

# Biosynthesis of sulfur-containing amino acids in *Streptomyces venezuelae* ISP5230: roles for cystathionine $\beta$ -synthase and transsulfuration

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**A 0.5 kb fragment of *Streptomyces venezuelae* ISP5230 genomic DNA was amplified by PCR using primers based on consensus sequences of cysteine synthase isozyme A from bacteria. The deduced amino acid sequence of the PCR product resembled not only cysteine synthase sequences from prokaryotes and eukaryotes but also eukaryotic cystathionine  $\beta$ -synthase sequences. Probing an *Str. venezuelae* genomic library with the PCR product located a hybridizing colony from which pJV207 was isolated. Sequencing and analysis of the *Str. venezuelae* DNA insert in pJV207 detected two ORFs. The deduced amino acid sequence of ORF1 matched both cysteine synthase and cystathionine  $\beta$ -synthase sequences in GenBank, but its size favoured assignment as a cystathionine  $\beta$ -synthase. ORF2 in the pJV207 insert was unrelated in function to ORF1; in its sequence the deduced product resembled acetyl-CoA transferases, but disruption of the ORF did not cause a detectable phenotypic change. Disruption of ORF1 failed to elicit cysteine auxotrophy in wild-type *Str. venezuelae*, but in the *cys-28* auxotroph VS263 it prevented restoration of prototrophy with homocysteine or methionine supplements. The change in phenotype implicated loss of the transsulfuration activity that in the wild-type converts these supplements to cysteine. This study concludes that disruption of ORF1 inactivates a *cbs* gene, the product of which participates in cysteine synthesis by transsulfuration. Enzyme assays of *Str. venezuelae* mycelial extracts confirmed the formation of cysteine by thiolation of *O*-acetylserine, providing the first unambiguous detection of this activity in a streptomycete. Enzyme assays also detected cystathionine  $\gamma$ -synthase, cystathionine  $\beta$ -lyase and cystathionine  $\gamma$ -lyase activity in the extracts and showed that the substrate for cystathionine  $\gamma$ -synthase was *O*-succinylhomoserine. Based on assay results, the *cys-28* mutation in *Str. venezuelae* VS263 does not inactivate the cysteine synthase gene but impairs expression in cultures grown in minimal medium.**

Keywords: cysteine auxotroph, sulfur metabolism, gene disruption, streptomycetes

## INTRODUCTION

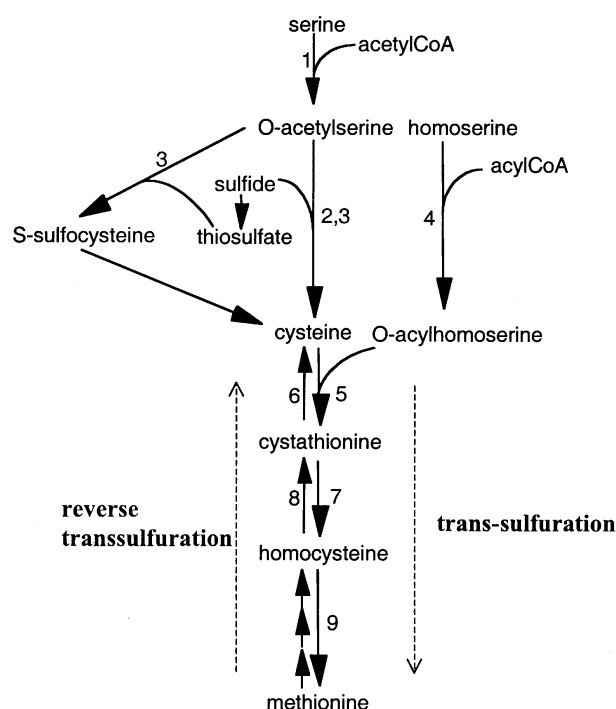
Filamentous bacteria of the genus *Streptomyces* produce a large variety of secondary metabolites. The bio-

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**Abbreviations:** CBL, cystathionine  $\beta$ -lyase; CBS, cystathionine  $\beta$ -synthase; CGL, cystathionine  $\gamma$ -lyase; CGS, cystathionine  $\gamma$ -synthase; CS, cysteine synthase; OAH, *O*-acetylhomoserine; OSH, *O*-succinylhomoserine.

The GenBank accession number for the sequence reported in this paper is AF319543.

synthetic processes by which these metabolites are formed depend ultimately on the primary metabolism of producer organisms, so knowledge of the essential primary activities is necessary for full understanding of an area of secondary metabolism such as antibiotic production. Compared with that of enteric bacteria, bacilli and other well-studied micro-organisms, the primary metabolism of streptomycetes is not well known. In *Streptomyces venezuelae*, which produces the antibiotics chloramphenicol and jadomycin B, biochemical and molecular genetic investigations have



**Fig. 1.** Metabolic reactions of sulfur-containing amino acids in bacteria. Enzymes: 1, serine acetyltransferase; 2, *O*-acetylserine thiolase (*cysK* product); 3, *S*-sulfocysteine synthase (*cysM* product); 4, homoserine acyltransferase; 5, CGS; 6, CGL; 7, CBL; 8, CBS; 9, methionine synthase.

shown (Vining & Stuttard, 1994; Wang *et al.*, 2001; He *et al.*, 2001) that genes for the biosynthesis of these antibiotics are located in separate clusters on the chromosome. Those for chloramphenicol are flanked by the auxotrophic markers *pdx-4* and *cys-28* (Doull *et al.*, 1986; Vats *et al.*, 1987; Stuttard, 1988). Our initial interest in the *cys-28* marker led us to explore the molecular biology and biochemistry of cysteine biosynthesis in *Str. venezuelae*, and its relationship to the relatively unexplored metabolism of sulfur-containing amino acids in this and other streptomycetes.

Studies of the biosynthesis and metabolism of sulfur-containing amino acids in *Escherichia coli* and numerous other micro-organisms have established that bacteria form cysteine from serine by a two-step process initiated by serine acetyltransferase (Fig. 1). The *O*-acetylserine intermediate reacts with  $H_2S$  to give cysteine by the action of *O*-acetylserine thiolase. Although both enzyme activities should properly be included in the term cysteine synthase (CS), this designation will be used below, as elsewhere (Kredich, 1997), to refer only to the second reaction, releasing cysteine. In enterobacteria, isozymic forms (A and B) of the thiolase are encoded by *cysK* and *cysM* respectively (Hulanicka *et al.*, 1986; Sirko *et al.*, 1987). Isozyme B accepts thiosulfate as an alternative substrate to sulfide, forming *S*-sulfocysteine in the initial condensation, but this eventually yields cysteine (Nakamura *et al.*, 1984).

Although *cysK* has been cloned from a wide variety of bacteria in addition to enterics: e.g., *Rhodobacter sphaeroides* 2.4.1 (O'Gara *et al.*, 1997), *Flavobacterium K3-15* (Muller *et al.*, 1996), *Synechococcus* sp. (Nicholson *et al.*, 1995) and *Streptococcus suis* (Osaki *et al.*, 2000), *cysM* has a more limited distribution (Hensel & Trüper, 1976). Also, some bacteria (e.g., *Pseudomonas aeruginosa*) possess alternative reactions, commonly called reverse transsulfuration, that generate cystathionine and then cleave it with cystathionine  $\gamma$ -lyase (CGL) to form cysteine (see Fig. 1; Gunther *et al.*, 1979). In *Saccharomyces cerevisiae* cystathionine is available from the cystathionine  $\beta$ -synthase (CBS)-catalysed condensation of homocysteine and serine (Cherest & Surdin-Kerjin, 1992), whereas in bacteria cystathionine is usually formed, along with the homocysteine required for methionine biosynthesis, in a cystathionine  $\gamma$ -synthase (CGS)-catalysed transsulfuration reaction between cysteine and *O*-acetylhomoserine (reviewed by Greene, 1997).

Only limited information is available on the formation and interconversion of sulfur-containing amino acids in streptomycetes. From the growth responses of various *Streptomyces griseus* mutants disturbed in sulfur metabolism, Kitano *et al.* (1985) concluded that thiosulfate is an intermediate in the reduction of sulfate en route to cysteine. This was supported by evaluations in *Streptomyces coelicolor* and *Streptomyces lividans* of the growth requirements of cysteine auxotrophs (Lydiat *et al.*, 1988). The role postulated for thiosulfate as a direct precursor of cysteine in actinomycetes was consistent with results obtained by Donadio *et al.* (1990) when genes for thiosulfate formation in *Saccharopolyspora erythraea* were cloned and disrupted. However, a principal role for this pathway is challenged by evidence that labelled methionine is incorporated into the cysteine component of the antibiotic cephamycin C in *Streptomyces clavuligerus* (Whitney *et al.*, 1972). Cysteine itself is a direct precursor of cephamycin C, but incorporation of methionine implicates the operation of a reverse transsulfuration pathway (see Fig. 1). Although there is no evidence for primary as distinct from secondary metabolic functions of a pathway from methionine to cysteine in *Str. clavuligerus*, the key transsulfuration enzyme CGL has been characterized from *Streptomyces lactamdurans* (Kern & Inamine, 1981). Subsequent discovery of the enzyme in *Streptomyces phaeochromogenes*, and other evidence of its wide distribution in actinomycetes (Nagasawa *et al.*, 1984), suggests that transsulfuration may be as important as thiolation for the formation of sulfur-containing amino acids in these bacteria. We here report the first cloning and characterization of a gene for CBS from a prokaryote, and confirm the role of transsulfuration in the generation of sulfur-containing amino acids in a streptomycete.

## METHODS

**Strains and plasmids.** These are listed in Table 1.

**Culture conditions.** Stock *E. coli* strains were routinely grown

**Table 1.** Strains and vectors

Strain/plasmid	Genotype/phenotype	Source/reference
<b><i>Str. venezuelae</i> strains</b>		
ISP5230	Wild-type	Stuttard (1982)
VS263	<i>cys-28</i>	Doull <i>et al.</i> (1986)
VS1021/1022	ISP5230(pJV215/pJV216)	This study
VS1025	ISP5230(pJV218)	This study
VS1028	ISP5230 with <i>cbs</i> disrupted	This study
VS1030	VS263(pJV218)	This study
VS1032	VS263 with <i>cbs</i> disrupted	This study
VS1034	ISP5230(pJV222)	This study
VS1036	ISP5230 with ORF2 disrupted	This study
<b><i>E. coli</i> strains</b>		
DH5 $\alpha$	F $^{-}$ $\Phi$ 80d <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> )U169 <i>endA1 recA1 hsdR17</i> ( <i>r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup></i> ) <i>deoR thi-1 phoA supE44 <math>\lambda</math><sup>-</sup> gyrA96 relA1</i>	Gibco-BRL
ET12567	<i>dam<sup>-</sup> dcm<sup>-</sup> hsdM<sup>-</sup></i>	MacNeil <i>et al.</i> (1992)
<b>Plasmids</b>		
pHJL400	<i>tsr, amp, lacZ</i>	Larson & Hershberger (1986)
pBluescript II SK +	Phagemid: <i>amp lacZ</i> (usually abbreviated to pSK +)	Stratagene
pUC18	<i>amp lacZ</i>	Pharmacia
pJV205	PCR-amplified 0.5 kb <i>cbs</i> fragment of <i>Str. venezuelae</i> cloned in pSK +	This study
pJV207	9.2 kb fragment of ISP5230 DNA containing ORFs 1 and 2 cloned in pSK +	This study
pJV208/209	4.0 kb <i>PstI</i> fragment of pJV207 subcloned in pSK + (both orientations)	This study
pJV210/211	3.0 kb <i>PstI</i> fragment of pJV207 subcloned in pSK + (both orientations)	This study
pJV213	pJV208 with 1.5 kb <i>SalI</i> cassette from pJV225 inserted at <i>SalI</i> site	This study
pJV215/216	4.0 kb <i>PstI</i> fragment of pJV207 subcloned in pHJL400 (both orientations)	This study
pJV217	pJV216 with 0.8 kb <i>BglIII</i> segment replaced with 1.5 kb <i>NcoI</i> cassette from pUC120A containing <i>apr</i>	This study
pJV218/219	pJV213 insert subcloned in pHJL400 (both orientations)	This study
pJV220/221	4.0 kb <i>XhoI-BglIII</i> fragment of pJV207 subcloned in pHJL400 (both orientations)	This study
pJV222	pJV220 with 1.5 kb <i>NcoI</i> cassette from pUC120A inserted at the <i>NcoI</i> site in ORF2	This study
pJV223	<i>PstI-EcoRI</i> fragment of pR4 cloned in <i>XhoI</i> site of pSK +	This study
pJV224	pSK + containing 0.6 kb <i>Str. venezuelae</i> DNA fragment flanked on each side by multi-cloning site of pSK +	This study
pJV225	pSK + containing <i>NcoI</i> cassette with <i>apr</i> flanked on each side by multi-cloning site of pSK +	This study
pJV226	pJV225 with <i>apr</i> replaced by <i>vph</i> in <i>PstI</i> fragment from pUC1169	This study
pJV227	pHJL400 with <i>vph</i> cloned in <i>PstI</i> site	This study
pR4	pSK + containing <i>apr</i> in <i>BamHI-PstI</i> fragment subcloned from pR3	He <i>et al.</i> (2001)
pUC120	pBluescript II containing <i>apr</i> from pKC462a	Paradkar & Jensen (1995)
pUC120A	pUC120 containing <i>apr</i> in an <i>NcoI</i> cassette	This work

overnight on LB agar or in LB broth (Sambrook *et al.*, 1989) supplemented with ampicillin (100  $\mu$ g ml $^{-1}$ ) or apramycin (50  $\mu$ g ml $^{-1}$ ) when needed for strains harbouring plasmids. Cultures used for isolating single-stranded DNA were grown in 2  $\times$  YT broth (Sambrook *et al.*, 1989). Cultures of *Str. venezuelae* were grown routinely in MYM medium, which contained (per l): maltose (4 g), yeast extract (Difco; 4 g) and malt extract (Difco; 10 g) at pH 7.3 (Stuttard, 1982). Thio-strepton (25  $\mu$ g ml $^{-1}$ ) or apramycin (50  $\mu$ g ml $^{-1}$ ) was included when needed to retain a plasmid. To determine the growth requirements of auxotrophic strains, cultures were grown on minimal agar (MM) from washed spore suspensions (Hop-

wood *et al.*, 1985); for strains that produced few spores on MYM agar, TOY sporulation agar (Chang *et al.*, 2001) was used. To obtain mycelium for enzyme assays, cultures were grown from a vegetative inoculum prepared by aerating a spore suspension (100  $\mu$ l) with MYM medium (20 ml) in a 250 ml Erlenmeyer flask on a rotary shaker at 27  $^{\circ}$ C overnight. A portion (1 ml) of the inoculum culture was added to each 50 ml MYM or MMY medium in a 500 ml Erlenmeyer flask. The latter medium contained (per l): glucose (10 g; autoclaved separately), yeast extract (1 g), asparagine (0.5 g), MgCl $_2$ ·6H $_2$ O (0.2 g), FeSO $_4$ ·7H $_2$ O (0.01 g), pH 7.2–7.4. In cultures of VS263 and VS1032 used to prepare mycelium

extracts for assays of CS activity, it was supplemented with methionine and cystathionine (50 µg ml<sup>-1</sup> each).

**Preparation of cell extracts.** The mycelium from a 50 ml *Str. venezuelae* culture was collected by centrifugation (10000 g at 4 °C for 10 min) and washed with TEPD buffer (100 mM Tris/HCl, 1 mM EDTA, 0.1 mM pyridoxal phosphate, 0.1 mM DTT, pH 8.0). For homocysteine synthase assays, PEPD buffer (TEPD with Tris replaced by 100 mM phosphate, pH 7.6) was used. The washed mycelium was disrupted with a Branson Sonifier (model 210) for 6 × 15 s at 4 °C. The sonicate was centrifuged (12000 g for 20 min at 4 °C and the supernatant solution was used as the cell extract. Protein was measured with the bicinchoninic acid reagent (Pierce Chemical). Cell extracts of strains compared for enzyme activity were adjusted with TEPD or PEPD buffer to the same protein concentration.

**Assay of CS activity.** The reaction mixture (500 µl) contained cell extract (250 µl), 30 mM O-acetylserine and 20 mM Na<sub>2</sub>S, all prepared in TEPD buffer. After incubation at 30 °C for 30 min, activity was stopped by adding 50 µl 50% (w/v) trichloroacetic acid. The mixture was clarified by centrifugation and its cysteine content was determined by the specific colorimetric method described below.

**Assay of CBS and CGS.** Cystathionine can be formed by CBS-catalysed condensation of homocysteine with serine, or by CGS-catalysed condensation of O-acyl (succinyl- or acetyl-) homoserine with cysteine. To measure CBS activity, the reaction mixture (500 µl) contained 250 µl cell extract, 20 mM homocysteine and 20 mM serine, all in PEPD buffer. CGS activity in mycelial extracts of *Str. venezuelae* was measured in a reaction mixture containing 20 mM O-succinyl-DL-homoserine, synthesized as described by Nagai & Flavin (1967), and 20 mM cysteine as substrates, again all in PEPD buffer. Both assay mixtures were incubated for 30 min at 30 °C and terminated by freezing (-20 °C); the cystathionine formed was measured by HPLC using the procedure described below for amino acid analysis.

**Assay of CBL and CGL.** Cystathionine is cleaved to form pyruvic acid, homocysteine and ammonia by cystathionine β-lyase (CBL), or α-ketobutyric acid, cysteine and ammonia by CGL. The reaction mixture (500 µl) for either assay consisted of 250 µl cell extract, 200 µl cystathionine (10 mM in 10 mM HCl) and 50 µl TEPD buffer. After incubation at 30 °C for 30 min, reactions were terminated by acidification with 500 µl 2 M HCl and clarified by centrifugation for analysis. Pyruvic and α-ketobutyric acids in enzyme incubation mixtures were derivatized by reaction with 1,2-diamino-4,5-dimethoxybenzene, synthesized as described by Ohmori *et al.* (1992). The 3-methyl-2-hydroxy-6,7-dimethoxyquinoxaline formed from pyruvic acid, and 3-ethyl-2-hydroxy-6,7-dimethoxyquinoxaline from α-ketobutyric acid were measured by HPLC with a detector monitoring absorbance at 360 nm (Ohmori *et al.*, 1992).

**Specific measurement of cysteine.** The colorimetric method of Gaitonde (1967) was modified as follows: the cysteine-containing solution (0.5 ml) was mixed in a test tube with 0.5 ml glacial acetic acid and 0.5 ml ninhydrin reagent. The solution was heated in boiling water for exactly 90 s, cooled on ice for 2 min and diluted with 2 ml 95% ethanol before the absorbance was measured at 560 nm.

**Measurement of amino acids by HPLC.** Each sample (40 µl) was neutralized and mixed with 40 µl o-phthalaldehyde reagent (Pierce Chemical). After 1 min, 120 µl 0.1 M sodium

acetate buffer (pH 6.2) was added and a portion (20 µl) of the mixture was injected on to a 4.6 × 45 mm Beckman Ultrasphere ODS column. Elution of fluorescent amino acid derivatives by a programmed methanol/solvent A gradient was monitored with a Beckman fluorescence detector (excitation 305–395 nm, emission 420–650 nm). Solvent A contained 100 ml of a 19:1 mixture of methanol and tetrahydrofuran, brought to 1000 ml with 0.1 M sodium acetate, pH 6.2. The percentage of solvent A in the gradient was decreased from 100% to 25% in 6.74 min, then to 0% in 0.26 min, and kept at 0% for an additional 0.5 min. To reequilibrate the column for another injection, solvent A was increased to 100% in 0.5 min and kept at 100% for another 2 min.

**Chloramphenicol assays.** Production of chloramphenicol was bioassayed as described by Doull *et al.* (1985). For specific and more precise measurement of chloramphenicol produced in liquid cultures, the HPLC procedure of Brown *et al.* (1996) was used.

**DNA manipulation.** The procedures of Sambrook *et al.* (1989) were used to isolate or modify DNA, and to prepare and transform competent *E. coli* cells. For rapid screening of *E. coli* plasmids, the Sekar (1987) procedure was adopted. DNA fragments were recovered from agarose gels with the QIAEX II Gel Extraction Kit (Qiagen) using the supplier's protocol. Routine plasmid isolation from streptomycetes was based on the alkaline lysis method for isolating *E. coli* plasmids, but Solution I was replaced with P-buffer (Hopwood *et al.*, 1985) containing 2 mg lysozyme ml<sup>-1</sup> (Solution I\*), and the mycelium was incubated for 30 min at 37 °C instead of on ice. Isolation of streptomycete genomic DNA and protoplast procedures for transforming streptomycetes followed the protocols of Hopwood *et al.* (1985).

**Gene disruption.** To inactivate ORF1, a disruption plasmid was prepared by inserting into pJV208 at its *Sall* site near the middle of ORF1, a fragment (both orientations) conferring apramycin resistance (Am<sup>R</sup>) that had been excised with *Sall* from pJV225. From the resultant plasmid (pJV213) a *Pst*I fragment conferring Am<sup>R</sup> was recloned in pHJL400 to give the disruption plasmid pJV218. This was passaged through *E. coli* ET12567 to avoid restriction barriers (MacNeil *et al.*, 1992) during its subsequent use in transforming protoplasts of *Str. venezuelae* ISP5230 and VS263. From these transformants strains resistant to both thiostrepton and apramycin (Am<sup>R</sup> Ts<sup>R</sup>), and therefore presumed to contain pJV218 either as the free plasmid or integrated into chromosomal DNA by a single crossover, were isolated (VS1025 derived from the wild-type, and VS1030 derived from the *cys-28* mutant). To promote allele replacement in single-crossover strains by a second crossover between the integrated disruption plasmid and homologous chromosomal DNA, strains VS1025 and VS1030 were grown through two rounds of sporulation on MYM agar without antibiotic selection. Spores from single colonies were screened to detect strains resistant to apramycin and sensitive to thiostrepton (VS1028 arising from VS1025, and VS1032 from VS1030). To disrupt ORF2 the apramycin resistance gene from pUC120A was inserted into an *Nco*I site near the centre of the ORF in pJV220, giving pJV222. The plasmid was used to transform ISP5230 and Am<sup>R</sup> colonies were selected.

**PCR amplification of *Str. venezuelae* genomic DNA.** Primers ch3 (5'-GAGACCATCGGCAACACCCC-3') and ch4 (5'-GTGATCGTGCCGCCGGTGCC-3') were designed from a CLUSTAL W alignment of the *cysK* sequences in a variety of Gram positive and Gram negative bacteria. Consensus sequences in two conserved regions 500 bp apart (Fig. 3) were

modified for streptomycete codon usage (Wright & Bibb, 1992) and used to amplify a DNA fragment from the *Str. venezuelae* ISP5230 genome by PCR. The procedure in the first 6 cycles involved denaturing at 96 °C for 1 min, annealing at 67 °C for 1 min and extension at 72 °C for 1 min. Denaturing and annealing were each reduced to 45 s for the next 30 cycles. Fragments of DNA amplified in the reaction were cloned in the *Sma*I site of pUC18 with the SureClone kit (Pharmacia Biotech) and sequenced.

**Construction of a library of *Str. venezuelae* genomic DNA.** The vector pBluescript II SK+ (10  $\mu$ g) was digested with *Bam*HI and treated with thermosensitive alkaline phosphatase (Gibco-BRL). After purification, the DNA was ligated with *Str. venezuelae* ISP5230 DNA partially digested with *Sau*3AI and size fractionated (5–15 kb fragments). The ligation mixture was used to transform *E. coli* DH5 $\alpha$ .

**Sequencing and sequence analysis.** Nested deletions (Sambrook *et al.*, 1989) were introduced into DNA fragments cloned in pBluescript II SK+, and both strands of the DNA were sequenced. Restriction sites were located with Gene Runner (Hastings Software). Potential ORFs were detected with CODONPREFERENCE (GCG), and the internet-based FramePlot 2.3 (Ishikawa & Hotta, 1999). CLUSTAL W (Thompson *et al.*, 1994) was used for multiple sequence alignments, and BLAST searches (Altschul *et al.*, 1997) were used to compare cloned sequences with DNA and protein sequences in GenBank.

## RESULTS

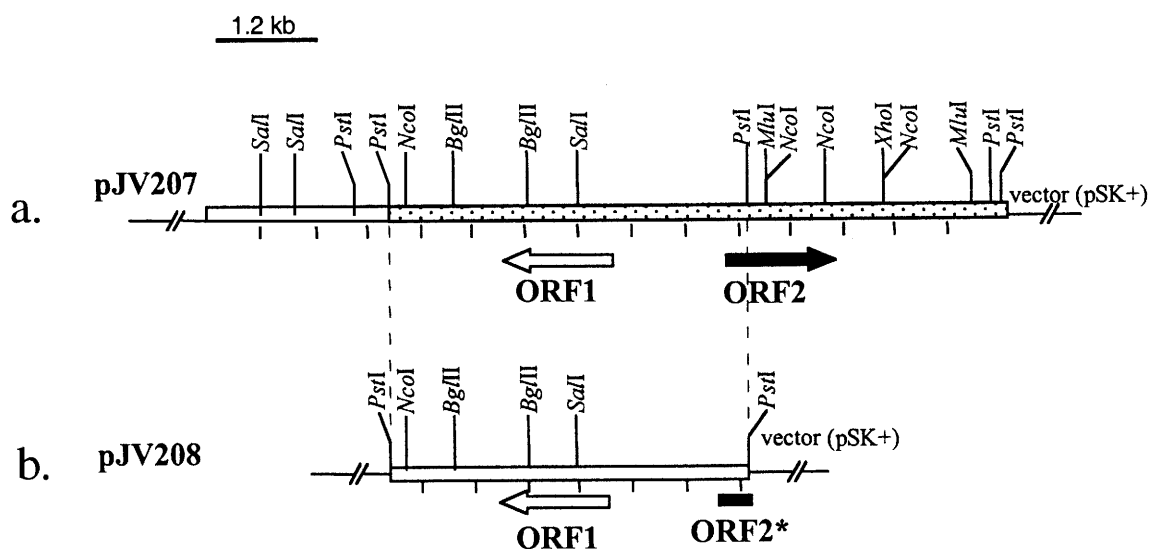
### Cloning the CBS gene from *Str. venezuelae*

Amplification of *Str. venezuelae* genomic DNA by PCR with primers ch3 and ch4 containing conserved bacterial *cysK* sequences gave a 500 bp fragment. When the amplicon, cloned in pUC18 as pJV205, was used with BLAST X to query GenBank, matches were obtained between its deduced amino acid sequence and both CBS

and CS sequences in prokaryotes and eukaryotes. It resembled most closely the sequences of mammalian and putative bacterial CBSs. While *cysK* was not excluded as the source of the amplified DNA, the evidence favoured derivation from a *Str. venezuelae* CBS gene. Screening a genomic library of *Str. venezuelae* ISP5230 in *E. coli* for hybridization with the pJV205 insert identified a colony from which the recombinant plasmid pJV207 was isolated. A restriction map (Fig. 2a) of the 9.2 kb *Str. venezuelae* DNA fragment cloned in pJV207, and hybridization probing with the pJV205 insert indicated that the PCR amplicon originated from within the 4.0 kb *Pst*I segment of pJV207. Subcloning the 4.0 kb segment in pBluescript II SK+ generated pJV208 (Fig. 2b) and pJV209, which differed only in the orientation of the insert. An adjacent 3.0 kb *Pst*I segment (see Fig. 2a) was similarly subcloned in both orientations (pJV210 and pJV211), and the combined 7.0 kb DNA region was sequenced (GenBank accession no. AF319543). The amplicon sequence is present at nt 4484–5019.

### Sequence analysis

The G + C content of the 7.0 kb *Pst*I segment in pJV207 was 72.5 mol%, typical of streptomycete genes (Wright & Bibb, 1992). Analysis of the sequence for codon usage (Devereux *et al.*, 1984) and third base bias (Ishikawa & Hotta, 1999) located ORF1 (third base 97.8 mol% G + C; GTG start codon in-frame +2 at nt 4460; preceded with a 10 bp separation by the putative RBS GGAG) and ORF2 (third base 98.8 mol% G + C; ATG start codon in-frame -3 at nt 1974; preceded by the putative RBS GGAG). No potential -10 or -35 promoter hexamers were detected upstream of ORF1 or ORF2, and the GCG TERMINATOR program did not



**Fig. 2.** Restriction map of the pJV207 insert. The stippled area was sequenced; the 4.0 kb *Pst*I fragment containing ORF1 and incomplete ORF2\* was subcloned in pJV208.

CBS- <i>S. venezuelae</i>		VQFHDSM	7
CBS-yeast		MTKSEQQADSRHNV	14
CBS-human	MPSETPQAEVGTGCPHRSGPHSAKGSLEKGSPEDEKAKEPLWIRPDAPSRTWQLGRPASESPPHHTAPAKSPKILPDI		80
CSA- <i>B. subtilis</i>		MVRVANSI	8
CSA- <i>M. leprae</i>		MSIAEDV	7
cysM2- <i>M. tuberculosis</i>		MRIAQHI	7
CSA- <i>E. coli</i> K12		MSKIFEDN	8
CSA- <i>Flavobacterium</i> sp		MKFQNA	6
CBS- <i>S. venezuelae</i>	ISLVGNTPLVKLNVTAGI--QATVLAKVEYFNPGG SVKDR AVRMI EAAEQSGELKPG-GTIVEPTSGNTGVGLAIVAQ		84
CBS-yeast	IDLVGNTPLIALKKLPAKALGKPKQIYAKLELYNPGG SIKDR AKSMVEEAEASGRHPSRSTLIEPTSGNTGIGLALIGA		94
CBS-human	LKKIGDTPMVRINKIGKKFGLKCELLAKCEFFNAGG SVKDR SLRMI EDAERDGLKPG-DTII EPTSGNTGIGLALAAA		159
CSA- <i>B. subtilis</i>	TELIGNTP IVKLNRLADEN--SADVYLKLEYMNP GG SVKDR GLAMI EAAEKEGKLGAG-NTII EPTSGNTGIGLAMVAA		85
CSA- <i>M. leprae</i>	TQLVGNTP LVRLRRVTEGA--LADVAKLESFN P SVKDR GVAMIDTAE EAGLIKPD-TVILEPTSGNTGIALAMVCA		84
cysM2- <i>M. tuberculosis</i>	SELIGGTP LVRLNSVVPDG--AGTVAAKVEYL NPGG SSKDR AVKMI EAAEASGQLKPG-GTIVEPTSGNTGVGLALVAQ		84
CSA- <i>E. coli</i> K12	SLTIGHTP LVRLNRIGN----GRILAKVESRNP S SVKCR GANMI WDAEKRGLKPG-VELVEPTSGNTGIALAYVAA		82
CSA- <i>Flavobacterium</i> sp	LETIGNTP VVKINLNFN-S--DHEIWKLEKSN P GG SIKDR ALAMI EDAEAKGLLNKD-STII EPTSGNTGIGLALVAA		82
	* * * * *		
CBS- <i>S. venezuelae</i>	QKGYKCIFVCPDKVSLDKINVLRYGAEVVVCP AVDPEHPDSY Y NVSDRLVRETPGAWKPDQY SNPN NPRSHYETTGP		164
CBS-yeast	IKGYRTIITLPEKMSNEKVSVLKALGAEI IRTP TAAAWDS PESHIGVAKLEKEI PGA VILDQY NMMN PEAHYFGTGRE		174
CBS-human	VRGYRCIIVMPEKMSSEKVDVLRALGAEI V RTPT NARFDS PESHVGVAWRLKNEI PNSHI LDQY RNA SNPLAHYDTTAE		239
CSA- <i>B. subtilis</i>	AKGLKAILVMPD TMSMERRN LLRAYGAE LV TPGAEG--MKGAIKAEELAEKH--GYFVPQ FNN PSNPEI HRQTGKE		161
CSA- <i>M. leprae</i>	ARGYHC V L T MP TMSI ERRML LRAYGAE L I TPGADG--MSGAIKAEELAKSD-KRYFI PQ FEN PANPA IHAATTAE		161
cysM2- <i>M. tuberculosis</i>	RRGYKCVFVCPDKVSEDKRNVLIA YGAELV V CPTAV PHDPAS Y SVSDRLV RDIDGAWKPDQY ANPEGPASHYVTTGPE		164
CSA- <i>E. coli</i> K12	ARGYK L T MP TMSI ERRK L K L GAN LV L TEGAKG--MKGAIQKAEI VASNPEK Y LL QQ F SN PANPE THEK TTGPE		160
CSA- <i>Flavobacterium</i> sp	VKGYKL L V MP ES MSI ERRK I ME YGA E F V L TPREKG--MKGAIKAEELAEETP-NSWI P Q F D N PANV K I H V T T AQE		159
	* * * * *		
CBS- <i>S. venezuelae</i>	LWEQT-DGK----ITHFVAGV TGGT ISCTGNYLK-EASGGSV KI GADPEGS-VYSGGS-----R PYL VEGV GEDFW		231
CBS-yeast	IQRQLEDL N F D N L RAV VAG AGT GGT ISG ISKY LK--EQNDKI Q I V G AD P F G S-ILAQ PEN LN K T D I DYK VEG I G Y D F V		251
CBS-human	ILQQC-DGK----LDMLVASV TGGT ITGIAR K LK--EKCPG C R I G V D P E G S-ILAE PEEL N Q T E Q T Y E V E G I G Y D F I		311
CSA- <i>B. subtilis</i>	I VE Q F G D D Q----L D A F V A G I T G G T I T G A G E V L K--EAY P S I K I Y A V E P S D S P V L S G G K P-----G P H K I Q G I G A G F V		229
CSA- <i>M. leprae</i>	V W R D T-DGK----V D I F V S G I T G G T I T G V A R I K--E RR P A T Q F V A V E P A A S P V L S G G QK-----G P H P I Q G I G A G F V		228
cysM2- <i>M. tuberculosis</i>	I W A D T-EGK----V T H F V A G I T G G T I T G A G R Y L K-EV S G G R V R I V G A D P E G S-VY S G G A G-----R PYL VEGV GEDFW		231
CSA- <i>E. coli</i> K12	I W E D T-DGQ----V D V F I A G V T G G T I T G S R Y I K G T K G K T D L I S V A V E P T D S P V I A Q A L A G E E I K P G H K I Q G I G A G F I		235
CSA- <i>Flavobacterium</i> sp	I L Q D F P E G-----L D Y V I T G V T G G H I G I A K A L K--E K Y P N L K V I A V E P L S P V L S G G S P-----A P H P L Q G I G A G F V		226
	* * * * *		
CBS- <i>S. venezuelae</i>	PTAYDRNV TDR I V A V S D K D S F Q M R R L A K E E G L L V G G S C G M A V V A A L E V A R E L G---P D D V V V L L P D S G R G Y M S K I F S D		308
CBS-yeast	PQVLD R K L I D V Y K T D D K P S P K Y A R Q L I S N E G V L V G G S S G S T F A V V K Y C E D H P E L T E D D V I V A I F P D S I R S Y L T K F V D D		331
CBS-human	P T V L D R T V D K W F K S N D E E A F T F A R M L I A Q E G L L C G G S A G S T V A V A V K A A Q E L Q---E G Q R C V V L L P D S V R N Y M T K F L S D		388
CSA- <i>B. subtilis</i>	P D I L N T E V Y D E I F P V K N E E A F E Y A R R A A R E E G I L G I S S G A I Y A A L Q V A K K L G---K G K K V L A I P S N G E R Y L S T P L Y Q		306
CSA- <i>M. leprae</i>	P S V L V L D L V D E I V T V G N E E A L D L R R L A K E E G L L V G I S A G A V C A A L H V A R R P E N---T G K L I V V L P D F G R Y L S T M L F A		306
cysM2- <i>M. tuberculosis</i>	P A A Y D P S V P D E I I A V S D S D S F D M R R L R A R E E A L V G G S C G M A V V A A L K V A E E A G---P D A L I V V L P D G G R G Y M S K I F N D		308
CSA- <i>E. coli</i> K12	P A N L D L K L V D K V I G I T N E A I S T A R R L M E E E G I L A G I S S G A V A A L K L Q E D E S F---T N K N I V V I L P S S G R Y L S T A L F A		313
CSA- <i>Flavobacterium</i> sp	P S I L D I T L D G V I T G K D E A Y E Y A I N A A K E G L P F C S P H R S R L S R Y R K T F T G N T A---W S K N S L P L I T P E K I F L R D S S		304
	* * * * *		
CBS- <i>S. venezuelae</i>	E W M A G H-----G F L E D-----T S S A T V D V L R H K E G G T P S L V H M H P D E T V G Q A I E V L R E Y G V S Q M P I V K P G A S H P		374
CBS-yeast	E W L K N N L W D D D V L R F D S K L E A S T T K Y A D V F G N A T V K D L H L K P V V S K E T A K V T D V I K I L K D N G F D Q L P V L T E D G K L S		411
CBS-human	R W M L Q K-----G F L K E E---D L T E K K P W W H L R V Q E L G L S A P L T V L P T I T C G H T I E I L R E K G F D Q A P V D E A G V L		456
CSA- <i>B. subtilis</i>	FD		308
CSA- <i>M. leprae</i>	DLAN		310
cysM2- <i>M. tuberculosis</i>	A W M S S Y-----G F L R S R L D G S T E Q S T V G D V L R R K S G-AL P A L V H T H P S E T V R D A I G I L R E Y G V S Q M P V V G---A E P		375
CSA- <i>E. coli</i> K12	D L F T E K-----E L Q Q		323
CSA- <i>Flavobacterium</i> sp	K I L		307
CBS- <i>S. venezuelae</i>	D V M A E V V G S V V E R D V L D---A L F T Q R A S L E D S L E S H S A P L P Q V G S G E P V A A L S V L G D A A D A I V L V E G-----		442
CBS-yeast	G L V T L S E L R K L S I N N S N-ND N T I K G Y L D F K L N N F N D V S Y N E N K S G K K K I K F D E N S K L D L N R F F E K N S A V I T D G		490
CBS-human	G M V T L G N L S S L L A G K V Q P S D Q V G K V I Y K Q F Q I R L T D T L G R L S H I E M D H F A L V H E Q I Q Y H S T G K S S Q R Q-----		528
CSA- <i>B. subtilis</i>			308
CSA- <i>M. leprae</i>			310
cysM2- <i>M. tuberculosis</i>	P V M A G E V A G S V S E R E L L S---A V F E G R A K L A D A V S A H S P L R M I G A G E L V S A A G K A L R D-W D A L M V V E E G-----		442
CSA- <i>E. coli</i> K12			323
CSA- <i>Flavobacterium</i> sp			307
CBS- <i>S. venezuelae</i>	-K P T G V V R Q D L L A F L A N G G A K	463	
CBS-yeast	L K P I H I V T K M D L L S Y L A	507	
CBS-human	-M V F G V V T A I D L N F V A A Q E R D Q K	551	
CSA- <i>B. subtilis</i>		308	
CSA- <i>M. leprae</i>		310	
cysM2- <i>M. tuberc</i>	-K P V G V I T R Y D L L G F L S E G A R R	464	
CSA- <i>E. coli</i> K12		323	
CSA- <i>Flavobacterium</i> sp		307	

**Fig. 3.** Alignment of the ORF1 product sequence (CBS-*Str. venezuelae*) with GenBank CS and CBS sequences. Below the alignment asterisks identify amino acids identical in all sequences; dots identify similar amino acids. The pyridoxal phosphate binding motif is boxed. Arrows show the regions used to design PCR primers and the 5'-3' orientations; bold letters identify conserved amino acids included in the primers.

**Table 2.** Effect of supplements on the growth of *Str. venezuelae* strains in minimal agar

ISP5230, wild-type; VS1028, wild-type with ORF1 disrupted; VS263, *cys-28* mutant; VS1032, *cys-28* mutant with ORF1 disrupted. +, Normal growth; -, no growth;  $\pm$ , poor growth.

Supplement	Strain			
	ISP5230	VS1028	VS263	VS1032
O-Acetylserine	+	+	-	-
Cysteine	+	+	+	+
Cystathionine	+	+	+	+
DL-Homocysteine	+	+	+	-
Methionine	+	+	+	-
O-Acetylhomoserine	+	+	-	-
O-Succinylhomoserine	+	+	-	-
Na <sub>2</sub> S	+	+	-	-
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>	+	+	$\pm$	$\pm$

detect a termination sequence immediately downstream of the ORF1 stop codon. However, several 6 nt inverted repeats were present 30–100 nt further downstream, and almost 80 nt downstream an inverted repeat followed by a run of Ts could signal termination. Also, several high A + T regions in this area might contain transcription signals (Rosenberg & Court, 1979).

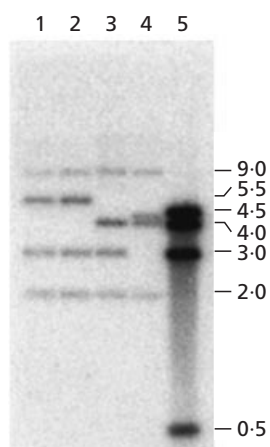
In BLAST P searches of GenBank the deduced amino acid sequence of the 48.82 kDa (463 aa) product of ORF1 resembled CBS and CS sequences in both prokaryotes and eukaryotes. It most closely matched (82% identity) the putative CBS (CAB89449) encoded by the *Str. coelicolor* A3(2) gene cloned in cosmid E25; next in similarity was the *cysM2* product (CAA17193) of *Mycobacterium tuberculosis* (62% identity). Mammalian CBSs were much less similar (37–42% identity), but in general CBS sequences ranked higher than CS sequences. The closest CS match (42% identity) was with the *cysK* product (GenBank T43792) of *Clostridium perfringens*. Although CBS genes are more common in eukaryotes than prokaryotes, similarities between the sequence of the ORF1 product and sequences in the genomes of *Str. coelicolor* A3(2) ([http://www.sanger.ac.uk/Projects/S\\_coelicolor/](http://www.sanger.ac.uk/Projects/S_coelicolor/)), *M. tuberculosis* (Cole *et al.*, 1998), *Mycobacterium leprae* (Cole *et al.*, 2000) and *P. aeruginosa* (Stover *et al.*, 2000) indicate that a CBS gene in *Str. venezuelae* would not be unique. However, none of the prokaryote CBSs postulated from genome sequencing has been functionally characterized.

A multiple sequence alignment of CBSs and CSs from various organisms (Fig. 3) indicated that CBSs are about 150 aa longer than CSs, with the extra length in the C-terminal region. The sequence of the ORF1 product (CBS-*Str. venezuelae*) matched CBS sequences in length (~450 aa) and overall alignment, whereas only the N-terminal region aligned with CS sequences. All of the sequences contained a conserved pyridoxal phosphate-binding motif. The highly conserved lysine 42 in both

the SVKCRI motif of the *E. coli cysK* product (Rege *et al.*, 1996) and the SIKDRI motif of the *Flavobacterium* sp. *cysK* (Muller *et al.*, 1996) corresponded to lysine 44 in the SVKDRI motif of the *Str. venezuelae* ORF1 product. The results implied that the ORF1 product, like other transsulfuration enzymes, is pyridoxal phosphate-dependent, and supported assignment of the GTG at nt 4460 as the start codon for ORF1. On the evidence from sequence analysis, ORF1 was predicted to be a CBS rather than a CS gene, but confirmation was sought from complementation, gene disruption and enzyme analysis.

#### Test for complementation of the *cys-28* mutation with ORF1

The cysteine auxotroph (VS263) obtained by mutagenesis with ethyl methanesulfonate of a *cml*<sup>-</sup> strain of *Str. venezuelae* ISP5230 (Vats *et al.*, 1987) was examined for growth requirements imposed by the unidentified *cys-28* mutation. When colonies on MM agar were supplemented with substances potentially involved in cysteine biosynthesis, positive responses were obtained with cysteine, methionine, homocysteine and cystathionine (Table 2). Thiosulfate restored weak growth, but sulfite, sulfide, serine, O-acetylserine, homoserine, O-acetylhomoserine (OAH) and O-succinylhomoserine (OSH) had no effect. These results implied that the step in cysteine biosynthesis converting O-acetylserine to cysteine, catalysed by CS (also called O-acetylserine thiolase) was blocked in VS263, and pointed to a mutation in either a gene encoding CS or a regulatory gene as the cause of the *cys-28* phenotype. To determine whether ORF1 could complement the mutation, the 4.0 kb *Pst*I insert from pJV208 was subcloned in the shuttle vector pHJL400 to give pJV215 and pJV216 (alternative orientations). Transformation of VS263 with pJV215, in which ORF1 had the same orientation as the vector promoter, and screening for growth on MM agar did not detect prototrophs, indicating that



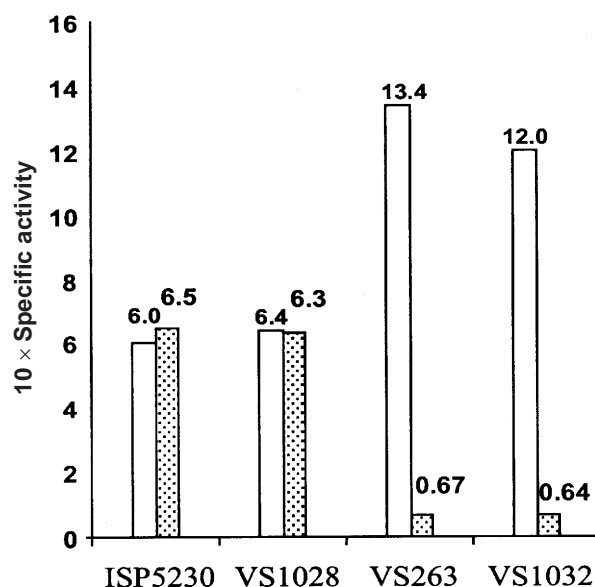
**Fig. 4.** Southern hybridization of *Pst*I digests of pJV207 and genomic DNA from wild-type, ORF1-disrupted, and ORF2-disrupted strains of *Str. venezuelae*. The pJV207 insert was used as the probe: lane 1, VS1028 (ORF1-disrupted ISP5230); lane 2, VS1032 (ORF1-disrupted VS263); lane 3, ISP5230; lane 4, VS1032 (ORF2-disrupted ISP5230); lane 5, pJV207.

either the *cys-28* mutation was not in the CS gene, or that the DNA cloned in ORF1 lacked a functional CS gene.

#### Disruption of ORF1 and ORF2

Strain VS1028 was obtained by introducing a disrupted allele of ORF1 into wild-type *Str. venezuelae* ISP5230, and selecting for strains in which a double crossover had occurred. Strain VS1032 was obtained similarly from the *cys-28* mutant. Both VS1028 and VS1032 were confirmed by Southern hybridization to have exchanged ORF1 for its disrupted counterpart (Fig. 4). Examination of each strain for growth requirements showed that the ORF1-disrupted wild-type remained prototrophic, whereas the ORF1-disrupted *cys-28* mutant differed from its parent in being unable to grow on MM supplemented with methionine or homocysteine (Table 2). However, it grew when supplemented with cystathionine or cysteine, implying that ORF1 encodes CBS, which converts homocysteine to cystathionine. The ability of the *cbs*-disrupted wild-type strain VS1025 to grow on MM agar indicated that CBS is not needed for cysteine biosynthesis in *Str. venezuelae* ISP5230, but provides an alternative pathway when the direct route via CS is blocked (see Fig. 1). Both VS263 and the *cbs*-disrupted VS1032 grew when supplemented with concentrations of thiosulfate at or above  $10 \mu\text{g ml}^{-1}$ .

The deduced amino acid sequence of ORF2 showed 40–70% identity to acetyl-CoA acyltransferases of bacteria. Since these enzymes participate in the oxidation of fatty acids, ORF2 seemed not to have a specific connection with the biosynthesis or metabolism of sulfur-containing amino acids. The possibility that it might function in chloramphenicol biosynthesis as a dichloroacetyl transferase was investigated by dis-

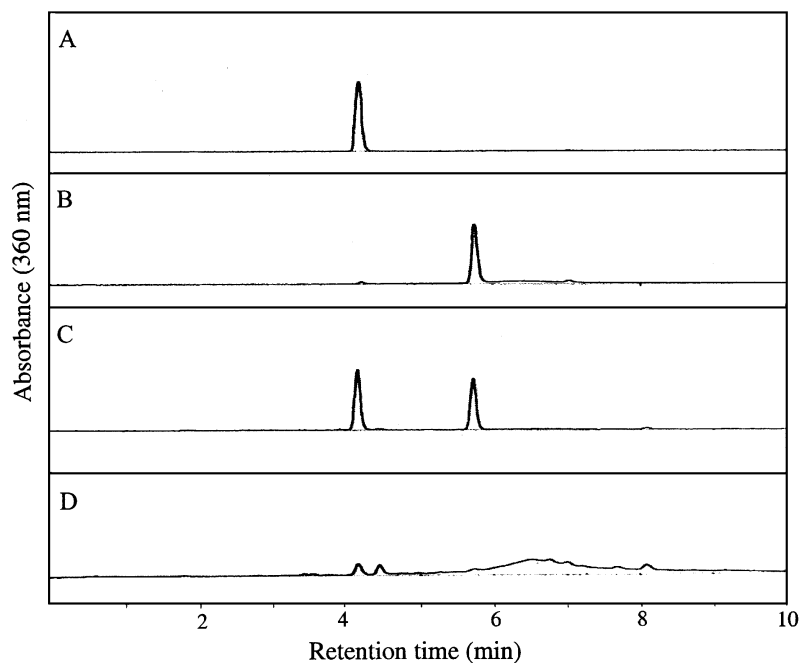


**Fig. 5.** Assays of CS activity in mycelium extracts of *Str. venezuelae* ISP5230, VS1028, VS263 and VS1032 grown in MYM (white bars) and MMY (shaded bars) media. Extracts were adjusted with TEPD buffer to the same total protein concentration. Values shown are specific activities [ $\text{mM cysteine (mg protein)}^{-1}$ ] obtained by direct measurement of cysteine formed in CS reaction mixtures.

rupting the gene and analysing cultures for altered acylation products. Disruption was confirmed by Southern hybridization (see Fig. 4). Cultures of the parent and disrupted strains compared by bioassay and HPLC analysis for production of chloramphenicol or related products showed no differences (data not shown). Since the disrupted strain also grew prototrophically on MM agar, the results imply that ORF2 is not essential for primary metabolism or chloramphenicol production in *Str. venezuelae* ISP5230.

#### Enzyme assays

The activities of five enzymes that participate in the biosynthesis and interconversion of sulfur-containing amino acids were assayed in mycelium extracts of *Str. venezuelae*. CS activity was determined in *Str. venezuelae* strains ISP5230, VS1028, VS263 and VS1032. Cultures were grown in the nutrient-poor MMY medium supplemented, for those strains carrying the *cys-28* mutation (VS263 and VS1032), with methionine and cystathionine to allow growth comparable to that in ISP5230 and VS1028 cultures. Assays directly measuring the amount of cysteine synthesized showed similar CS activity in ISP5230 and VS1028 (Fig. 5), indicating that disruption of *cbs* had no effect, and thereby confirming that ORF1 does not encode CS. The assays also showed that CS activities in VS263 and VS1032 were 10-fold lower than in ISP5230 and VS1028, respectively. That the lower activities were not due to cystathionine or methionine in the supplement used to improve growth in



**Fig. 6.** CBL and CGL activities assayed by HPLC in mycelial extracts of *Str. venezuelae* ISP5230. A, pyruvic acid standard (0.5 mM); B, 2-ketobutyric acid standard (0.5 mM); C, pyruvic acid and 2-ketobutyric acid formed from cystathionine (10 mM) incubated with mycelium extract; D, control with trichloroacetic acid added before cystathionine to the assay mixture used in C.

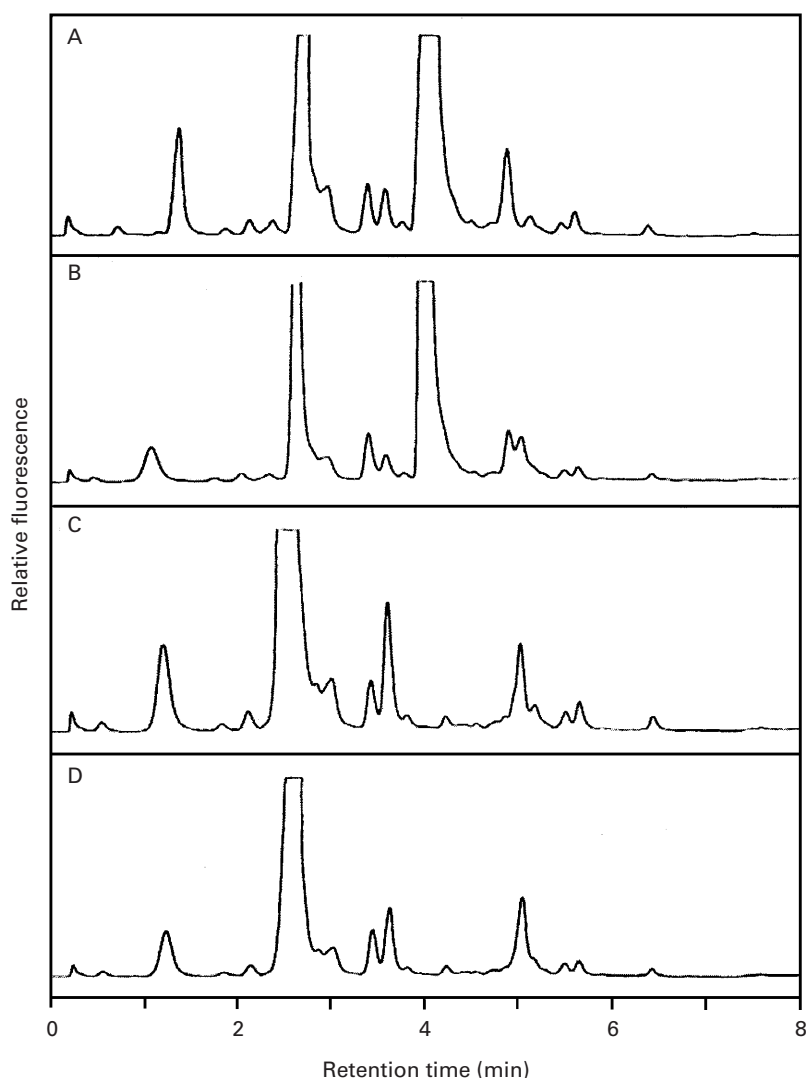
the nutrient-poor MMY medium was shown by the similar, low CS activities [0.07, 0.04 and 0.03 mM cysteine (mg protein)<sup>-1</sup>] in VS263 mycelium grown in MMY medium alone or with cystathionine or methionine supplements, respectively. The high activities in MYM medium [1.2–1.3 mM cysteine (mg protein)<sup>-1</sup>; see Fig. 5] were observed in both of the *cys-28* mutant strains (VS263 and VS1032), and were well above activities in the wild-type strains (ISP5230 and VS1028), which were not influenced by the culture medium. Low CS activity in the nutrient-poor MMY medium was consistent with the cysteine requirement of *cys-28* mutant strains tested for auxotrophy on MM agar. Since enzyme assays with extracts from mycelium grown in MYM medium indicated that the structural CS gene in the *cys-28* mutant was intact, the results suggested that auxotrophy was caused by mutation of a regulatory gene controlling CS expression. Systematically varying the composition of MYM medium showed that yeast extract and malt extract in combination strongly stimulated CS activity in mycelium extracts, but neither substance alone was effective. The presence of glucose markedly lowered activity.

Assays for CBL and CGL in mycelial extracts from strains ISP5230, VS1028, VS263 and VS1032 grown in MYM medium showed both enzymes present at similar levels in each strain; Fig. 6 shows the assay results for ISP5230. CBS, which catalyses the formation of cystathionine from homocysteine and serine in eukaryotes, has previously been reported in *Streptomyces* by Nagasawa *et al.* (1984), but without evidence for a substantive role. In the present study where enzyme reaction mixtures containing mycelial extracts from strains ISP5230, VS1028, VS263 and VS1032 were analysed by HPLC to detect the formation of cystathionine, the presence of CBS activity could not be confirmed. The

high threshold for detecting the activity of this enzyme by the HPLC assay method could be partly responsible, but low intrinsic CBS activity and rapid breakdown of the reaction product by the cystathionine lyases abundant in *Str. venezuelae* may be the principal reason for failure to detect accumulation of cystathionine. With the cloning of *cbs* from *Str. venezuelae*, the consequence of disrupting *cbs* in wild-type and *cys*-auxotrophic strains was assessed to determine the role of the gene in this streptomycete. In wild-type *Str. venezuelae*, the gene product has a supplementary role in cysteine biosynthesis through reverse transsulfuration (see above). Cystathionine can also be generated by an alternative route via CGS, an enzyme required for the biosynthesis of methionine in enterobacteria, but not hitherto demonstrated to have a role in streptomycetes. When cysteine and OAH or OSH were supplied as co-substrates to mycelial extracts of *Str. venezuelae* ISP5230, VS1028, VS263 and VS1032, assays for CGS gave similar results with each strain. The data for ISP5230 (Fig. 7) were representative, and indicated that OSH, but not OAH, condenses with cysteine in the CGS-catalysed biosynthesis of cystathionine.

## DISCUSSION

Although the main themes of streptomycete metabolism are similar to those of other bacteria, there are inevitable differences reflecting the unique position of streptomycetes in the microbial world. The research described here addresses an aspect of streptomycete primary metabolism – the biosynthesis and metabolism of sulfur-containing amino acids – that has not yet been fully explored. Our attempt to use a PCR procedure to clone a gene complementing the *cys-28* mutation led instead to cloning and characterization for the first time



**Fig. 7.** CGS activity assayed by HPLC analysis of cystathionine in reaction mixtures. A, reaction mixture containing 20 mM O-acetyl-DL-homoserine (OAH) and 20 mM L-cysteine as substrates; B, same reaction mixture as A with 10 mM cystathionine added as a reference; C, reaction mixture with OAH replaced by 20 mM O-succinyl-DL-homoserine; D, same reaction mixture as C with 10 mM cystathionine added as a reference.

of a streptomycete CBS gene. This is the first example of a CBS gene cloned and functionally characterized from a prokaryotic organism. Its discovery pointed to the existence of a transsulfuration pathway in *Str. venezuelae* ISP5230, and further investigation of the enzymes involved in cysteine and methionine metabolism has clarified the role of transsulfuration in interconverting sulfur-containing amino acids in this streptomycete.

#### Cysteine biosynthesis in streptomycetes

The presence of CS activity in a streptomycete has been demonstrated here for the first time by a direct enzyme assay. The cysteine auxotrophy of the *Str. venezuelae* mutant VS263 was correlated with low intracellular CS activity, and the resulting impaired output of cysteine from the thiolation pathway. Evidence that CS activity in the mutant depended markedly on growth conditions, and was high in the nutrient-rich MYM medium, indicated that the gene encoding CS in *Str. venezuelae*

VS263 was intact, and suggested that the *cys-28* mutation unmasks a repressor preventing gene expression. The possibility that a gene regulating cysteine biosynthesis might be involved in the *cys-28* mutation is also suggested by restoration of slow growth with thiosulfate. Regulation of CS by thiosulfate varies from the strong repression exerted in *Rhodopseudomonas palustris* to the derepression of enzyme synthesis found in *Rhodopseudomonas sulfidophila* (Hensel & Trüper, 1976). Thus thiosulfate might have a role as an effector in the cysteine biosynthesis pathway of *Str. venezuelae*. However, an alternative explanation for the ability of thiosulfate to support slow growth of *Str. venezuelae* VS263 is the potential existence of the *S*-sulfocysteine pathway for biosynthesis of cysteine in this streptomycete. Actinomycetes vary in the contributions of the direct thiolation and *S*-sulfocysteine pathways to cysteine biosynthesis: whereas in *Streptomyces griseus* the *S*-sulfocysteine pathway is the major contributor (Kitano *et al.*, 1985), in *Str. venezuelae* direct thiolation appears to be dominant, and both pathways are present

in *Sacp. erythraea*. This versatility in cysteine biosynthesis has been associated (Donadio *et al.*, 1990) with differences in the distribution of the CSB isozyme (*cysM* product) in actinomycete species. Like the *cysM* product in enterobacteria, CSB exhibits dual functions supporting both the direct thiolation and *S*-sulfocysteine pathways. The exclusive presence of *cysM* and absence of *cysK* in *Str. venezuelae* may account for the amplification of a *cbs* fragment by PCR with primers ch3 and ch4, which contained consensus sequences of both cysteine synthase isozyme A and B genes. Cloning and sequencing 100 fragments amplified from *Str. venezuelae* ISP5230 DNA with these primers did not yield a single product that matched the sequence of *cysK* (data not shown).

### CBS and other transsulfuration enzymes in *Str. venezuelae*

Since disrupting *cbs* in wild-type *Str. venezuelae* did not prevent growth on minimal medium, the gene appeared at first to have no role in primary metabolism. However, a function was revealed by insertional inactivation of *cbs* in the *cys-28* mutant, which in minimal medium cannot synthesize cysteine by thiolation of *O*-acetylserine. Disrupting *cbs* created cysteine auxotrophy by interrupting the alternative supply of this amino acid via reverse-transsulfuration. This route to cysteine relies on the availability of cystathionine from CBS-catalysed condensation of homocysteine and serine, and is lost when *cbs* is inactivated. Besides confirming the function of the gene, the disruption provided evidence unobtainable from enzyme assays for reverse transsulfuration in a streptomycete. Although assay values for CBS activity in mycelial extracts were below the limits of detection by the procedure used, the activity presumably meets the needs of the organism *in vivo*. Overall, the results indicated that, although cysteine is mainly synthesized *de novo* in *Str. venezuelae* by thiolation of *O*-acetylserine, interconversion of pre-existing sulfur-containing amino acids has a supplementary role that can become important under some circumstances.

### Methionine biosynthesis

In the transsulfuration reaction between cysteine and *O*-acylhomoserine for the biosynthesis of methionine, the acyl group and the fate of the acylated product can vary. In Gram-negative bacteria, with some exceptions, OSH is the substrate, whereas in Gram-positive bacteria such as bacilli, corynebacteria and brevibacteria, the substrate is OAH. The evidence that OSH but not OAH is the substrate for CGS in *Str. venezuelae* is consistent with previous observations that streptomycetes differ from other Gram-positive bacteria, and resemble Gram-negative bacteria, in using OSH for this reaction (Kanzaki *et al.*, 1986). Some micro-organisms convert *O*-acylhomoserine directly to homocysteine in an alternative route to methionine. Genes for the thiolase (homocysteine synthase) catalysing this reaction have

been cloned and sequenced from *P. aeruginosa*, where this is the main route (Fogliano *et al.*, 1995), and the gene products have been identified from genome sequencing of several bacterial genomes, including that of the actinomycete *M. tuberculosis* (GenBank CAA17112). The presence of CGL in *Str. venezuelae* was predictable in view of the report (Nagasawa *et al.*, 1984) that this enzyme is widely distributed in streptomycetes. Since CBL activity was also detected in *Str. venezuelae*, a dual-function cystathionine  $\beta,\gamma$ -lyase of the type discovered in *Lactococcus lactis* (Dobric *et al.*, 2000) is not excluded. This enzyme catalyses  $\alpha,\beta$  and  $\alpha,\gamma$ -elimination reactions equally well, and could have been responsible for the similar levels of the two lyases detected in *Str. venezuelae*. However, information currently available does not distinguish between the activities of a dual function enzyme or two individual enzymes. The similar amounts of CBL and CGL in mycelial extracts suggest that conversion of cystathionine to cysteine must be tightly regulated. The high level of CGS activity is consistent with this reaction supplying the relatively large amount of cystathionine needed for conversion to methionine. Since the formation of cysteine from cystathionine is not a principal supply route in *Str. venezuelae*, most of the cystathionine would be expected to provide homocysteine by the action of CBL, and then be *S*-methylated to methionine.

### Relationships among streptomycete genes for reactions involving sulfur amino acids

Although *cysK* is widely distributed in bacteria, it has not been identified in the completely sequenced genome of *Str. coelicolor* A3(2). Its apparent absence, and our failure to obtain a *cysK* fragment by PCR amplification from the *Str. venezuelae* ISP5230 genome, suggest that this gene is not universally present in streptomycetes. The importance of transsulfuration reactions supporting cysteine and methionine biosynthesis in these organisms is implied by the putative assignment of six genes in the *Str. coelicolor* A3(2) genome to such functions (Redenbach *et al.*, 1996). They include the *cysM* cloned in cosmid E19A, the CBS gene in cosmid E25, the CGS gene in cosmid K13, the CGL gene in cosmid Q11, and probably also the putative lyase gene in cosmid G20A responsible for the CBL activity that forms homocysteine from the cystathionine generated by CGS. BLAST searches of GenBank showed close similarity between protein sequences deduced from these genes and their counterparts in other organisms. A BLASTX search of the *Str. coelicolor* A3(2) genome with the cloned *Str. venezuelae* *cbs* showed a striking 82% similarity in deduced amino acid sequences to a gene in the *Str. coelicolor* cosmid E25, indicating that *cbs* is well conserved in streptomycetes. Examination of sequence relationships suggests early divergence of CBS and CS groups. The CBSs from *Str. venezuelae* and *Str. coelicolor* A3(2) cluster with the *cysM2* products of *M. tuberculosis* and *M. leprae* to form a prokaryotic subgroup that has diverged from the subgroup of eukaryotic CBSs. Probably because eukaryotic CBSs

have received more attention than those from prokaryotes, the *Str. venezuelae* CBS gene is the first in the latter group to have been cloned and characterized.

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