Characterization of the hom–thrC–thrB cluster in aminoethoxyvinylglycine-producing Streptomyces sp. NRRL 5331

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Three genes from the aminoethoxyvinylglycine (AVG)-producing Streptomyces sp. NRRL 5331 involved in threonine biosynthesis, hom, thrB and thrC, encoding homoserine dehydrogenase (HDH), homoserine kinase (HK) and threonine synthase (TS), respectively, have been cloned and sequenced. The hom and thrC genes appear to be organized in a bicistronic operon as deduced by disruption experiments. The thrB gene, however, is transcribed as a monocistronic transcript. The encoded proteins are quite similar to the HDH, HK and TS proteins from other bacterial species. The overall organization of these three genes, in the order hom–thrC–thrB, differs from that in other bacteria and is similar to that reported in the Streptomyces coelicolor genome sequence. This is the first time in which the gene cluster for the three last steps of threonine biosynthesis has been characterized from a streptomycete. Disruption of thrC indicated that threonine is not a direct precursor for AVG biosynthesis in Streptomyces sp. NRRL 5331 and suggested that the branching point of the aspartic acid-derived biosynthetic route of this metabolite should lie earlier on the threonine biosynthetic route.

Keywords: threonine biosynthesis, homoserine dehydrogenase, homoserine kinase, vinylglycine, actinomycetes

INTRODUCTION

Vinylglycines have been traditionally studied as phytoxins responsible for the foliar chlorosis resulting from plant infection by certain micro-organisms. Examples include rhizobitoxine [1,2-amino-4-(2-amino-3-hydroxypropoxy)-trans-but-3-enoic acid] produced by the root-nodulating bacterium Bradyrhizobium japonicum (La Favre & Eaglesham, 1986) and the broad host range plant pathogen Burkholderia andropogonis (Mitchell & Frey, 1988), methoxyvinylglycine [1,2-amino-4-methoxy-trans-but-3-enoic acid] produced by the opportunistic human pathogen Pseudomonas aeruginosa (Goodwin & Mercer, 1983), and aminoethoxyvinylglycine [AVG; 1,2-amino-4-(2-amino-ethoxy)-trans-but-3-enoic acid] produced by the soil dweller Streptomyces sp. NRRL 5331 (Fig. 1). All of them are enol ether amino acids derived from aspartic acid (Mitchell & Coddington, 1991). Vinylglycines are analogues of cystathionine and constitute potent inhibitors of β-cystathionase (Giovanelli et al., 1971; Owens et al., 1971) in the methionine biosynthesis pathway. Additionally, their characteristic vinyl group makes these metabolites irreversible inhibitors of other pyridoxal phosphate-dependent enzymes (Rando, 1974; Gehring et al., 1977; Soper et al., 1977). Vinylglycines thus interfere, at very low concentrations, with the biosynthesis of the senescence hormone ethylene in plants through mechanism-based inhibition of two key enzymes on ethylene production, namely, the 1-aminocyclopropane-1-carboxylate synthase (Sato & Yang, 1989; Feng & Kirsch, 2000), and the 1-aminocyclopropane oxidase (Barry et al., 1996; Have & Woltering, 1997). Therefore, the normal function of the plant, and in particular ethylene biosynthesis, should be drastically affected in the presence of vinylglycines.

The biosynthetic pathway of vinylglycines has been only

Abbreviations: AVG, aminoethoxyvinylglycine; HDH, homoserine dehydrogenase; HK, homoserine kinase; PLP, pyridoxal phosphate; TS, threonine synthase.

The GenBank accession number for the sequence reported in this paper is AJ312095.
studied in the case of rhizobitoxine. In _Bur. andropogonis_ it has been established that aspartate is the first precursor of the pathway, homoserine being an essential intermediate (Mitchell & Coddington, 1991). This result suggests that the vinylglycine pathway should branch somewhere from the threonine biosynthetic pathway. Additionally, hydroxythreonine has been proposed as a likely biosynthetic intermediate on the pathway from homoserine to rhizobitoxine (Mitchell & Frey, 1988). On the other hand, studies with _Bra. japonicum_ have led to the cloning of two genes whose products are putatively involved in the other branch of the route, namely RtxA, a dehydroxyacetone phosphate aminotransferase involved in serinol production, and RtxB, the dihydrorhizobitoxine synthase responsible for the condensation of serinol and homoserine (Ruan et al., 1993). The biosyntheses of AVG and methoxyvinylglycine, however, remain totally obscure although they should have some steps in common since the main part of the molecule is shared.

To gain knowledge about AVG biosynthesis in _Strepto- myces_ sp. NRRL 5331 we decided to study the threonine biosynthetic genes of this bacterium. Threonine biosynthesis from aspartate has been well characterized in _Escherichia coli_, _Bacillus_ corynebacteria and other bacteria (for a review see Malumbres & Martin, 1996), but not from _Streptomycetes_. Here we report the cloning and characterization of the genes encoding the enzymes involved in the conversion of aspartate semialdehyde to threonine in the AVG-producing _Streptomycetes_ strain NRRL 5331. Promoter analysis and transcript disruption techniques were used to reveal their transcriptional organization.

**METHODS**

**Bacterial strains, cloning vectors and cultivation.** _Streptomyces_ sp. NRRL 5331 was used as the source of DNA in the construction of the genomic library. _Escherichia coli_ strain XL-1 Blue MR was used for obtaining SuperCos 1 cosmid (Stratagene) recombinant derivatives, and also served as a host for subcloning in plasmids pBluescript (Stratagene), pUC18 and pUC19. _Streptomycetes_ sp. NRRL 5331 was routinely grown in TSB, YEME medium without sucrose (Kieser et al., 2000) or YEPEG medium [glucose, 10 g l\(^{-1}\); Bacto peptone, 5 g l\(^{-1}\); yeast extract, 3 g l\(^{-1}\); Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\)·6H\(_2\)O, 0.03 g l\(^{-1}\)]. Sporulation was achieved in TBO medium (Aparicio et al., 2000) at 30 °C. Auxotrophs were tested on _Streptomyces_ minimal medium (Hopwood, 1967). -threonine or -homoserine (0–4 mM) were added, when required, to the minimal medium.

**Genetic procedures.** Standard genetic techniques with _E. coli_ and _in vitro_ DNA manipulations were as described by Sambrook & Russell (2001). Recombinant DNA techniques in _Streptomycetes_ species and isolation of _Streptomyces_ total and plasmid DNA were performed as previously described (Kieser et al., 2000). For construction of the genomic library, _Streptomycetes_ sp. NRRL 5331 genomic DNA was partially digested with Sau3AI and fragments in the 35–40 kb size range were cloned into SuperCos 1 digested with BamHI and XbaI. The ligation mixture was packaged with Gigapack III XL (Stratagene) and used to transfect _E. coli_ XL-1 Blue. Southern hybridization was carried out with probes labelled with digoxigenin by using the DIG DNA labelling kit (Roche Biochemicals).

**Generation of DNA probes.** DNA probes for screening of the library of size-fractionated genomic DNA were obtained by PCR amplification of _Streptomycetes_ sp. NRRL 5331 chromosomal DNA by using oligonucleotides derived from conserved stretches of several microbial homoserine dehydrogenases.
(HDHs), homoserine kinases (HKs) and threonine synthases (TSs). The oligonucleotide pairs used were the following: HDH1 (5′-GTGTSACCGCAGCAAGG-3′) and HDH2 (5′-GATGATGGTGTTTTGTCGCTT-3′) for the hom gene (encoding HDH); HK1 (5′-AACCTGGCCC-CAGGCTTCGAC-3′) and HK2 (5′-GGTTTGTCSGGGTG-GCCCTC-3′) for the thrB gene (encoding HK); TS1 (5′-CCSACCCGTCTCCTGACG-3′) and TS2 (5′-TCGTCGTCGAAATGGCCTG-3′) for the thrC gene (encoding TS). DNA fragments of the expected size (198 nt for hom; 300 nt for thrB; 210 nt for thrC) were obtained.

DNA sequencing and analysis. Sequencing templates were obtained by random subcloning of fragments generated by controlled partial HaeIII digestions. DNA sequencing was accomplished by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using the Perkin Elmer AmpliTaq Dye-terminator sequencing system on double-stranded DNA templates with an Applied Biosystems model 310 sequencer. Each nucleotide was sequenced a minimum of three times on both strands. Alignment of sequence contigs was performed using the University of Wisconsin Genetics Computer Group software programs (Devereux et al., 1984) and the NCBI Worldwide Web BLAST server (www.ncbi.nlm.nih.gov/blast).

AVG determination. AVG production was determined by a bioassay using Bacillus subtilis as the test organism. Solutions of pure AVG (Sigma) were used as reference values for halo formation.

AVG assay in cell-free extracts. Intracellular levels of AVG during growth were determined by analysing supernatants prepared by high-speed centrifugation of sonicated mycelial pellets. Sonication was carried out at 14 µm frequency for 30 s periods, until complete rupture, in the extraction buffer (50 mM Tris/HCl pH 8.0, 100 mM NaCl, 1 mM EDTA). The ratio between the weight of the mycelium sample and the volume of the extraction buffer was kept constant throughout the experiment. The disrupted mycelium was centrifuged (40000 g for 1 h at 4 °C) and the supernatant used for analysis.

Transcript disruption. The transcript responsible for hom and thr genes expression was disrupted by pHZ1351Km plasmid-mediated single-crossover integration as follows. A 1.3 kb BamHI fragment encompassing the neomycin phosphotransferase gene from transposon Tn5 (Beck et al., 1982) was cloned into a BamHI-digested pHZ1351 vector to yield pHZ1351Km. This plasmid retained the broad host-range and high copy number properties of the parental vector isolated from Streptomyces sp. FR-005 (Bao et al., 1997), and is prone to chromosomal integration mediated by homologous recombination. Although pHZ1351 was originally described as useful for gene replacement, and its replicon was never found to be integrated into the Streptomyces hygroscopicus KMP3 chromosome (Bao et al., 1997), we have observed that the replicon of pHZ1351Km is stable upon integration into Streptomyces sp. NRRL 5331 chromosome (not shown). pHZ1351Km was therefore used as a vector for DNA delivery into Streptomyces sp. NRRL 5331 by transformation.

Subcloning in promoter-probe vectors. Promoter activity of selected DNA fragments was assessed by cloning them upstream of the promoterless kanamycin resistance gene present in the promoter-probe vector pJI486 (Kieser et al., 2000). In particular, for the putative thrB promoter two DNA fragments were used, a 708 bp Smal fragment including the 3′ end of thrC and the 5′ end of thrB genes, and a 275 bp NruI–Smal fragment extending 205 bp upstream from the thrB start codon. Both DNA fragments were subcloned in the EcI36II site of pJI486. Following transformation of Streptomyces lividans 66 (Kieser et al., 2000) with the resulting plasmids, promoter strength was assessed with increasing concentrations of kanamycin.

RESULTS

Cloning and identification of hom, thrB and thrC genes forming a single gene cluster

Despite the high degree of conservation among microbial HDHs, HKs and TSs, we were unable to identify their corresponding genes in Streptomyces sp. NRRL 5331 by hybridization using heterologous probes internal to the homologous genes from Nocardia lactam-durans and Corynebacterium lactofermentum. Therefore, the three biosynthetic genes had to be identified by using homologous DNA probes PCR-derived from Streptomyces sp. NRRL 5331 chromosomal DNA (see Methods). A cosm library was constructed in the SuperCos 1 vector and several positively hybridizing cosmids were selected independently with the three probes. Further characterization of all of them by cross-hybridization suggested that the three genes were linked.

Two of the cosmids (Cos 27 and Cos 13) were further selected and mapped with restriction enzymes NolI, SacI, BamHI and EcoRI. The two cosmids covered a contiguous 60 kb region of the Streptomyces sp. NRRL 5331 chromosome. Interestingly, the three genes were linked in a 3.7 kb region, most of it covered by a BamHI fragment (Fig. 2). Internal BamHI and SacI fragments of the cosmids were the same size as their homologous fragments of Streptomyces sp. NRRL 5331 total DNA, suggesting that the cloned DNA was not rearranged.

The complete sequence of the 3.7 kb region, which encompasses the three genes, was determined. Computer-assisted analysis of the sequenced region revealed
that characterise the NADP-dependent reduction of aspartate β-semialdehyde into homoserine. These values ranged from 39.6% for the HDH of *P. aeruginosa* (swal accession no. P29365) to 89% for the *S. coelicolor* HDH (swal accession no. Q9ADB4) (Table 1). An alignment of the regions of bacterial HDH proteins that show the greatest degree of sequence conservation is shown in Fig. 3(a). These include the N-terminal end fingerprint region GXGXXG common to the vast majority of NAD(P)H binding sites (Wierenga et al., 1986) and the central consensus patterns for these enzymes (Thomas et al., 1993).

Located 8 bp downstream from the TAA stop codon of *hom* lies the ATG start codon of *thrC*, an ORF encoding a protein of 356 aa. This gene product was easily assigned as the enzyme that catalyses the transformation of homoserine phosphate into threonine because of its significant sequence similarity over its entire length (up to 93% identity) to other TS enzymes from both bacteria and archaea, including *S. coelicolor* (swal accession no. Q9ADB3), *Mycobacterium leprae* (swal accession no. P45837), *Aquifex aeolicus* (swal accession no. O66740) and *Archeoglobus fulgidus* (swal accession no. O28953) (Table 1). The enzyme shows in its N-terminal end the characteristic lysine residue that constitutes the pyridoxal phosphate attachment site (Fig. 3b), a feature also shared by other pyridoxal phosphate-dependent enzymes like serine and threonine dehydratases ( Parsot, 1986).

Downstream (347 bp) from the TGA stop codon of *thrC* is the ATG start codon of the ORF (*thrB*) needed to phosphorylate homoserine, which in turn will serve as

### Table 1. Percentage identity between the HDHs, TSs and HKs of *Streptomyces* sp. NRRL 3551 and other microorganisms

<table>
<thead>
<tr>
<th>Species</th>
<th>Streptomyces sp. NRRL 5331</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HDH</td>
</tr>
<tr>
<td><em>Streptomyces coelicolor</em></td>
<td>89.9</td>
</tr>
<tr>
<td><em>Mycobacterium leprae</em></td>
<td>60.8</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>60.8</td>
</tr>
<tr>
<td><em>Corynebacterium glutamicum</em></td>
<td>58.6</td>
</tr>
<tr>
<td><em>Bacillus halodurans</em></td>
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</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>42.1</td>
</tr>
<tr>
<td><em>Aquifex aeolicus</em></td>
<td>39.4</td>
</tr>
<tr>
<td><em>Synechocystis</em> sp.</td>
<td>NA</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>39.6</td>
</tr>
<tr>
<td><em>Thermotoga maritima</em></td>
<td>37.1</td>
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</tbody>
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Three potential complete ORFs (Fig. 2), corresponding to the *hom*, *thrB* and *thrC* genes sought. The G+C content of the nucleotide sequence is 72.2 mol%, well within the range of the reference values for *Streptomyces* DNA (Wright & Bibb, 1992).

Seventy-nine base pairs from the 5′ end of the BamHI fragment (Fig. 2) lies the ATG start codon of the *hom* gene (1290 bp), whose product showed a very high end-to-end sequence identity with bacterial HDHs, enzymes.

![Fig. 3](image-url)
A strong promoter controls the expression of thrB

Computer-assisted analysis of the 347 bp intergenic region between thrC and thrB revealed a putative transcription initiation codon (Met) and the putative −10 and −35 regions for the potential promoter of thrB are shown in black bold type and underlined (see text). The potential RBS is boxed. The possible terminator (inverted repeat) is indicated by convergent arrows. The Smal and NruI sites used for promoter activity assessment are also indicated.

Fig. 4. Nucleotide sequence of the intergenic region between thrC and thrB in Streptomyces sp. NRRL 5331. The translation initiation codon (Met) and the putative −10 and −35 regions for the potential promoter of thrB are shown in black bold type and underlined (see text). The potential RBS is boxed. The possible terminator (inverted repeat) is indicated by convergent arrows. The Smal and NruI sites used for promoter activity assessment are also indicated.

hom and thrC are organized as a single transcriptional unit

The closeness of hom and thrC in the Streptomyces sp. NRRL 5331 chromosome, only 8 bp apart, suggested that the two genes were transcriptionally linked. Since the mRNA isolated from the parental strain turned out to be highly unstable for primer extension or S1 mapping studies, the study of that putative transcriptional linkage was addressed by chromosomal gene disruption of the possible bi-cistronic transcript controlling the expression of both genes. A 870 bp Smal DNA fragment, encompassing the 3' end of hom and the 5' end of thrC genes, was cloned into the EcoRI site of pHZ1351Km (a vector with a highly unstable ori, see Methods), and the resulting plasmid used to transform strain NRRL 5331.

Several transformants were obtained by selection for thiostrepton and kanamycin resistance and tested for their inability to grow on minimal medium. One of these disrupted mutants was randomly selected and named HDH-TS. The identity of the mutant was confirmed by Southern hybridization (Fig. 5). Chromosomal DNAs isolated from Streptomyces sp. NRRL 5331 and mutant HDH-TS were digested with BamHI and probed with the 870 bp Smal fragment used to construct the pHZ1351Km derivative utilized for gene disruption. A hybridizing band of 3-5 kb was found for the wild-type as expected (Fig. 5). However, in the disrupted mutant, two new bands of 11 kb and 1-6 kb were detected, indicating that a single crossover event had occurred. The observed hybridizing bands corresponded exactly to those bands expected according to the integration shown in Fig. 5.

As a result of plasmid integration into the chromosome, the disrupted mutant retains intact copies of hom and thrC genes, although a putative bi-cistronic transcript governing the expression of both genes would be interrupted. Therefore, in such a case, the expression of thrC would be blocked and the mutant would display a threonine auxotrophy phenotype. To test this possibility the mutant strain was grown in minimal medium with and without added threonine, showing an absolute requirement for added threonine to sustain growth. Furthermore, the disrupted mutant could not be complemented with homoserine. These results indicate that
Fig. 5. Disruption of the transcript that controls the expression of thrC. (a) Predicted restriction enzyme polymorphism caused by gene disruption. The BamHI restriction pattern before and after disruption is shown. The probe is indicated by thick dashed lines. B, BamHI; S, Smal. Note that only the Smal sites relevant for obtaining the fragment used in the gene disruption are shown. (b) Southern hybridization of the BamHI-digested chromosomal DNA of the wild-type (lane 1) and the mutant (lane 3). Digoxigenin labelled lambda DNA digested with HindIII is shown in lane 2.

Production of AVG in the thrC (TS) mutant and the wild-type

To determine the growth phase where we could detect a maximum of AVG production, Streptomyces sp. NRRL 5331 cells were grown for 72 h in TSB medium and used as inoculum (1 ml in 50 ml) of cultures in YEPEG medium. Cultures were then incubated for different periods of time and the AVG content of the broth was measured (see Methods). A clear peak of maximum yield [80 µg ml⁻¹; 1.25 µg AVG (mg dry weight)⁻¹] was observed at 48 h of incubation, during the second half of the exponential phase, decreasing rapidly afterwards. Such conditions were then used to test AVG levels in the mutant strain. No significant differences were found from the production of the wild-type strain (not shown).

To determine if AVG was retained in the cell, AVG levels were also measured in cell-free extracts of the wild-type and the thrC mutant grown in the same media. No traces of AVG were found in the cytoplasm or the cell-wall fractions, even at a threefold concentration relative to the broth. Therefore, AVG does not seem to be retained inside the cells either in the wild-type or in the thrC mutant.

DISCUSSION

This paper constitutes the first characterization of the threonine biosynthetic gene cluster for a Streptomyces species. The first conclusion from sequence analysis of the Streptomyces sp. NRRL 5331 cluster is that the overall organization of the genes, in the order hom–thrC–thrB, is identical to the one displayed by M. tuberculosis (Cole et al., 1998) and to that found recently after completion of the S. coelicolor genome. Furthermore, the genes upstream and downstream from this cluster, lysA and rho, respectively, also match the genes found in both bacteria. Such an organization would imply a different strategy for the regulation of gene expression in these two genera of actinomycetes as compared with enterobacteria such as E. coli (Thèze & Saint-Girons, 1974) or Serratia marcescens (Komatsu-bara et al., 1979), where the thrA gene, which encodes a polypeptide with aspartate kinase and homoserine dehydrogenase activities, is organized in an operon along with the thrB and thrC genes. The organization in Streptomyces sp. NRRL 5331 is also different from what has been observed in lower actinomycetes.
such as *Brevibacterium lactofermentum* (Malumbres et al., 1994) [renamed as * Corynebacterium lactofermentum* (Amador et al., 1999)] or *Corynebacterium glutamicum* (Peoples et al., 1988), or in other Gram-positive bacteria such as *Lactococcus lactis* (Madsen et al., 1996), where *hom* and *thrB* are linked and *thrC* is located elsewhere in the chromosome. However, a similar gene arrangement has been found in other, more distantly related, microorganisms such as *Bacillus* ( Parsot & Cohen, 1988; Malumbres et al., 1995), where the three genes are linked together in the same operon, or *Pseudomonas* (Clepet et al., 1992) and *Methyllobacillus* (Motoyama et al., 1994; Marchenko et al., 1999), where the *hom* and *thrC* genes seem to be co-transcribed while the *thrB* gene is not linked to the other two (see Malumbres & Martin, 1996 for a review).

Functional promoter analysis and chromosomal disruption have been used in this work to prove that *Streptomycyes* sp. genes are organized in two operons, a situation resembling that observed in *P. aeruginosa* (Clepet et al., 1992) or *Methyllobacillus flagellatus* (Marchenko et al., 1999), but in these Gram-negative bacteria the *thrB* gene is located far apart from the other two. Although a similar overall arrangement of the threonine genes has been found in *Myc. tuberculosi* (Cole et al., 1998) and *S. coelicolor* genomes, no studies have been undertaken to establish whether the three genes are transcriptionally linked or not.

The other two genes needed to complete the threonine biosynthetic route from aspartate, namely *ask* and *asd*, encoding aspartate kinase and aspartate semialdehyde dehydrogenase, respectively, are not within the 60 kb DNA stretch covered by cosmids 27 and 13 ([unpublished results]). This separate arrangement of the genes responsible for threonine biosynthesis from aspartate in two different clusters has been also observed in *Myc. tuberculosi* (Cole et al., 1998). A cluster containing the *ask–asd* genes unlinked to the threonine genes has been found recently in *Amycolatopsis lactamundans* (Hernando-Rico et al., 2001). In the *S. coelicolor* genome *ask* and *asd* are also organized in an operon separated from *hom–thrC–thrB* (Redenbach et al., 1996); it is therefore tempting to speculate that the same situation will be found in other *Streptomycyes* species. However a discrete *asd* gene has been reported in *Streptomycyes akiyoshiensis* (Le et al., 1996).

If AVG biosynthesis in *Streptomycyes* follows a route equivalent to that of rhizobitoxine formation in *Bur. andropogonis*, homoserine would constitute an essential intermediate (Mitchell & Coddington, 1991), thus suggesting that the route should branch somewhere from the threonine biosynthetic pathway (Fig. 1). Furthermore, vinylglycine has been described as substrate for TS in the formation of threonine (Laber et al., 1994).

The levels of AVG displayed by the *thrC* mutant suggest that threonine is not a direct precursor for AVG in *Streptomycyes* sp. 5331 since disruption of the *thrC* gene should have resulted in the absence of AVG formation as compared to the wild-type. The branching should, therefore, lie earlier in the threonine route.

The availability of the threonine genes should prove useful in the future in elucidating the AVG biosynthetic route in *Streptomycyes* sp. NRRL 5331, and therefore establish the basis for a rational manipulation of AVG production.

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