (accession codes in brackets): AcbMAsp = AcbM protein from *Actinoplanes* sp. SE50 (Y18523); GlkASli = glucokinase from *Streptomyces lividans* TK24 (AAF42869); GlcKBsu = putative glucokinase from *Bacillus subtilis* 168 (P54495); MLR7234 = glucokinase from *Rhizobium loti* (AP003011); GlkARsa = glucokinase from *Renibacterium salmoninarum* (X89964).

7.5: Sequence alignment of the AcbL protein with other dehydrogenases

```
SCF91.05c       ------------------------------------------------------------
AdhTsp          MVTKQDVEAAYLLLLGRPAEPGAAEYWMENVYHTIDLVTGLMASDEFRINRLPRLVEAGL  60
SC6A5.31c       ------------------------------------------------------------
AcbLAsp         ------------------------------------------------------------


SCF91.05c       ----------------MAQMLAARLHVPSRTLRLEEVPRPQPGPGEVLVKVEAAGVCLSD  44
AdhTsp          SLSDFAPAAAPKALADNGPPMQAALHRGIGQIEVVEINRPEALPGTVVVKVGASGICGTD  120
SC6A5.31c       ----------------MRRARAAVVREPGAPFTVLEVELEDPRPREVLVRMTAAGVCHTD  44
AcbLAsp         ------------------MSRHRAIVRTGTGVVVADVPTPVPGPGELLVGTEWAGLCGTD  42
                                       :      . : ::    . *  ::*    :*:* :*


SCF91.05c       VHLIDGTLTPLLLRGDTVTLGHEVSGTVAGTGAGVT-AWSPGQRVVLHAGE---------  94
AdhTsp          LRAYRQDPN-----SQDLPHGHEFAGTIVEVGEGVS-RDRIGKRVTADLFLNAMCGNCQF  174
SC6A5.31c       LGIQAGWPR----RLTPMVFGHEGAGRVEAVGAEVT-GLVPGDTVCLTFASCGGCGQCTA  99
AcbLAsp         IQMLRGLRD-----DPEPIIGHEGIARVIAAGDGVPGTLRPGTLVAVNPTHR--------  89
                :                  ***  . :  .*  *.     *  *


SCF91.05c       ---RRDGVTYTR---------------------GVDY-DGGWAEYALCAADAMTPLPDAI  129
AdhTsp          CISGHAYHCIDK---------------------ALPFRSGGFAEYLRVRNAATFDLPDSI  213
SC6A5.31c       GHPAYCDAARDLNLSGGRGDGSTPLRLDGAPLHGGFFGQSSFATYAVVHEHGVVRVPADL  159
AcbLAsp         ---DDPSFLLGHN-------------------VPGLLQERTLLPATAVSGGLVLPLPDGT  127
                                                          .              :*


SCF91.05c       PFEQGAIIPDAVSTPWGAITETGEVRPAEAVGVWGVGGLG-VHAVQLLRAIGACPVVAVD  188
AdhTsp          DDALGALVEPLAVSTHAIRLVG--VQPGMIGVVIGAGTIG-LCAIAAALHAGATNVFVVA  270
SC6A5.31c       PATLAAPLGCGGQTGAGTVLNRLRPEPGTSLVVLGAGGVG-LSALMAAVAVGCDPVLAVD  218
AcbLAsp         DVTLGPLLEPLAVVSYALSQLG--AVRPATLVIVGDGTVGHLAARAAVTWLAERPRVVLV  185
                    .. :        .               : * * :* : *       .    ..:


SCF91.05c       PSPVARERALAAG-------------ADLALDSADPEFRQKVGAATGGAGLAAAFDFAGV  235
AdhTsp          RHAFQGELARAIG-------------ATDILPDDIGSAINRIREVAPHGADFVIEAVGGG  317
SC6A5.31c       PVASRRDLARALG-------------ARAALPPDD-ALVAAVRQLTDGGAHHVVDTT-GR  263
AcbLAsp         HHTAAGRDFSAAGPHPADVLLTTGELAGRPLPGPVAALLATPRDAT-VEALEAVLAAAGA  244
                  .       * *             *   *              :   .  .     *


SCF91.05c       PPVREQAVSVLAPKGRLVLAGLTDKPLTVTDG--------TRFSYLQQRILGHYGSDMP-  286
AdhTsp          QATIDQATRFLAPRGRVGIVGETGPGIKVVEAY---PALMKELTFLYSNCYGYLDGKHD-  373
SC6A5.31c       PGMLDRAVAALRPRGALALLGLGGEVTFDMMR----------LMTKGVRLHGVMEGDSDP  313
AcbLAsp         DVLIDVLGGLPPGARSAMLPGVDLTAVRAANCGGFPEPALVTTTSGGTRLFGHRGVGNAH  304
                    :            : *                            .  *


SCF91.05c       VALPQLLRLIQGGRLDFSGSVSGVLP-LAEAAEAVARLEKKEGDPIRLVLRP--------  337
AdhTsp          FEVAIENLALDGERLRK--LITHEFL-IADAPLAFQTANNKKFSSVKVQLKP--------  422
SC6A5.31c       ARFVPELIALHRRGLFPVDRLVTTFA-FEEIGAAVAAMRDGSAVKPVLTFS---------  363
AcbLAsp         LRAAAGELARDPDRYRDLVTHRNDLAGAARIMTALRSGRDRIIDGRRLVKLAIQVNVRED  364
                          .            :       *.   ..       :


SCF91.05c       --
AdhTsp          --
SC6A5.31c       --
AcbLAsp         HR 366
```

The AcbL sequence is significantly related to members of the zinc-dependent alcohol dehydrogenase (COG1062). Identical (*) and conserved (:) amino acids are indicated. Gaps are indicated by "-". The sequences aligned are from the following sources (accession codes in brackets): AcbLAsp = AcbL protein from *Actinoplanes* sp. SE50/110 (Y18523); SCF91.05c = putative zinc-containing alcohol dehydrogenase from *Streptomyces coelicolor* (AL132973); AdhTsp = alcohol dehydrogenase from *Thiobacillus* sp. (AJ007958); SC6A5.31c = probable zinc-containing dehydrogenase from *Streptomyces coelicolor* (AL049485).

7.6: Sequence alignment of the AcbN protein with other dehydrogenases

```
SC9A4.23c       MTTTGSALSGALPFAGLTALVTGGASGIGLATARLLTAQGARVAVLDLS-EPQPAGFAAA  59
SCF41.15        MPHTPTPPS--PELAGLAAVVTGGASGIGLATARLLARRGARVAVLDLAPDAVPATDLEA  58
YHG             --MKYGSLLNKFRLDGKVALITGGTRGIGLATAYAFGEAGAKLYLSARREEYEDAGAILT  58
AcbNAsp         --MSG--------TLVGRRALVVGGTTGIGRGIADAWAAAGAEVTVCGRSEPAGDGAAGLH  51
CC2836          -------MIDDTDFTGRRVLVVGGSSGIGNGIAHAFKARGAAVAVWGTRASAGDYDPADG  53
                             : *  .::.**: *** . *      ** : :

SC9A4.23c       LR-------ADIRDDASVVAAVDAAVSRLGGLDIVVNNAGIGAQGGVEDNSDEEWHRVLD  112
SCF41.15        LLPVG----CDVTDDASVRAGLDRAVDLLGGLDILVNNAGVGAQGTVADNDDAEWHRVLD  114
YHG             AGYDVTFYPADLKTREAACALVKRVADDAGRLDILINNAGIANGGDTPLFTEQQWRDVIA  118
AcbNAsp         WEP------LDLTRTGEAGARLERIAGDRVDLACFAAVSYGARRAVFEDVAVDEWRTQLD  105
CC2836          SDLSG---LAYAQVDVGDPDAIEAAPAPFDTLDVLVLCQGTVVYK-RGEFERPGWDRVMA  109
                                      :.       *  .                   *   :

SC9A4.23c       TNVVGMVRVTRAALPALRASRHAAVVNVGSIAATAG-LPQR-VLYSAGKGAVVAMTRAMA  170
SCF41.15        VNVLGIVRTTRAALPHLRASRHAAVVNTCSVAATAG-LPRR-ALYSASKGAVQSLTLAMA  172
YHG             TNVETVFWCSQAAIPVMREGGRGAIVNVGSMSGIVSNIPQNQVAYNSSKAAVHMMTKSLA  178
AcbNAsp         INVHGLWLTLRAALPGLRAARPGLFVGVSSEVVYNAGPGRS--GYTASKAAAKALLESVA  163
CC2836          VNLDSLMHCARRFKPALTASR-GSVIIVSSISGLSANIGNP--AYAASKAGAISLTKTLG  166
                 *:  :    :   * :  .  . .: . *     .   .    * :.*...  :  ::.

SC9A4.23c       ADLLPEGIRVNAVNPG-TADTPWIGRLLDRAADPAAEYTALAARQPHGRLVAADEVAAAI  229
SCF41.15        ADHIREGIRVNCVNPG-TADTPWIDRLLSSADDPAAERTALNARQPTGRLVSAEEVAAAI  231
YHG             SELALDNIRVNAVAPG-YIDT----DMSRGGMVHPVRGPIWLEMTPMKRFGRPDEIATAI  233
AcbNAsp         QEEDESRVRIVQVLPAGMVDTP----SIRRRRPAGFDYSDYMVPADFARNALELAATAGA  219
CC2836          QAWAPEGVRVNGLAPG-LVETK----LTKVTTESPERLAGALAAIPQRRMGTPADMAGAA  221
                     . :*:  : *.   :*                  .         *       : .

SC9A4.23c       AYLASPAAGSTTGTCLAVDGGMDGLRLRKEG-  260
SCF41.15        GYLASPAAASVTGTALAVDGGMQGLRLRPDGG  263
YHG             LFLASEASSYVTGDILVVDGGYTTR-------  258
AcbNAsp         GHHGDSLVVDRGGDWWSVRERMPVSQSRPVRT  251
```

The AcbN sequence is significantly related to members of the short-chain dehydrogenases/reductases family (SDR) (COG1028). Identical (*) and conserved (:) amino acids are indicated. Gaps are indicated by "-". The sequences aligned are from the following sources (accession codes in brackets): AcbNAsp = AcbN protein from *Actinoplanes* sp. SE50/110 (Y18523); SC9A4.23c = putative oxidoreductase from *Streptomyces coelicolor* (AL391072); SCF41.15 = probable oxidoreductase from *Streptomyces coelicolor* (AL117387); YHG = short-chain dehydrogenases/reductases from *Agrobacterium tumefaciens* (AF242881).