The putative biosynthetic gene cluster for the α-glucosidase inhibitor acarbose was identified in the producer Actinoplanes sp. 50/110 by cloning a DNA segment containing the conserved gene for dTDP-α-glucose 4,6-dehydratase, acbB. The two flanking genes were acbA (dTDP-α-glucose synthase) and acbC, encoding a protein with significant similarity to 3-dehydroquinase synthases (AroB proteins). The acbC gene was overexpressed heterologously in Streptomyces lividans 66, and the product was shown to be a C7-cyclitol synthase using sedoheptulose 7-phosphate, but not ido-heptulose 7-phosphate, as its substrate. The cyclization product, 2-epi-5-epi-valiolenone (2S,3S,4S,5R)-5-(hydroxymethyl)cyclohexanone-2,3,4,5-tetrol, is a precursor of the valienamine moiety of acarbose. A possible five-step reaction mechanism is proposed for the cyclization reaction catalyzed by AcbC based on the recent analysis of the three-dimensional structure of a eukaryotic 3-dehydroquinase synthase domain (Carpenter, E. P., Hawkins, A. R., Frost, J. W., and Brown, K. A. (1998) Nature 394, 299–302).

The α-glucosidase inhibitor acarbose (part of the amylostatin complex) (Fig. 1), produced by strains of the genera Actinoplanes and Streptomyces, is a member of an unusual group of bacterial (mainly actinomycete) secondary metabolites, all of which inhibit various α-glucosidases, especially in the intestine (1, 2). Acarbose is produced industrially using developed strains of Actinoplanes sp. SE50/110. It is used in the treatment of diabetes patients, enabling them to better utilize starch- or sucrose-containing diets by slowing down the intestinal release of α-d-glucose. The acarbose-like natural products contain, as a unifying structural feature, a pseudodisaccharide based on the C7-cyclitol valienamine bound via an imino bridge to a hexose derivative, which in acarbose is 4-amino-4,6-dideoxyglucose (cf. Fig. 1). Biosynthetically, these compounds resemble aminoglycoside antibiotics (3, 4). Also, the C7-amino-cyclitol units are considered to be similar to other C7-N units, a common structural motif more frequently observed in bacterial secondary metabolites (5). From the labeling patterns of variously 13C-labeled α-glucoses, fed to cultures of validamycin-producing Streptomyces sp. or to acarbose-producing Actinoplanes sp., it was suggested that the valienamine moiety is derived from a C7-sugar precursor formed in reactions of the pentose phosphate cycle (6, 7).

The genetics and biochemistry of acarbose biosynthesis have not yet been studied in the producing strains. Only speculations are available on the possible enzymatic mechanism(s) by which the C7-cyclitol unit could be formed. However, the 6-deoxyhexoses are frequent building units or side chains in many actinomycete secondary metabolites and are mostly synthesized via a dTDP-hexose pathway (3, 8). Therefore, we used the highly conserved gene sequences of the dTDP-α-glucose 4,6-dehydratase to probe for related genes in the acarbose producer Actinoplanes sp. 50/110. In this way, a gene cluster was isolated that contains several genes putatively involved in the biosynthesis of this natural product. Besides genes for dTDP-6-deoxyhexose formation, such as acbA (dTDP-α-glucose synthase) and acbB (encoding dTDP-α-glucose 4,6-dehydratase), a third gene, acbC, was found that encodes an AroB-like protein (dehydroquinase synthase (DHQS); 1). The acbC gene was expressed heterologously in Streptomyces lividans, 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 sedoheptulose 7-phosphate, but not ido-heptulose 7-phosphate, as a substrate.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are listed in Table I. S. lividans 1326 was used as the host strain for the protein expression experiments. The strain was routinely cultured at 28 °C on SNA agar plates (13) or in tryptic soy broth liquid medium (9). To maintain the plasmid pIJ6021, these media were supplemented with kanamycin (50 μg/ml). The thioesterase-inducible expression of the cloned acbC gene in S. lividans was carried out according to Takano et al. (11) with the exception that 7.5 μg of thioesterase/ml of YE medium liquid medium (9) was used, and the incubation time after induction was prolonged to 20 h. Actinoplanes sp. chromosomal DNA was prepared by standard procedures (9). Subcloning experiments with Escherichia coli were performed with the vector pUC18 and the host strain DH5α, which was grown at 37 °C in LB broth or on LB agar plates.

General DNA Manipulation Techniques—Restriction enzymes and T4 DNA ligase were purchased from Gibco (Eggenstein, Germany) and used in accordance with the manufacturer’s instructions. Agarose gel

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† The abbreviations used are: DHQS, dehydroquinase synthase; ESI-MS, electrospray ionization mass spectrometry; kb, kilobase pair(s); PCR, polymerase chain reaction; DAHP, 3-deoxy-α-arabino-heptulosonate 7-phosphate.
electrophoresis and DNA manipulations of \( E. \) coli were done as described by Sambrook et al. (14); transformations of \( E. \) coli were carried out by the method of Hanahan (10). The size fractionation of restriction endonuclease-cleaved chromosomal DNA was done on 12-ml 20% sucrose gradients by centrifugation at 74,200 \( \times \) g for 15 h at 20 °C. Fractions were collected, and DNA was precipitated and concentrated by ethanol precipitation. Protoplast preparation and plasmid transformation techniques for \( S. \) lividans were performed according to published procedures (9, 15). For Southern hybridization, the genomic DNA was immobilized on a Hybond N membrane (Amersham Pharmacia Biotech, Braunschweig, Germany). Hybridization was performed in 50% formamide solution overnight using 20 \( \mu \)g/ml digoxigenin-labeled DNA (Amer- sham Pharmacia Biotech)-labeled DNA fragments as shown in Fig. 2. Stringency washes were done with 2 to 0.1× SSC at 65 °C.

**Instrumentation**—Electrospores ionization mass spectrometry (ESI-MS) was carried out on a Perkin-Elmer-Sciex API-3 or a Kratos Profile mass spectrometer, and gas chromatography-mass spectrometry on a Hewlett-Packard 5890 gas chromatograph with a 5071A mass selective detector. Proton NMR spectra were recorded on a Bruker AF 300 NMR spectrometer with a MocNMR 5.5 PCI as the instrument controller and data processor, and \(^{13}C\) NMR spectra on a Bruker AC 400 NMR spectrometer. An ISF-4-V culture shaker (Adolf Kuhner AG, Birsfelden, Switzerland) was used for the fermentation of the acarbose producer. Radioactive samples were counted in Bio-Safe II biodegradable scintil- lation fluid (Wallac, Turku, Finland) in a Wallac 1450 liquid scintillation counter. Proton NMR spectra were recorded on a Bruker AF 300 NMR spectrometer. An ISF-4-V culture shaker (Adolf Kuhner AG, Birsfelden, Switzerland) was used for the fermentation of the acarbose producer. Radioactive samples were counted in Bio-Safe II biodegradable scintillation fluid (Wallac, Turku, Finland) in a Wallac 1450 liquid scintillation counter.

**Strategy for the Identification of the Acarbose Biosynthesis Gene Cluster**—Two different strategies were used to identify the acarbose biosynthesis gene cluster in the genome of Actinoplanes sp. (1). The \( d\) and \( st\) \( E. \) genes from Streptomyces griseus (8), encoding \( d\)TDP-\( \alpha \)-glucose synthase and \( d\)TDP-\( \alpha \)-glucose 4,6-dehydratase, respectively, were used as heterologous probes to identify the equivalent gene(s) in the genomic DNA of Actinoplanes sp. by means of DNA-DNA hybridization experiments. For this purpose, a 0.70-kb EcoR/BglII fragment, containing most of the \( st\) \( E. \) gene (16), and a 0.76-kb KpnI fragment, containing most of the \( st\) \( E. \) gene (17), were amplified by PCR using primers AS2 (5′-GCCGCCGCAAGTTCTCGATGCACG-3′) and AS5 (5′-CCGCGTATTGGTTGAGCAGCGGTG-3′). Amplification was performed in a Biometra Personal Cycler using 2.5 units of Taq polymerase (Gibco). The reaction mixtures (100-µl volume) contained 200 ng of chromosomal DNA, 50 pmol of each primer, 0.2 mM dNTPs (Boehringer Mannheim, Germany), 10 mM Tris-HCl (pH 8.3), 500 mM potassium glutamate, and 5% dimethylformamide. The following conditions were used for the reactions. The enzyme was added after an initial denaturation for 5 min at 95 °C, followed by 25 cycles (95 °C for 1 min, 54 °C for 30 s, and 72 °C for 30 s) at 72 °C for 5 min (ramping rate of 1 °C/s). The PCR product was cloned into pUC18, resulting in pAS1. The amplified 300-base pair DNA fragment was sequenced by the MaxyTech Products International Corp.) in a Beckman LS 1801 scintillation counter.

**DNA Sequencing and Computer Analysis of Protein and DNA Sequences**—Various overlapping restriction fragments from the 10.7-kb \( S s t I \) and 12.4-kb BglII DNA fragment inserts in pAS5 and pAS6, respectively, were subcloned into pUC18 and sequenced by the A.L.F DNA sequencer (Amersham Pharmacia Biotech) was used to construct unidirectional deletions in DNA fragments in accordance with the manufacturer's instructions. Homology searches were done as described by Sambrook et al. (15). Primer AS-C1 was designed for the in- duction of the \( N d e I \) site in place of the native stop codon and for the ability to create a start codon fusion of \( abcC \) into the promoter-ribosome-binding site cassette of expression vector pJLB201. Primer AS-C2 was designed for the introduction of an \( E c o R I \) site at 117 base pairs downstream of the \( abcC \) stop codon for the ligation of the acbC DNA fragment into pJLB201 NdeI/EcoRI. PCR was performed as described above, and the following conditions were used: an initial denaturation for 5 min at 95 °C and then 25 cycles (95 °C for 1 min, 50 °C for 20 s, and 72 °C for 40 s) and 72 °C for 5 min (ramping rate of 1 °C/s). The PCR product was cloned into pUC18 HinClII, resulting in pAS8/5.1. The insert was sequenced (1) and concentrated by ethanol precipitation. Prototax preparation and plasmid transformation techniques for \( S. \) lividans were performed according to published procedures (9, 15). For Southern hybridization, the genomic DNA was immobilized on a Hybond N membrane (Amer- sham Pharmacia Biotech, Braunschweig, Germany). Hybridization was performed in 50% formamide solution overnight using 20 \( \mu \)g/ml digoxigenin-labeled DNA (Amer- sham Pharmacia Biotech)-labeled DNA fragments as shown in Fig. 2. Stringency washes were done with 2 to 0.1× SSC at 65 °C.

The reaction product was detected by the TLC assay described above, and the following conditions were used for the reaction: an initial denaturation for 5 min at 95 °C and then 25 cycles (95 °C for 1 min, 54 °C for 30 s, and 72 °C for 30 s) at 72 °C for 5 min (ramping rate of 1 °C/s). The PCR product was cloned into pUC18, resulting in pAS1. The amplified 300-base pair DNA fragment was sequenced by the MaxyTech Products International Corp.) in a Beckman LS 1801 scintillation counter.

**Characterization of the \( abcC \) Protein**—The product of the \( abcC \) gene was cloned into pUC18 and sequenced by the MaxyTech Products International Corp.) in a Beckman LS 1801 scintillation counter. Homology searches were done as described by Sambrook et al. (15). Primer AS-C1 was designed for the in- duction of the \( N d e I \) site in place of the native stop codon and for the ability to create a start codon fusion of \( abcC \) into the promoter-ribosome-binding site cassette of expression vector pJLB201. Primer AS-C2 was designed for the introduction of an \( E c o R I \) site at 117 base pairs downstream of the \( abcC \) stop codon for the ligation of the acbC DNA fragment into pJLB201 NdeI/EcoRI. PCR was performed as described above, and the following conditions were used: an initial denaturation for 5 min at 95 °C and then 25 cycles (95 °C for 1 min, 50 °C for 20 s, and 72 °C for 40 s) and 72 °C for 5 min (ramping rate of 1 °C/s). The PCR product was cloned into pUC18 HinClII, resulting in pAS8/5.1. The insert was sequenced (1) and concentrated by ethanol precipitation. Prototax preparation and plasmid transformation techniques for \( S. \) lividans were performed according to published procedures (9, 15). For Southern hybridization, the genomic DNA was immobilized on a Hybond N membrane (Amer- sham Pharmacia Biotech, Braunschweig, Germany). Hybridization was performed in 50% formamide solution overnight using 20 \( \mu \)g/ml digoxigenin-labeled DNA (Amer- sham Pharmacia Biotech)-labeled DNA fragments as shown in Fig. 2. Stringency washes were done with 2 to 0.1× SSC at 65 °C.

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The reaction product was detected by the TLC assay described above, and the following conditions were used for the reaction: an initial denaturation for 5 min at 95 °C and then 25 cycles (95 °C for 1 min, 50 °C for 20 s, and 72 °C for 40 s) and 72 °C for 5 min (ramping rate of 1 °C/s). The PCR product was cloned into pUC18, resulting in pAS1. The amplified 300-base pair DNA fragment was sequenced by the MaxyTech Products International Corp.) in a Beckman LS 1801 scintillation counter.
CD3OD) &c 46.0 (t, C-6), 67.6 (t, C-7), 70.7 (d, C-4), 76.0 (d, C-2), 79.7 (d, C-3), 81.6 (s, C-5), and 209.8 (s, C-1). Trimethylsilyl derivative: derivatization procedure, mass spectrometry, T-ret = 11.01 min (same as trimethylsilyl-2-epi-5-epi-valiolone; different from trimethylsilyl-2-epi-valiolone (T-ret = 11.06 min); fragment ions: m/z 276 and 480 (M + 3 trimethylsilyl) (2-epi-5-epi-[6-^13^C]valiolone, m/z 278 and 482).

**Precursor Role of the AcbC Product and Mechanism of Cyclization**

p-sedo-[1-^13^C]Heptulose 7-phosphate (82.5% ^13^C) was prepared from L-[3-^13^C]serine (containing a trace of L-[3-^14^C]serine to guide the isolation of products) and n-ribose 5-phosphate as described (41). This material (21.25 mg) was incubated with S. lividans 1326/pAS8/7 extract in five 1-ml reaction mixtures to give, after Sephadex LH-20 purification, 12 mg of 2-epi-5-epi-valiolone characterized by ^1H NMR and showing the expected strongly enhanced ^13^C NMR signal for C-7. A 10-mg sample of this material was fed to two 60-ml resting cell cultures of Actinoplanes sp. strain SN223/29, and acarbose (6 mg) was isolated and purified following previously described procedures (25). ^13^C NMR and ES-MS analysis of the resulting acarbose showed 2% incorporation of the labeled precursor with ^13^C enrichment specifically at C-7.

p-sedo-[1-^13^C,7-^14^C,7-^3^H]Heptulose 7-phosphate was prepared from n-[6-^13^C]- and n-[6-^2^H]glucose as described (41). A sample of this material (75 nCi, ^3^H/^14^C = 5.5) was incubated with S. lividans 1326/pAS8/7 cell-free extract under the assay conditions described above. Purification of the resulting 2-epi-5-epi-valiolone by preparative TLC as described above gave a product of ^3^H/^14^C = 3.7.

**RESULTS**

**Identification and Partial Cloning of the Acarbose Biosynthesis Gene Cluster**—To isolate the putative biosynthetic gene cluster for acarbose from the genomic DNA of Actinoplanes sp. 50/110, we chose the widely used strategy to screen the DNA for 6-deoxyhexose-specific genes, which has been described earlier (8). For this purpose, restriction digests of the genomic DNA of Actinoplanes sp. were hybridized with DNA probes from the strD and strE genes of S. griseus. The strE probe hybridized weakly but specifically with only one band in all cases, e.g. ~2.2-kb BamHI, 13-kb BglII, and 11-kb SstI fragments (data not shown). The strD probe did not give a signal at all. Therefore, first a PCR approach was used to clone an ~300-base pair segment of the gene homologous to strE (pAS1; see “Materials and Methods”). This fragment was used to hybridize against genomic DNA of Actinoplanes sp. variably restricted with single endonucleases and combinations thereof. The result was that hybridization was found only in a single genomic region that was identical to that detected with the strE probe (data not shown). The 300-base pair insert of pAS1 was also used as a specific probe to screen size-fractionated genomic DNA libraries of 2–3-kb BamHI, 10–12-kb SatI, and 12–15-kb BglII fragments, cloned in vectors pUC18 or Bluescript II KS(−), for hybridizing plasmids. In each library, hybridizing plasmids were found that contained overlapping genomic DNA segments; they are called pAS2, pAS5, and pAS6 (Fig. 2; cf. Table I).

**Identification of the Genes acbABC—Sequence analysis of the 2.2-kb BamHI DNA fragment inserted into pAS2 revealed the presence of the full-length reading frame of a dTDP-D-glucose 4,6-dehydratase-encoding gene, called acbB, and two incomplete additional reading frames, each oriented in opposite direction relative to acbB, which were named acbA and acbC (cf. Fig. 2). The sequences of the acbA and acbC genes were completed by subcloning and sequencing overlapping segments from pAS5 and pAS6 and were found to encode a member of the family of dTDP-D-glucose synthases and a protein
related to the AroB family of proteins (3-dehydroquinate syn-
thases) of bacteria, respectively.

Protein sequence comparisons revealed that the AcbA pro-
tein is more related to the RfbA proteins of enterobacteria
(57.8% identity in a 218-amino acid overlap to *E. coli* RfbA)
than to the StrD protein of *S. griseus* (37.0% identity in a
208-amino acid overlap). In contrast, the neighboring acbB
gene encodes a protein clearly more related to the strepomy-
cete homolog StrE (57.9% identity in a 218-amino acid overlap)
to the enterobacterial counterpart RfbB (37.0% identity in
a 345-amino acid overlap to *E. coli* RfbB). This explains why
the *strD* gene did not give a hybridization signal. The deduced
sequence of the AcbC protein is only distantly similar to the
AroB proteins, which among themselves are more strongly
conserved (Fig. 3). AcbB shows the highest degree of similarity
to the AroB protein of *Mycobacterium tuberculosis* (26.8% iden-
tity in a 340-amino acid overlap), which in turn shows signifi-
cantly higher similarity to the AroB proteins of other bacteria,
e.g. *E. coli* (40.6% identity in a 345-amino acid overlap), *Coryne-
bacterium pseudotuberculosis* (50.1% identity in a 353-amino
acid overlap), and *Bacillus subtilis* (36.7% identity in a 341-
amino acid overlap). However, the eukaryotic DHQS proteins
are more distant, e.g. the DHQS domain of the multifunctional
AROM protein of *Emericella* (formerly *Aspergillus*) nidulans
shows only 26.8% identity (in a 340-amino acid overlap) to
the RfbB of *M. tuberculosis* and a very similar sequence diver-
gence (26.7% identity in a 315-amino acid overlap) to AcbC. How-
ever, the eukaryotic DHQS proteins are more distant, e.g. the DHQS domain of the multifunctional

**FIG. 2. Cloning and sequence analysis of the genes acbABC.** The inserts of the three recombinant plasmids pAS2, pAS5, and pAS6, containing independently isolated overlapping genomic segments of ~17 kb total length, are given together with a restriction map. The sequence of the DNA around the *acbB* gene, used as a tag, was determined. The genes encode proteins related to the following enzyme families: *acbA*, dTDP-n-glucose synthase; *acbB*, dTDP-n-glucose 4,6-dehydratase; and *acbC*, AroB proteins (3-dehydroquinase synthases).
the DHQS proteins all have strictly conserved amino acid residues in those positions shown to be involved in catalysis and substrate binding, whereas this is the case only for part of those in AcbC (Ref. 26; cf. Fig. 3). This suggested that AcbC and AroB do not have identical functions, but that they catalyze similar reactions.

The C7-cyclitol Synthase AcbC: Overproduction and Characterization of the Enzyme Reaction—The possible involvement of AcbC in the cyclization of the precursor of the C7-cyclitol moiety of acarbose led us to test the hypothesis that this could be formed from a C7-keto sugar phosphate, such as sedo-heptulose 7-phosphate (6, 7). However, when the conversion of sedo-heptulose 7-phosphate was tested in crude extracts of Actinoplanes sp., using reaction conditions suitable for the AroB-catalyzed reaction, no formation of cyclitols could be detected. We then expressed the AcbC protein heterologously in both E. coli and S. lividans 1326. Overexpression in E. coli under control of the T7 promoter was achieved only in the form of insoluble proteins (data not shown). However, induction of expression by thiostrepton in S. lividans 1326/pAS8/7 under control of the tipAp promoter yielded large quantities of soluble protein (Fig. 4). When the crude extracts from induced cells of S. lividans 1326/pAS8/7 were incubated with sedo-heptulose 7-phosphate in the test system developed for the AroB-catalyzed reaction, a rapid conversion of the substrate occurred. sedo-Heptulose 7-phosphate was converted to a substance migrating much faster in the analytical TLC system employed, indicating loss of the phosphate group (data not shown). Co-chromatography with sedo-heptulose, valiolone, and valienone revealed that none of these comigrated with the reaction product. The diastereomeric substrate ido-heptulose 7-phosphate (41) was not converted under the same conditions. Also, induced extracts from S. lividans 1326/pJL6021 (control) or heat-inactivated extract (5 min at 95 °C) from S. lividans 1326/pAS8/7 did not cyclize sedo-heptulose 7-phosphate, thereby proving the specificity of the AcbC protein for catalyzing the observed reaction.

Identification of the AcbC Reaction Product—The initial preparative synthesis and partial purification of the AcbC product yielded a substance, the first NMR and mass spectrometry analyses of which were consistent with its being a valiolone of yield. We then expressed the AcbC protein heterologously in both E. coli and S. lividans 1326. Overexpression in E. coli under control of the T7 promoter was achieved only in the form of insoluble proteins (data not shown). However, induction of expression by thiostrepton in S. lividans 1326/pAS8/7 under control of the tipAp promoter yielded large quantities of soluble protein (Fig. 4). When the crude extracts from induced cells of S. lividans 1326/pAS8/7 were incubated with sedo-heptulose 7-phosphate in the test system developed for the AroB-catalyzed reaction, a rapid conversion of the substrate occurred. sedo-Heptulose 7-phosphate was converted to a substance migrating much faster in the analytical TLC system employed, indicating loss of the phosphate group (data not shown). Co-chromatography with sedo-heptulose, valiolone, and valienone revealed that none of these comigrated with the reaction product. The diastereomeric substrate ido-heptulose 7-phosphate (41) was not converted under the same conditions. Also, induced extracts from S. lividans 1326/pJL6021 (control) or heat-inactivated extract (5 min at 95 °C) from S. lividans 1326/pAS8/7 did not cyclize sedo-heptulose 7-phosphate, thereby proving the specificity of the AcbC protein for catalyzing the observed reaction.

Identification of the AcbC Reaction Product—The initial preparative synthesis and partial purification of the AcbC product yielded a substance, the first NMR and mass spectrometry analyses of which were consistent with its being a valiolone of unknown stereochemistry. Its Rf in the standard TLC system, between those of synthetic valiolone and valienone, was identical to that of an authentic sample of synthetic 2-epi-5-epi-[6,2H2]-valiolone, [(2S,3S,4S,5R)-5-(hydroxymethyl)-[6,2H2]cyclohexanone-2,3,4,5-tetrol], recently recognized in feeding experiments as the precursor of the valienamine moiety of acarbose. We therefore purified the AcbC product further and compared it directly with authentic 2-epi-5-epi-[6,2H2]-valiolone by one- and two-dimensional NMR, ESI-MS, and gas chromatography-mass spectrometry of their trimethylsilyl derivatives. Except for the predictable spectral differences due to the presence of deuterium in the standard sample, the two compounds gave identical spectra and retention times. Incubation of AcbC with a sample of sedo-[1,13C]-heptulose 7-phosphate (41) gave 2-epi-5-epi-[7,13C]-valiolone (Fig. 5A), which was fed to a culture of the acarbose producer, Actinoplanes sp. strain SN223/29. The resulting sample of acarbose was enriched with 13C at C-7 of the valienamine moiety (Fig. 5C), confirming the precursor role of the enzymatically generated 2-epi-5-epi-valiolone in acarbose biosynthesis (Fig. 6). An analogous incubation of ido-[1,13C]-heptulose 7-phosphate with AcbC again gave no detectable cyclization product.

Cyclization Mechanism—Two principal mechanisms are known for the cyclization of open-chain sugar phosphates to cyclitols. One is exemplified by the DHQS reaction (27), and the other by the cyclization of glucose 6-phosphate catalyzed by myo-inositol-1-phosphate synthase (28). The latter mechanism applied to the synthesis of the acarbose precursor predicts the loss of both hydrogens from C-7 of the substrate, sedo-heptulose 7-phosphate, whereas in a DHQS-like cyclization, these two hydrogens would be retained in the product. We therefore incubated AcbC with a sample of sedo-[7,13C,7-3H]-heptulose 7-phosphate and measured the 3H/14C ratio of substrate and product. The change in this isotope ratio from 5.5 to 3.7 indicates 67% retention of tritium in the cyclization of the substrate to 2-epi-5-epi-valiolone, a result that rules out the myo-inositol synthase-like mechanism. The retention of <100% of the tritium is readily explained by nonenzymatic exchange via enolization.

**DISCUSSION**

The isolation of the putative biosynthetic gene cluster for acarbose from the producer Actinoplanes sp. is hampered by the lack of a genetic system in this organism, reflected by the fact that spore production is very low and a lack of protoplast regeneration and transformation systems. Also, enzymes with direct and specific involvement in the biosynthesis of this type of compounds are unknown so far. Therefore, an approach had to be chosen to isolate genomic DNA of the producing organism directly by means of indicative genetic probes, derived from more widely used genes with likely involvement in this pathway in related organisms. These were found in the genes for the common steps starting the versatile dTDP-hexose pathways used in many secondary metabolic contexts in streptomycetes (3, 8, 29). The two genes achA, encoding dTDP-n-glucose synthase, and achB, encoding dTDP-n-glucose 4,6-dehydratase, are members of gene families of widespread use in strain-
specific sugar modification pathways and therefore mostly occur in actinomycete secondary metabolic gene clusters or those for the formation of extracellular polysaccharides in Gram-negative bacteria (3, 30, 31). It is interesting to note that both genes and their products are conserved neither in a taxonomic nor in a pairwise coupled fashion, although they are always located in close proximity, often in the same operon, on the DNA (8, 29). No evidence for the existence of more than one copy of a dTDP-D-glucose 4,6-dehydratase gene in the genome of Actinoplanes sp. could be detected, although this is not a rare phenomenon since several bacterial strains, including streptomycetes, have been shown to contain more than one copy of the acbA- and acbB-related gene families, e.g. Streptomyces antibioticus Tu89 (32), E. coli K12 (33), and M. tuberculosis Rv (34) contain two to three copies of both gene families. Nevertheless, the three acb genes exhibit typical actinomycete G + C content (69%) and codon usage (35).

One of the aims of this work, the identification of biosynthetic genes in Actinoplanes sp. for its product acarbose, is accomplished in part since we could demonstrate the enzymatic activity of the AcbC protein to be that of a sedo-heptulose-7-phosphate cyclase producing the acarbose precursor, 2-epi-5-epi-valiolone (see below). The demonstration of this activity in Actinoplanes sp. under acarbose-producing conditions as well as its specific inactivation by insertional mutagenesis in the genome of this organism would be further proofs for the physiological function of acbC, but are not possible at present for the reasons mentioned above. However, the presence of additional genes involved in acarbose metabolism in the neighborhood of the three genes reported here was demonstrated by preliminary DNA sequencing on both sides of the acbABC locus. Thus, the genes for the acarbose 7-kinase (acbK) and the acarviosyltransferase (acbD) reported earlier (24, 36) have been detected in close proximity to this subcluster. Therefore, the conclusion is justified that we have identified the gene cluster for acarbose production and metabolism.

The sequence similarity of AcbC to the AroB-related DHQS proteins, cyclizing 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) to dehydroquinate, suggested that this enzyme catalyzes C7-cyclitol synthesis using a heptulose 7-phosphate as a substrate in accord with the in vivo labeling data reported earlier in biogenetic studies on valienamine-containing metabolites (6, 7). This was clearly proven by analysis of the enzyme

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4 A. Stratmann, M. Jarling, P. M. Diaz-Guardamino, H. Thomas, H. Apeler, and W. Piepersberg, unpublished observations.
reaction catalyzed by AcbC. Also, the activity of the AcbC protein is that of a sedo-heptulose-7-phosphate cyclase since it does not cyclize DAHP, nor does, in turn, the E. coli DHQS (AroB), overexpressed in a recombinant strain, cyclize sedo-heptulose 7-phosphate. The cyclization proceeds from sedo-heptulose 7-phosphate, in which C-5, the carbon undergoing transient oxidation to the ketone, has the same configuration as in DAHP, opposite to that in the final product, acarbose, at the same center. It takes place with retention of configuration at C-5 of the substrate to give a product, 2-epi-5-epi-valiolone, which still must undergo an epimerization at that carbon (now numbered C-2) during its conversion into acarbose. The C-5 epimer, ido-heptulose 7-phosphate, which would give the correct acarbose stereochemistry directly, is not a substrate for AcbC, presumably because its C-5 configuration does not allow oxidation to the ketone. A series of catalytic events during the cyclization of sedo-heptulose 7-phosphate to 2-epi-5-epi-valiolone is postulated in Fig. 7 in analogy to the recent proposal of Carpenter et al. (26). The stereochemistry of the product at the newly formed stereocenter, the quaternary carbon C-5, corresponds to that of dehydroquinate, the cyclization product of DAHP by DHQS. It differs from those of valiolone and natural valiolamine, which has been isolated from the validamycin fermentation (37). Another secondary metabolic use of the DHQS mechanism in actinomycetes seems to occur in the scyllo-inosose cyclase employed for the first step in the biosynthesis of 2-deoxystreptamine, a characteristic building block of a large subclass of the aminoglycosides (3, 38, 39). This enzyme catalyzes the cyclization of glucose 6-phosphate under equivalent conditions as the bacterial DHQS enzymes, namely in the presence of catalytic amounts of NAD$^+$ and cobalt ions and therefore could represent another member of the same enzyme family. However, details of the isolation of a gene for a scyllo-inosose cyclase have not yet been reported, and an internal DNA fragment of acbC does not hybridize to genomic DNA from actinomycetes producing streptamine-containing aminoglycosides.

Carpenter et al. (26) provided strong evidence that all catalytic steps occur in the same pocket of the DHQS proteins, based on their analysis of the three-dimensional structure of the DHQS domain of the filamentous fungus Emericella nidulans. This hypothesis is supported by the fact that the AcbC protein is especially conserved in the putative area of catalysis and sugar phosphate, cobalt (zinc in the fungal protein) ion, and NAD$^+$ binding (cf. Fig. 3). Also, 7 of the 13 amino acid residues of catalytic importance in the DHQS domain are conserved in AcbC and 4 out of the rest are conservatively exchanged. Of the catalytically active amino acid residues, just Lys-152 of DHQS (Glu-162 in...
AcbC, which is putatively involved in the phosphate elimination and cyclization steps, and His-275 (Pro-278 in AcbC), which seems to be engaged in de- and re-protonation of C-5, are not conserved; however, the function of Lys-152 (DHQS) could also be complemented by either Arg-161 or Arg-164 in AcbC. Also, the substrate-binding pocket in the environment of the C-1 and C-2 atoms of the C7-sugar precursors (formed by, for example, Lys-152, Lys-250, and Arg-264 in DHQS) seems to be engaged in de- and re-protonation of C-5, are not conserved; however, the function of Lys-152 (DHQS) could also be complemented by either Arg-161 or Arg-164 in AcbC.

Therefore, the cobalt ion and the 3 conserved amino acid residues in the Co2+-binding pocket are evident. The major differences between AcbC and DHQS proteins are rather suggestive alterations in the composition of the amino acid residues in the Co2+- (Zn2+-heptulose 7-phosphate (2-epi-5-epi-vali-clone) was not a surprise, although it deviates from the stereochirality of the cyclitol moiety at C-2 in the final product, acarbose (cf. Fig. 7). The AcbC protein clearly belongs to the family of bacterial DHQS (Arob) proteins, which in contrast to the fungal enzyme, use divalent cobalt ions as a cofactor instead of zinc ions (40); in the case of the bacterial DHQS enzymes, zinc ions are even inhibitory. This difference is quite surprising since the prokaryotic and eukaryotic DHQS proteins are much more similar to each other than to AcbC, and no suggestive alterations in the composition of the amino acid residues in the Co2+-binding pocket are evident. The major differences between AcbC and DHQS proteins are rather to be found in binding the substrates, which is reflected by the inverted stereochirality of the C-4 hydroxyl group and the one carbon extension at C-2 as well as by the lack of the carbonyl oxygen at C-1 in sedo-heptulose 7-phosphate relative to DAHP.

Therefore, the cobalt ion and the 3 conserved amino acid residues that are evidently involved in fixing the C-4 hydroxyl group in the DHQS proteins (cf. Fig. 3), Asp-146 (Asp-157 in AcbC), Glu-194 (Asp-157 in AcbC), Glu-194 (Glu-204), and Lys-197 (Lys-207), should have also be complemented by either Arg-161 or Arg-164 in AcbC. Nevertheless, Lys-152 (DHQS) seems to be involved in the phosphate elimination, and epimerization at C-4 cannot take place. These aspects should be investigated further, e.g., by amino acid replacement studies and determination of the crystal structure of the AcbC protein.

REFERENCES

1. Truscheit, E., Frommer, W., Junge, B., Müller, L., Schmidt, D. D., and Wingerder, W. (1981) Angew. Chem. Int. Ed. Engl. 20, 744–761
2. Müller, L. (1989) in Novel Microbial Products for Medicine and Agriculture (Demain, A. L., Komori, G. A., Hunter-Creva, J. C., and Rossomere, H. W., eds) pp. 109–116, Elsevier Science Publishers B.V., Amsterdam
3. Piepersberg, W., and Distler, J. (1997) in Bio/Technology: Products of Secondary Metabolism (Rehm, H.-J., and Reed, G., gener eds; Kleinkauf, H., and von Dihren, H., volume eds) 2nd Ed., Vol. 7, pp. 397–468, VCH Verlagsgesellschaft mbH, Weinheim, Germany
4. Piepersberg, W. (1997) in Bio/Technology of Actinomycetes (Strohl, W. R., ed) 2nd Ed., pp. 81–163, Marcel Dekker, Inc., New York
5. Floss, H. G. (1997) Nat. Prod. Rep. 14, 433–452
6. Toyokuni, T., Jin, W.-Z., and Rinehart, K. L., Jr. (1987) J. Am. Chem. Soc. 109, 3481–3483
7. Degwert, U., van Hulst, R., Pape, H., Herrald, R. E., Beale, J. M., Keller, P. J., Lee, J. P., and Floss, H. G. (1987) J. Antibiot. (Tokyo) 40, 855–861
8. Stockmann, M., and Piepersberg, W. (1992) FEMS Microbiol. Lett. 90, 185–190
9. Hopwood, D. A., Bish, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M., and Schrepffl, H. (1985) Genetic Manipulation of Streptomyces: A Laboratory Manual, John Innes Institute, Nofold, United Kingdom
10. Hanahan, D. (1983) J. Mol. Biol. 166, 557–580
11. Takano, E., White, J., Thompson, C. J., and Bish, M. J. (1996) Gene (Amst.) 166, 123–127
12. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene (Amst.) 33, 103–119
13. Distler, J., Klier, K., Piendl, W., Werbitzki, O., Böck, A., Kresze, G., and Piepersberg, W. (1985) FEMS Microbiol. Lett. 30, 145–150
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Bender, S. L., and Knowles, J. R. (1989) J. Bacteriol. 170, 2802–2808
16. Distler, J., Ebert, A., Mansouri, K., Pisovatotzki, K., Stockmann, M., and Piepersberg, W. (1987) Nucleic Acids Res. 15, 8041–8056
17. Pisovatotzki, K., Mansouri, K., and Piepersberg, W. (1991) Mol. Gen. Genet. 231, 113–123
18. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
19. Stuken, R. (1988) Nucleic Acids Res. 16, 1829–1836
20. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zheng Zhang, J. Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
21. Pearson, W. R., and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2444–2448
22. Rustenberg, B., Meijers, J., Peters, R., van der Hoeck, P., and van Alphen, L. (1975) FEBS Lett. 38, 254–258
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Dreyper, A., Pietzmann, R. and Pape, H. (1996) FEBS Lett. 388, 177–179
25. Lee, S., Sauerbrei, B., Niggemman, J., and Eggelkurt, E. (1997) J. Antibiot. (Tokyo) 50, 954–960
26. Carpenter, E. P., Hawkins, A. R., Frost, J. W., and Brown, K. A. (1998) Nature 394, 299–302
27. Widlanski, T., Bender, S. L., and Knowles, J. R. (1989) J. Am. Chem. Soc. 111, 2299–2300
28. Loewus, M. W., Loewus, F. A., Brilling, G. U., Otsuka, H., and Floss, H. G. (1980) J. Biol. Chem. 255, 11710–11712
29. Deckter, H., Gaisier, S., Pelzer, S., Schneider, P., Westrich, L., Wohlfelen, W., and Bechtold, A. (1996) FEMS Microbiol. Lett. 141, 195–201
30. Piepersberg, W. (1994) Crit. Rev. Biotechnol. 14, 251–285
31. Liu, H.-W., and Tharson, J. S. (1984) Annu. Rev. Microbiol. 48, 223–256
32. Sohng, J. K., and Yoo, J.-C. (1996) J. Biochem. Mol. Biol. 29, 183–191
33. Blattner, F. R., Plunkett, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A. Rose, D. J., Mau, B., and Shao, Y. (1997) Science 277, 1453–1474
34. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S., and Barrell, B. G. (1998) Nature 393, 537–544
35. Floss, H. G. (1997) in Pseudooligosaccharides and Metabolism of Starch in Actinomycetes (Rehm, H.-J., and Reed, G., general eds; Kleinkauf, H., and Koolsen, J. P., and Floss, H. G. (1999) J. Mol. Cytol., in press