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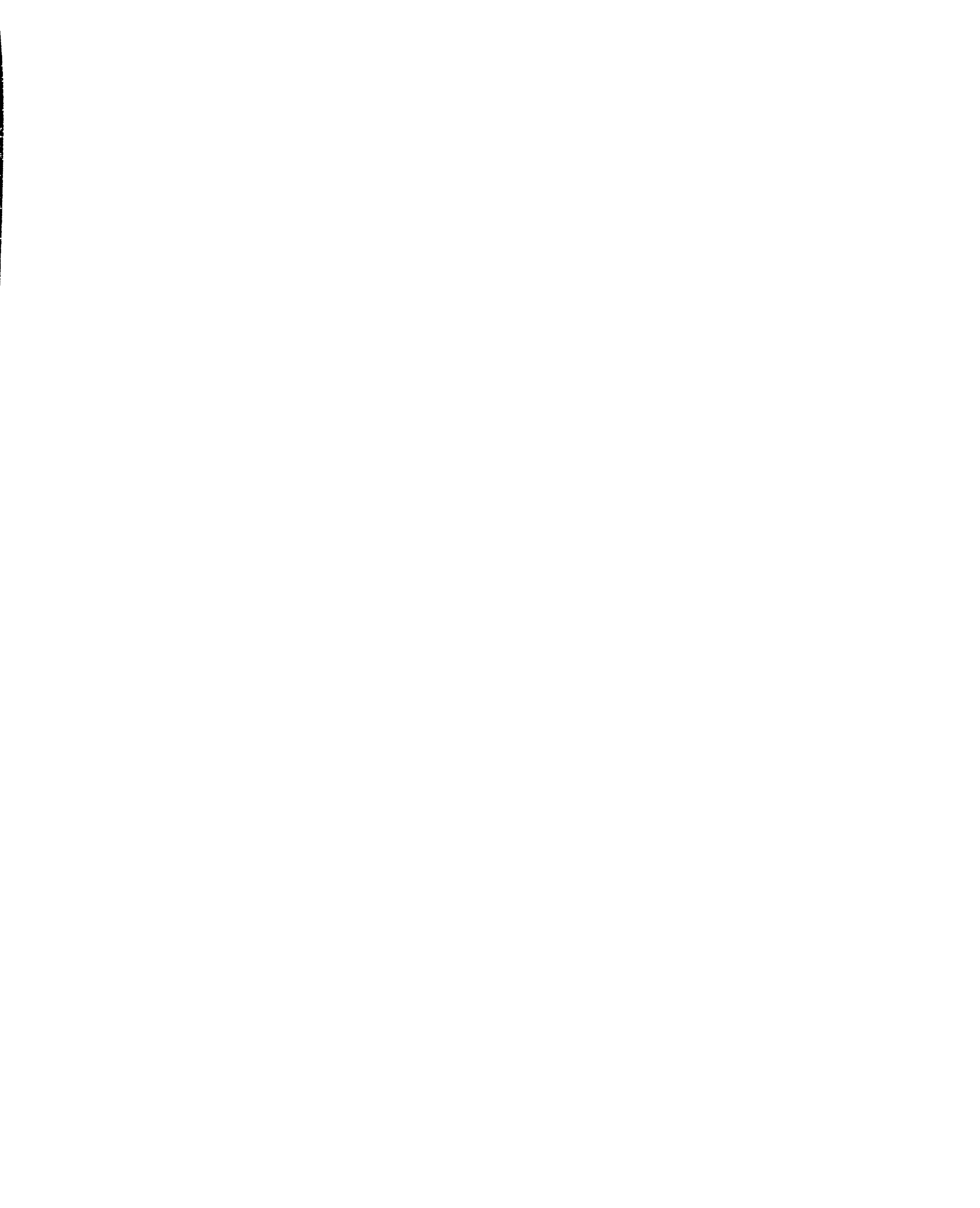

Genes for Cysteine Biosynthesis and Metabolism in *Streptomyces venezuelae* ISP5230: Cloning, Sequencing, Functional Analysis and Relevance to Chloramphenicol Biosynthesis

by

Zunxue Chang

Submitted in partial fulfilment of the requirements for the degree of doctor of philosophy

at

Dalhousie University
Halifax, Nova Scotia

July, 1999

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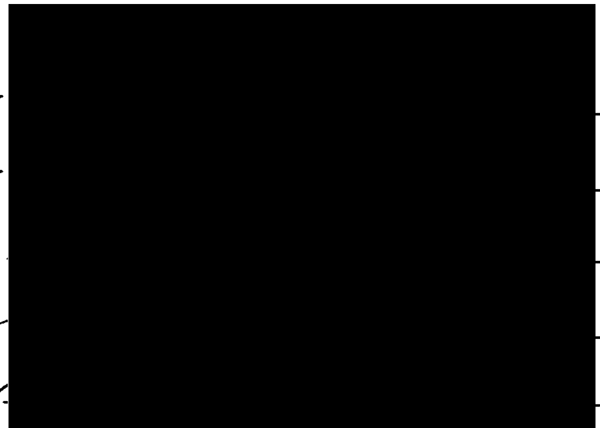

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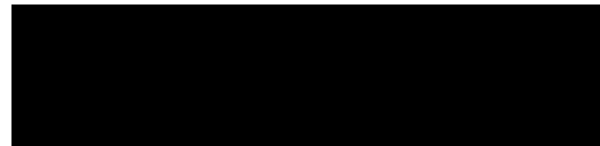

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ABSTRACT

The chloramphenicol (Cm) biosynthesis gene cluster (*cml*) in *Streptomyces venezuelae* is flanked by auxotrophic markers (*pdx* and *cys*) about 45 kb apart. By cloning *pdx* and walking the chromosome from this marker, a 4.0-kb *Sac*I fragment hybridizing with *bca* (a putative Cm chlorination gene) was discovered about 30-kb from *pdx*. Use of the 4.0-kb *Sac*I sequence as a marker to orient chromosome walking *vis-a-vis pdx* was anticipated, but was abandoned when analysis of the sequence failed to confirm the presence of an ORF associated with chlorination. As an alternative marker to orient chromosome walking, the *cys-28* mutation flanking the *cml* cluster was chosen, and its phenotype was characterized. The gene could not be cloned by complementation because of the high reversion frequency of available *cys* mutants, and attempts to isolate *S. venezuelae* Cm production mutants by transposon mutagenesis with Tn4560 gave disappointing results, attributed to non-random transposition of Tn4560 in the *S. venezuelae* genome.

Indications that the *cys-28* phenotype was due to loss of cysteine synthase (CS) activity directed attention towards cloning the CS gene (*cysK*) of *S. venezuelae*. A pair of PCR primers designed from the conserved amino acid sequences of CSs in the GenBanK database was used to amplify a *cysK* fragment from the *S. venezuelae* genome. Amino acid sequence deduced from the PCR product showed high similarity to CSs from both prokaryotes and eukaryotes, and to cystathionine β-synthases (CBSs) of eukaryotes. A genomic library of *S. venezuelae* constructed in the phagemid pBluescript II SK (+) was screened by colony hybridization using the PCR product as a probe. From a transformant that hybridized strongly to the probe, pJV207 containing a 9.2-kb insert of *S. venezuelae* DNA was isolated, and a 7.0-kb fragment subcloned from pJV207 was sequenced. Analysis of the 7.0 kb sequence detected two ORFs (ORF1 and ORF2). The deduced amino acid sequence of ORF1 resembled the sequences of both CS and CBS from various organisms. It showed evolutionary links to the CSs of prokaryotes and eukaryotes, and to the CBS of eukaryotes. In length it resembled CBS. Disruption of ORF1 in *S. venezuelae* blocked the pathway from homocysteine to cysteine, leading to the conclusion that ORF1 encodes cystathionine β-synthase. The product of ORF2 showed significant sequence similarity to the acetyl-CoA transferase (thiolase) of various organisms. Since disruption of ORF2 had no effect on Cm production, the possible role of this gene as a dichloroacetyl transferase in Cm biosynthesis was discounted.

Cell extracts of *S. venezuelae* strains were assayed for enzymes (O-acetyl-L-serine sulfhydrylase, cystathionine β- and γ-synthases, cystathionine β- and γ-lyases, and homocysteine synthase) potentially involved directly or by transsulfuration in cysteine synthesis. The results indicated that the structural gene for O-acetyl-L-serine sulfhydrylase was intact in the strain VS263 carrying the *cys-28* mutation, but was not expressed in minimal medium; both cystathionine β- and γ- lyase activities were detected. *S. venezuelae* contained high O-acetylhomoserine sulfhydrylase and cystathionine γ-synthase (with O-succinylhomoserine as a substrate) activities, but CBS activity was undetected by HPLC and was dispensable for cysteine biosynthesis in the wild-type strain.

LIST OF ABBREVIATIONS

aa	amino acid
AHS	O-acetylhomoserine sulfhydrylase
ASS	O-acetylserine sulfhydrylase
amp	ampicillin resistance gene
apr	apramycin resistance gene
bp	base pair
BSA	bovine serum albumin
CBS	cystathionine β-synthase
cfu	colony forming unit
Cm	chloramphenicol
cml	chloramphenicol biosynthesis gene
CoA	coenzyme A
CS	O-acetylserine sulfhydrylase (cysteine synthase)
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytosine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
ddATP	dideoxyadenosine 5'-triphosphate
ddCTP	dideoxycytosine 5'-triphosphate
ddGTP	dideoxyguanosine 5'-triphosphate
ddTTP	dideoxythymidine 5'-triphosphate

DDB	1,2-diamino- 4,5-dimethoxybenzene
DMSO	dimethylsulfoxide
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
DNase	deoxyribonuclease
EDTA	ethylenediamine tetraacetic acid
EHDQ	3-ethyl-2-hydroxy-6,7-dimethoxyquinoxaline
$_{f}M$	N-formylmethionine
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
MCS	multi-cloning site
MHDQ	3-methyl-2-hydroxy-6,7-dimethoxyquinoxaline
MIC	minimum inhibitory concentration
nt	nucleotide
NMR	nuclear magnetic resonance
NTG (MNNG)	N-methyl-N'-nitro-N- nitrosoguanidine
OAH	O-acetylhomoserine
OAS	O-acetylserine
OPA	o-phthalaldehyde
ORF	open reading frame

OSH	O-succinylhomoserine
PABA	p-aminobenzoic acid
PAPA	p-aminophenylalanine
pdx	pyridoxal phosphate
PEG	polyethylene glycol
pfu	plaque forming unit
pSK+	phagemid pBluescript II SK(+)
RBS	ribosome binding site
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SHS	O-succinylhomoserine sulfhydrylase
TEMED	N,N,N',N'-tetramethylenediamine
TES	N-tris-(hydroxymethyl) methyl-2-aminoethane sulfonic acid
THF	tetrahydrofuran
Tn	transposon
Tris	tris-(hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
TsAP	thermosensitive alkaline phosphatase
vph	viomycin resistance gene

v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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INTRODUCTION

I. Molecular Genetics of Chloramphenicol Biosynthesis

Our current understanding of chloramphenicol (Cm) biosynthesis has come from investigations of the pathway in *Streptomyces venezuelae*. A plausible biochemical process (Fig. 1) has been deduced from patterns of isotopic labeling in Cm isolated from cultures of *S. venezuelae* administered variously labeled precursors. Like all secondary metabolites, Cm is biosynthesized from primary metabolic precursors. For Cm, the primary precursors are derived from two different metabolic systems: one is the shikimic acid pathway of aromatic biosynthesis, and the other is a still unknown route that furnishes the dichloroacetyl substituent (Vining & Westlake, 1964; Munro et al., 1975). Although the part of the molecule originating in the shikimic acid pathway bears obvious similarity to the amino acids phenylalanine and tyrosine, Cm is not derived via these protein aromatic amino acids. Instead it is synthesized by branching of the pathway to divert some of the mainstream aromatic intermediate chorismic acid to 4-amino-4-deoxychorismic acid. This product is then converted to p-aminophenylalanine, either via 4-amino-4-deoxyprephenic acid (Teng et al., 1985; see Fig.1), or via an alternative series of reactions in which p-aminophenylpyruvic acid is formed first and is subsequently trans-aminated. p-Aminophenylalanine is a selective precursor of Cm, but it is not the only metabolite derived from 4-amino-4-deoxychorismic acid. Indeed, the latter is a biochemical branch point, leading on the one hand by a lyase reaction to p-aminobenzoic acid (PABA), and on the other hand by a mutase reaction to 4-amino-4-deoxyprephenic acid (see Fig. 1). In the latter route, 4-amino-4-deoxyprephenic acid is converted via p-aminophenylalanine and

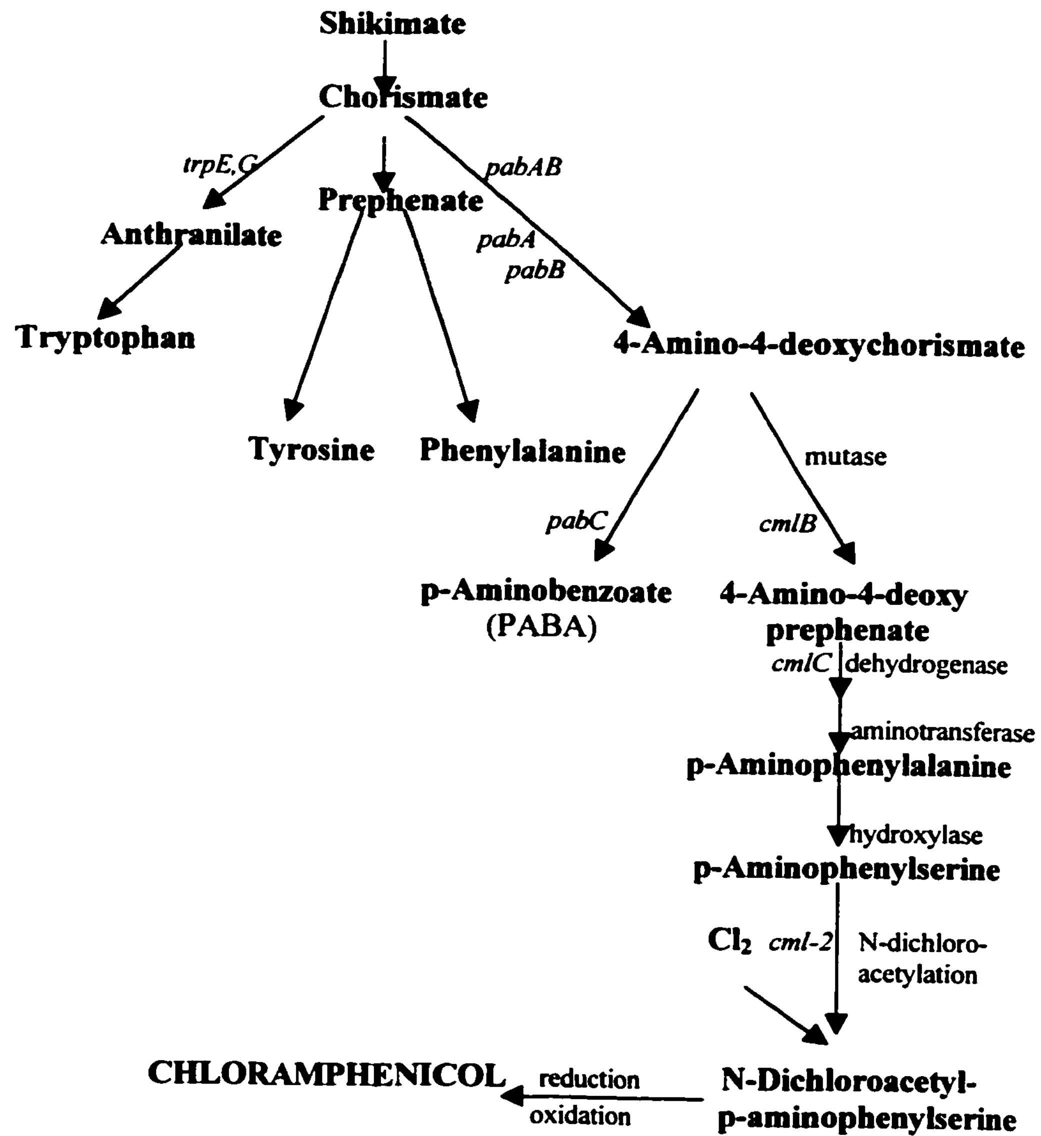


Fig. 1. Biosynthesis of chloramphenicol in *S. venezuelae*

subsequent secondary metabolic reactions to eventually give Cm.

Biochemical investigations of Cm biosynthesis have usually been carried out with *S. venezuelae* 13s, a strain that produces high yields of the antibiotic. However, for reasons not yet understood, strain 13s is genetically unstable, and to a large extent, the genetics and molecular biology of Cm biosynthesis have been studied in the more tractable *S. venezuelae* ISP5230. Although systems for manipulating genes in strain ISP5230 have been developed in recent years, only one Cm biosynthesis gene (*pabAB)* has yet been cloned and characterized.

II. Mapping the *cml* Gene Cluster in the *S. venezuelae* Chromosome

Stuttard and co-workers developed a fertility system for carrying out conjugational crosses in *S. venezuelae* ISP5230, and recombination data from such crosses with multiply marked strains have been used to establish a map of chromosomal genes involved in auxotrophic (biochemical) reactions required for growth, as well as of secondary metabolic genes required for Cm biosynthesis (Doull et al., 1986, Vats et al., 1987). By estimating recombination frequencies, the genes were placed in order on the chromosome of *S. venezuelae* ISP5230, and a linkage map was established (Vats et al., 1987, Fig. 2A). The Cm biosynthesis genes (*cml*) were located in a cluster near auxotrophic markers associated with requirements for arginine (*arg),* cysteine (*cys*) and pyridoxal (*pdx*). Later, transductional analysis with actinophage SV1 indicated that the *cml* cluster is located in a region of less than 45 kb on the chromosome of *S. venezuelae*, and is flanked at one end by *pdx* and at the other end by *cys-28* (see Fig. 2B, Vats et al., 1987; Stuttard, 1988).

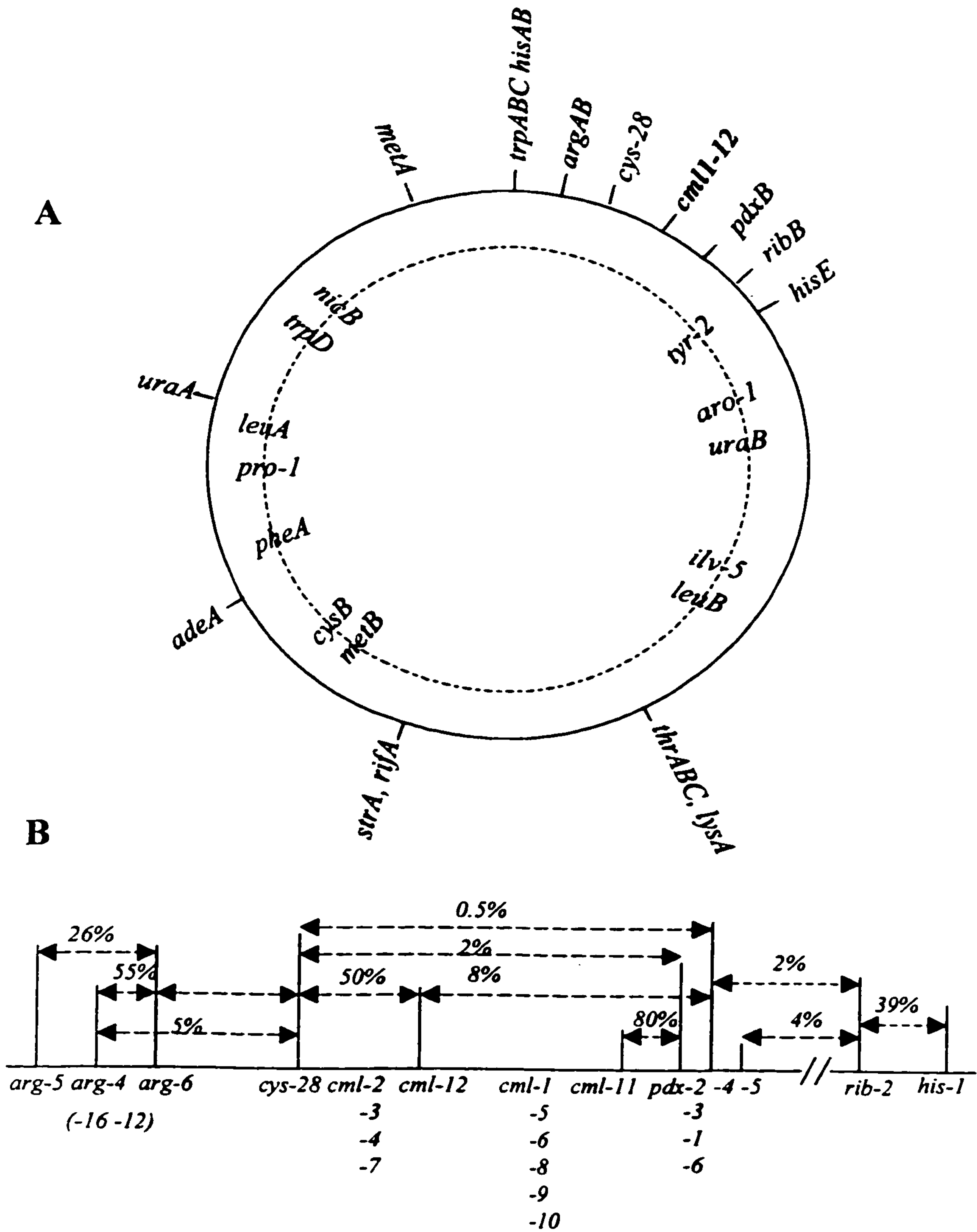


Fig. 2. Genetic map of *S. venezuelae* (Vining and Stuttard, 1994): (A) Genetic map of *S. venezuelae* based on recombination frequencies in conjugation experiments; (B) Fine structure of the *cml* region based on co-transduction frequencies.

III. Possible Association of *pab* Genes with a Cluster Encoding Early Steps in Cm Biosynthesis

Because 4-amino-4-deoxychorismic acid is an intermediate in the pathways leading to both *p*-aminobenzoic acid (PABA) and Cm, the genetic determinants (*pabA, pabB*) for 4-amino-4-deoxychorismic acid biosynthesis might be associated with other genes in biosynthetic pathways for either end-product, and if so they might be found in a cluster of genes responsible for the early steps forming Cm (the 'early *cml* cluster'). A gene, designated *pabAB,* that strengthened these expectations was cloned from *S. venezuelae* ISP5230 by complementing a mutant of *Streptomyces lividans* that required PABA for growth (Brown et al., 1996). Sequence analysis of the cloned DNA established that *pabAB* represented a single fused gene in which a *pabA* domain preceded (i.e., was 5' to) a *pabB* domain. The *pabA* and *pabB* domains showed similarity in their deduced amino acid sequences to *pabA* and *pabB* genes, respectively, previously isolated from other bacteria (Brown et al., 1996).

Upstream of *pabAB*, sequence analysis revealed two ORFs with deduced amino acid sequences similar to those of *rom* genes involved in the regulation of antibiotic resistance in Gram negative bacteria. Disruption of *pabAB* in *S. venezuelae* ISP5230 did not cause auxotrophy, and no evidence of a requirement for PABA was detected. Instead, the disrupted mutant exhibited severely reduced Cm production compared with the parent. This result indicated that the 4-amino-4-deoxychorismic acid synthase encoded by *pabAB* acts specifically in the secondary metabolic pathway (Brown et al., 1996). It implied that *S. venezuelae* ISP5230 contains parallel gene sets directing on the one hand the synthesis of a

primary 4-amino-4-deoxychorismic acid synthase involved in PABA formation, and on the other hand the synthesis of a secondary 4-amino-4-deoxychorismic acid synthase forming *p*-aminophenylalanine (PAPA), which is subsequently converted to Cm (see Fig. 1).

Downstream of *pabAB*, and with the same orientation as this gene, a series of three ORFs with deduced amino acid sequences resembling chorismate mutase, prephenate dehydrogenase and deoxy-*arabino*-heptulosonate-7-phosphate synthase (DAHPS) was present in the *S. venezuelae* chromosome. These ORFs plausibly represent genes in the pathway for PAPA biosynthesis. Analysis of the cloned DNA sequence further downstream from *pabAB* detected a fourth ORF encoding a protein with similarity to known membrane-associated Cm efflux proteins (K. A. Aidoo & L. C. Vining, personal communication).

IV. Evidence that *S. venezuelae* Has Primary Metabolic *Pab* Genes

In collaboration with Y. Sun, I cloned from *S. venezuelae* ISP5230 a second set of genes related to *pabA* and *pabB*. Analysis of their DNA sequence indicates that, in contrast to the *pabAB* gene associated with Cm biosynthesis, this second set of *pab* genes is not fused together. Instead, the *pabA* and *pabB* are discrete genes organized, like those of *Bacillus subtilis*, in an operon with *pabB* preceding *pabA*. The cloned *S. venezuelae* DNA fragment complemented a *pabB* mutant of *E. coli*. Disruption of these genes and further complementation experiments are needed to determine their functions, and to establish their relationship to other genes, such as those for *p*-aminobenzoic acid and anthranilic acid synthases (Lin et al., 1998), in this evolutionary super-family. Meanwhile, they are promising candidates for determinants of primary metabolic functions.

V. Complementation of *S. venezuelae* Mutants Blocked in Cm Biosynthesis

Aidoo (1989) and Aidoo et al.(1990) encountered difficulty in attempts to isolate *cml* genes from *S. venezuelae* ISP5230 by cloning restriction fragments from wild-type genomic DNA in available streptomycete vectors, and using the recombinant vectors to restore Cm production in *S. venezuelae cml* mutants by transformation. The difficulties were of several kinds: (i) the vectors available transformed *S. venezuelae* ISP5230 at very low efficiency; (ii) the procedure for detecting Cm-producing colonies among the transformants was very labor-intensive; and (iii) the transforming vectors inevitably carried chromosomal DNA fragments homologous with the host chromosome; therefore, they integrated readily into the chromosome by recombination. The consequent loss of free plasmid from the cytoplasm forestalled recovery of recombinant plasmid DNA containing the cloned gene fragment (Aidoo, 1989; Paradkar, 1991).

VI. Complementation of *S. lividans* Mutants with Auxotrophic Marker Genes

To circumvent the problem of DNA recovery that arose from introducing homologous DNA fragments into the cloning host, and at the same time to avoid the labour-intensive screening procedure needed to detect antibiotic-producing transformants, advantage was taken of the auxotrophic *pdx* marker flanking the *cml* cluster. The alternative strategy involved cloning *S. venezuelae* genomic DNA fragments in a streptomycete vector developed for efficient transformation of *S. lividans*, and using the recombinant plasmid library to transform the pdx-requiring *S. lividans* host to prototrophy. The *S. venezuelae* chromosomal DNA flanking the marker in the vector was analyzed for fragments containing

potential *cml* genes. Aidoo (1989) adopted this procedure to clone a putative *pdxH* of *S. venezuelae* ISP5230. Sequencing the cloned fragment and analyzing the DNA sequence located an ORF needed to complement the *pdxH* mutation in the *S. lividans* host strain. Using the amino acid sequence deduced from this ORF for a BlastX search of the GenBank database did not detect any protein known to catalyze a reaction in the biosynthesis of pyridoxal phosphate, but a potential match with a regulatory gene was found (N. Magarvey, personal communication).

VII. Chromosome Walking

The cloned DNA fragment containing the putative *S. venezuelae pdxH* gene was used as a starting point for chromosome walking aimed at extending the cloned region as successive contiguous fragments reaching beyond 45 kb on each side of *pdxH*

A. Walking procedure

The procedure involved hybridization of *Bam*HI-digested recombinant lambda DNA from successive isolations of hybridizing phage clones. The 'end fragment' identified from each walking step was used as the probe for the following hybridization.

B. Orienting markers

The success of a chromosome walking strategy depended on the presence within the *cml* cluster of a recognizable marker so that the direction of walking relative to the *pdxH* starting point could readily be determined. At the outset, the most suitable candidate for such

a marker in the *cml* cluster appeared to be *cml-2*, a mutation introduced into *S. venezuelae* ISP5230 by mutagenesis with NTG. The phenotype of this mutation is inability to introduce chlorine into the dichloroacetyl group of Cm, and a consequent accumulation of corynecins (unhalogenated analogues of Cm) in cultures of the mutant.

Two halogenating enzymes have been isolated from *S. venezuelae*, and characterized as bromoperoxidase-catalases (Knoch et al., 1989). The absence of one of these in a mutant accumulating corynecins pointed to its participation in Cm biosynthesis, and suggested that its gene corresponds to *cml-2*. The enzyme presumed to be responsible for the halogenation reaction yielding Cm was purified from *S. venezuelae* (Knoch et al., 1989), and a reverse genetics strategy was used to clone the gene encoding it. The N-terminal amino acid sequence of the enzyme was determined, and a corresponding oligonucleotide was synthesized. By using this to probe an *S. venezuelae* ISP5230 genomic library, the gene (*bca*) for the relevant bromoperoxidase-catalase was detected and cloned. Unexpectedly, disruption of *bca* in wild-type *S. venezuelae* did not affect Cm production (Facey et al., 1996).

Although *bca* is clearly not involved in the synthesis of enzymes for the Cm biosynthetic pathway, and therefore does not correspond to *cml-2*, it was considered to be potentially useful as a probe for other bromoperoxide-catalase genes that might well include the true *cml-2* halogenation gene. This possibility was emphasized by the detection during chromosome walking of a 4.0-kb *Sac*I fragment that, at reduced stringency, hybridized with the *bca* probe (Fig. 3). Moreover, the 4.0-kb hybridizing region was located about 30 kb to one side of '*pdxH*' (K.A. Aidoo, personal communication), a location anticipated from the

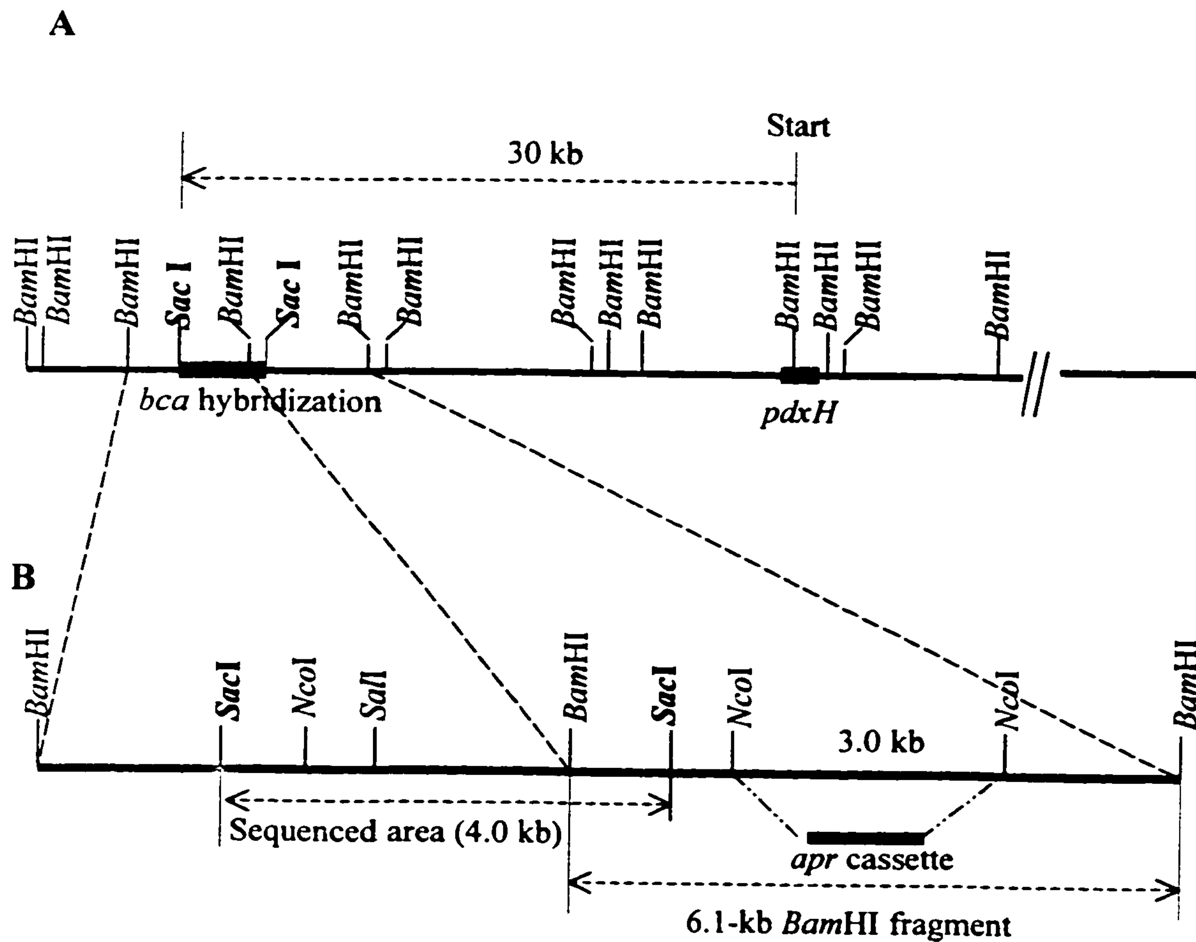


Fig. 3. Chromosome walking in *S. venezuelae*. (A) Chromosome walking outward from '*pdxH*. The start point and extent of chromosome walked over, and the area hybridizing to *bca* are indicated. (B) Expansion of the 12-kb BamHI-BamHI region, showing location of the 4.0-kb *Sac* I fragment sequenced, and of the 6.1 -kb *Bam*HI fragment disrupted by 'blind' insertion of an *apr* cassette.

relative positions of *pdxH* and *cml-2* on the genetic map of *S. venezuelae*.

To determine whether the region containing the 4.0-kb *Sac*I fragment included a gene needed for Cm biosynthesis, Aidoo carried out a "blind" disruption near its predicted location in the chromosome (see Fig. 3B). An apramycin resistance gene was inserted to replace a 3.0-kb *Nco*I segment of the 6.1-kb *Bam*HI fragment that encompassed the cloned 4.0-kb *Sac* I fragment. Southern hybridization of genomic DNA from the disrupted strain, using the apramycin resistance gene as a probe, showed that insertion of the apramycin resistance gene had occurred in the expected region. The disruption did not block Cm production, but it did cause a substantial delay in the onset of Cm biosynthesis, compared with the timing in strain ISP5230. It may, therefore, have affected a regulatory gene determining the shift between primary and secondary metabolism.

Indications from earlier work that the region of the chromosome containing the 4.0-kb *Sac*I fragment included DNA in the genetically mapped Cm biosynthesis gene cluster had been the principal reason for deciding, as the initial goal of my research, to subclone and sequence this region. Analysis of the sequence was expected to provide definitive information about the presence of Cm biosynthesis genes, and to establish not only whether the *bca* homologue corresponded to *cml-2*, but also whether it would be a useful marker to orient chromosome walking. The results obtained from sequence analysis, and the evidence (Facey et al., 1996) that *bca* was not essential for Cm biosynthesis cast doubt on the value of this gene as a marker for chromosome walking; therefore, alternative markers were investigated. The most promising of these was considered to be *cys-28*.

VIII. Investigation of *cys-28* as a Possible Marker for Chromosome Walking

To clone the gene responsible for the *cys-28* phenotype, it was first necessary to characterize the mutant. Relatively little information is available on the biosynthesis and metabolism of sulfur-containing amino acids in streptomycetes. Therefore, recent research on the molecular genetics and biochemistry of cysteine and related sulfur-containing amino acids, notably methionine and homocysteine, in a variety of organisms was consulted for useful background on cysteine metabolism in *S. venezuelae* (see Literature Review, Section III).

LITERATURE REVIEW

I. Chloramphenicol

Chloramphenicol (Cm) is a broad-spectrum antibiotic that inhibits protein synthesis by reversibly binding to the 50S subunit of prokaryotic ribosomes, blocking peptidyl transferase and thus preventing the formation of peptide bonds. It is one of the oldest antibiotics in current use (Ehrlich et al., 1947). Its structure was reported 50 years ago (Rebstock et al., 1949). Nevertheless, it is still a clinically important anti-infectious agent, with advantages such as rapid and efficient penetration of tissue. Its effectiveness against Gram-negative bacteria and Rickettsial infections still make it a good choice for treating life-threatening bacterial infections in some clinical situations, but it can cause serious side effects. The most dangerous of these is irreversible, lethal aplastic anaemia. The risk in some human populations of fatal reactions after prolonged treatment limits the widespread routine use of Cm as an antibacterial drug.

Although chloramphenicol was discovered as a natural product, and early investigations of its structure and therapeutic value were carried out with the product obtained from microbial fermentations, the chemical structure of the antibiotic proved to be relatively simple, and amenable to synthesis. Intensive development led to an efficient and inexpensive chemical process for manufacturing Cm. Consequently, industrial research into fermentation processes for large-scale Cm production using microorganisms discovered in antibiotic screening programs has been neglected. Most of the work done on Cm production has used *S. venezuelae*, the organism in which the antibiotic was originally discovered.

Investigations of Cm biosynthesis as well as research on the physiology, regulation and genetics of Cm production have also been carried out with cultures of this species (Vining & Stuttard, 1994).

II. Genetics of Cm Production

A. Genetic Mapping in *Streptomyces venezuelae*

Although not yet developed to the same extent as the genetics of enteric bacteria or bacilli, the genetics of streptomycetes, pioneered by David Hopwood, is nevertheless a well studied field. The model streptomycete on which most genetic research has focused is *Streptomyces coelicolor* A3(2), which has become the equivalent of *E. coli* in the enterobacteria. A coordinated effort is currently under way to clone and map the genome of *S. coelicolor* A3(2) (Redenbach et al., 1996).

Less is known about the genetics of other streptomycetes, but a rapid increase in knowledge of the *S. coelicolor* A3(2) system has accelerated research with less commonly used species, and some progress has been made with *S. venezuelae*. Earlier, Stuttard and co-workers (Doull et al., 1986; Vats et al., 1987) developed a fertility system for carrying out conjugational crosses in *S. venezuelae* ISP5230, and used recombination frequency data obtained with multiply marked strains, to develop a chromosomal map of genes involved in biochemical reactions required for growth, as well as of genes (*cml*) required for chloramphenicol biosynthesis (Doull et al., 1986). By estimating recombination frequencies, they were able to place the genes in order on the chromosome of *S. venezuelae* ISP5230, and

to establish a linkage map (Vats et al., 1987).

B. Mapping the *cml* gene cluster in the *S. venezuelae* chromosome

The Cm biosynthesis genes were located in a cluster near genes associated with nutritional requirements for arginine (*arg*), cysteine (*cys*) and pyridoxal (*pdx*). These auxotrophic markers in *S. venezuelae* correspond to analogous phenotypic markers in the chromosome of *S. coelicolor* A3(2). The *S. coelicolor* A3(2) markers have been mapped at positions in the 12 o'clock region of the chromosome (Redenbach et al, 1996).

The fine structure of the *cml* cluster was subsequently determined by generalized transduction with the temperate actinophage SV1 (Vats et al., 1987; Stuttard, 1988). Co-transduction analyses indicated that all of the available *cml* markers for Cm biosynthesis are confined to a narrow region between *cys-28* and a group of *pdx* markers in the *S. venezuelae* ISP5230 chromosome. The maximum length of the *cml* cluster can thus be predicted from the amount of infectious DNA that can be packaged by actinophage SV1. Stuttard (1982) estimated this to be about 45 kb (see Fig. 2B).

III. Metabolism of Sulfur-containing Amino Acids

As essential universal constituents of proteins, the sulfur-containing amino acids cysteine and methionine are of major importance in the assimilation of sulfur by all organisms. A general outline of the metabolic reactions used in sulfur assimilation and metabolism is shown in Fig. 4. Plants and microorganisms assimilate inorganic sulfate

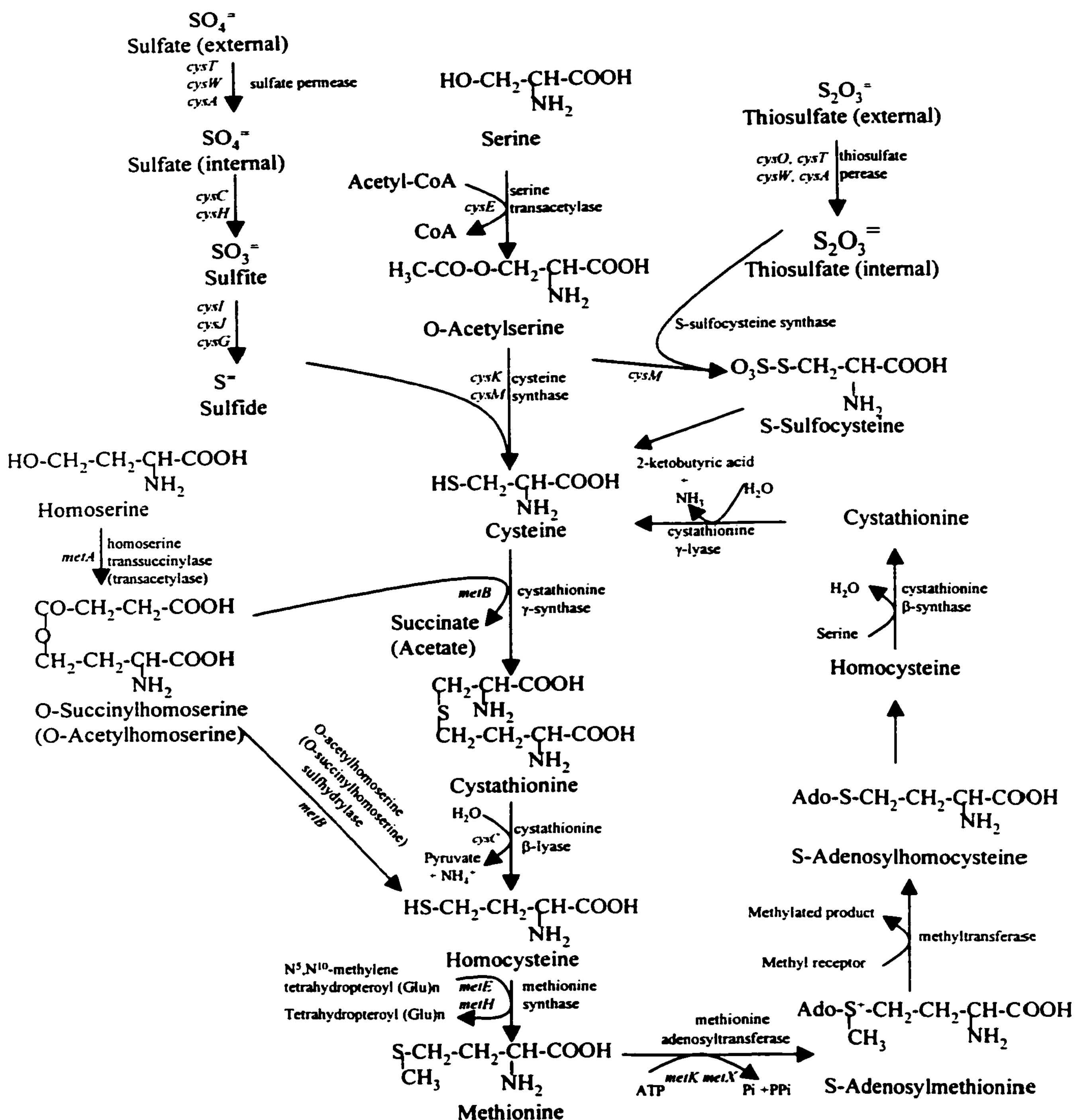


Fig. 4. The principal biochemical reactions involved in sulfur assimilation and metabolism of sulfur-containing amino acids in microorganisms. The genes listed are *E. coli* genes.

predominantly by reduction to sulfide, and incorporation of this reduced form of sulfur into L-cysteine and L-homocysteine. An essential early step in this process is the acylation of L-serine and L-homoserine to serve as the organic substrates for sulfhydrylation; although a preliminary acylation reaction is common to all organisms, the acyl group and the subsequent fate of the acylated product vary. Formation of methionine represents the culmination of the sulfur assimilation process, and in all organisms this occurs by addition of a methyl group to homocysteine. Thus differences between organisms in sulfur assimilation lie mainly in the interconversions of sulfur-containing amino acids that take place in the so-called "transsulfuration" pathway.

Inorganic sulfate can either be directly incorporated into L-cysteine by sulfhydrylation of O-acetylserine, or it can be assimilated via homocysteine formed in a reaction between hydrogen sulfide and O-substituted homoserine. Most of the L-homocysteine made in the latter reaction is methylated to give L-methionine in most bacteria, but in yeast and some other eukaryotes some of it is converted to L-cysteine. The homocysteine, cysteine and methionine formed in these organisms are converted into one another by transsulfuration with the intermediary formation of cystathionine. In mammals, the only pathway available for cysteine formation is from homocysteine.

A. Metabolic conversions of sulfur amino acids in enteric bacteria

The metabolism of sulfur-containing amino acids has been intensively investigated in *E. coli* and *S. typhimurium*. Biosynthesis of L-cysteine from L-serine is a simple two-step process (Fig. 5). Most of the enzymic machinery used in cysteine biosynthesis is needed for

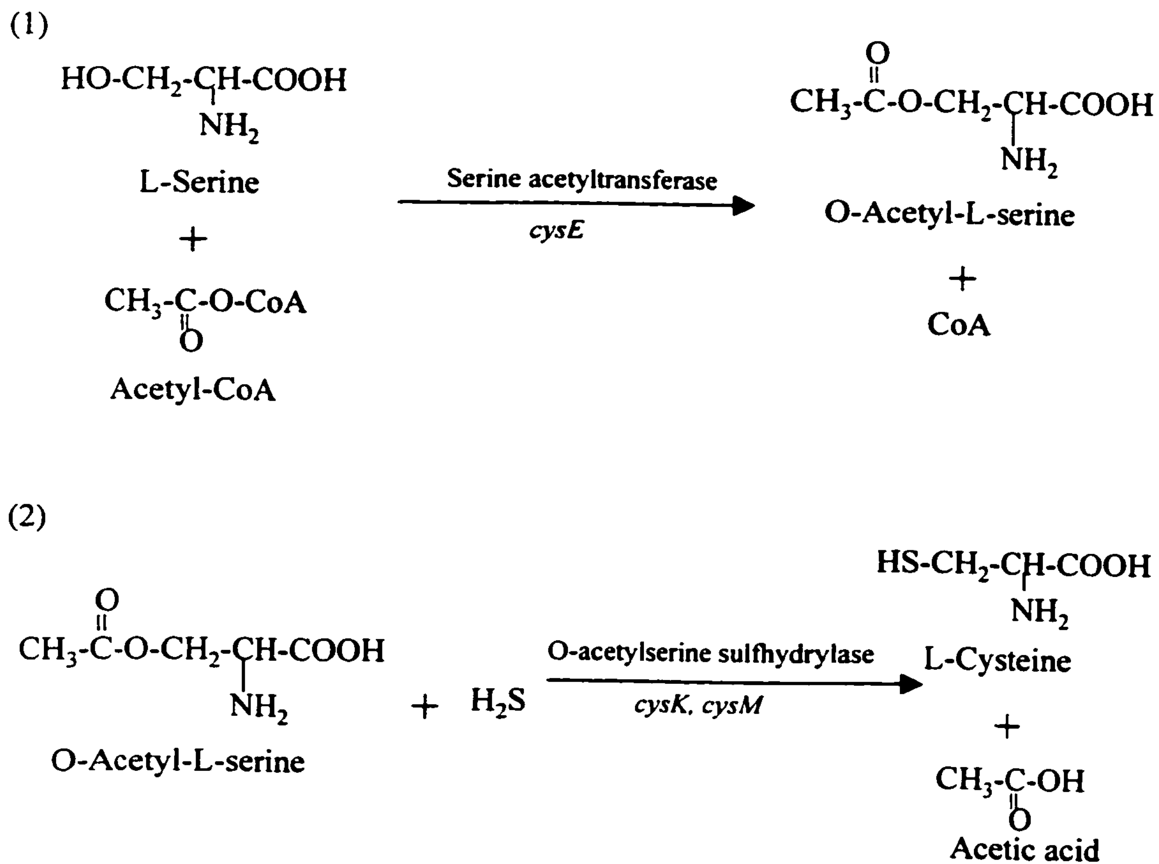


Fig. 5. Enzyme reactions participating in L-cysteine biosynthesis in *E. coli* and *S. typhimurium*

the transport and reduction of sulphate, and regulation of these processes. Therefore, a high proportion of *cys* mutants is defective in the genes governing such activities.

The final step in L-cysteine biosynthesis is mediated by a pair of enzymes: serine acetyltransferase (the product of *cysE*) and O-acetylserine sulfhydrylase (also termed cysteine synthase) (see Fig. 5). The acylating agent for serine acetyltransferase is acetyl-CoA; the acetylated intermediate can be sulfhydrylated by either of two isoforms (A or B) of O-acetylserine sulfhydrylase, encoded by *cysK* or *cysM*, respectively (Hulanicka et al., 1986; Sirko et al., 1987; Byrne et al. 1988).

The *cysK* and *cysM* products show marked sequence similarity (43% amino acid identity; Kredich, 1997); significantly, a mutation in *cysK* or *cysM* alone does not yield a *cys* auxotroph. Both isoenzymes A and B contain pyridoxal phosphate bound to lysine-42 at the active site as a cofactor (Nalabolu et al., 1992; Rege et al., 1996). O-Acetyl-L-serine sulfhydrylase A (the *cysK* product) and serine acetyltransferase form a multifunctional protein structure referred to as the cysteine synthase (CS) complex (Kredich et al., 1997). Isoenzyme A may have a principal role in aerobic growth on sulphate because it has more than 10-fold the activity of isoenzyme B under these conditions (Hulanicka et al., 1979). O-Acetylserine sulfhydrylase B may be important for L-cysteine biosynthesis during anaerobic growth. Noteworthy also is the activity of sulfhydrylase B on thiosulfate. With O-acetyl-L-serine it converts thiosulfate to S-sulfocysteine, which is then reduced to L-cysteine (Nakamura et al., 1984).

The formation of L-cysteine is well regulated in *E. coli* and *S. typhimurium*. Serine acetyltransferase is under strong feedback control by its end product (L-cysteine). Sulfide

and thiosulphate negatively regulate L-cysteine formation by acting as "anti-inducers". At high concentrations of O-acetylserine and sulfide, the activities of both O-acetylserine sulfhydrylase isozymes are inhibited (Tai et al., 1993).

The *cys* regulon includes genes participating in cysteine transport, cysteine synthesis and regulation of these activities. In controlling expression of the genes for cysteine biosynthesis *cysB* has an important role. The presence of N-acetyl-L-serine stimulates binding of CysB at a site upstream of the -35 region of the *cys* regulon promoter to activate transcription (Ostrowski & Kredich, 1989; 1990; Monroe et al., 1990; Hryniewicz & Kredich 1991). *cysB* itself is self-regulated by binding of the *cysB* product to its own repressor site; moreover; N-acetyl-L-serine lowers the binding affinity (Ostrowski & Kredich 1991; Hryniewicz & Kredich 1995). A mutation in *cysE* (the gene for serine acetyltransferase) results not only in defective transacetylation, but also in weak expression of the cysteine biosynthetic enzymes because N-acetyl-L-serine, the inducer of the cysteine regulon, is derived from the acetyltransferase product, O-acetyl-L-serine (Ostrowski & Kredich, 1990).

Methionine is essential in all cells. It not only has a role in initiating protein synthesis but it participates as S-adenosylmethionine as a methyl group donor in vital metabolic functions (Slany et al., 1993; Slany et al., 1994). In enterobacteria, L-methionine is synthesized by a transsulfuration reaction between L-cysteine and O-succinylhomoserine, with L-cystathionine and L-homocysteine being formed as intermediates. The enzymes responsible (Fig. 6) are cystathionine γ-synthase (encoded by *metB*), cystathionine β-lyase (encoded by *metC*) and homoserine succinyltransferase (encoded by *metA*).

The biosynthesis of L-methionine branches from other aspartic family amino acids

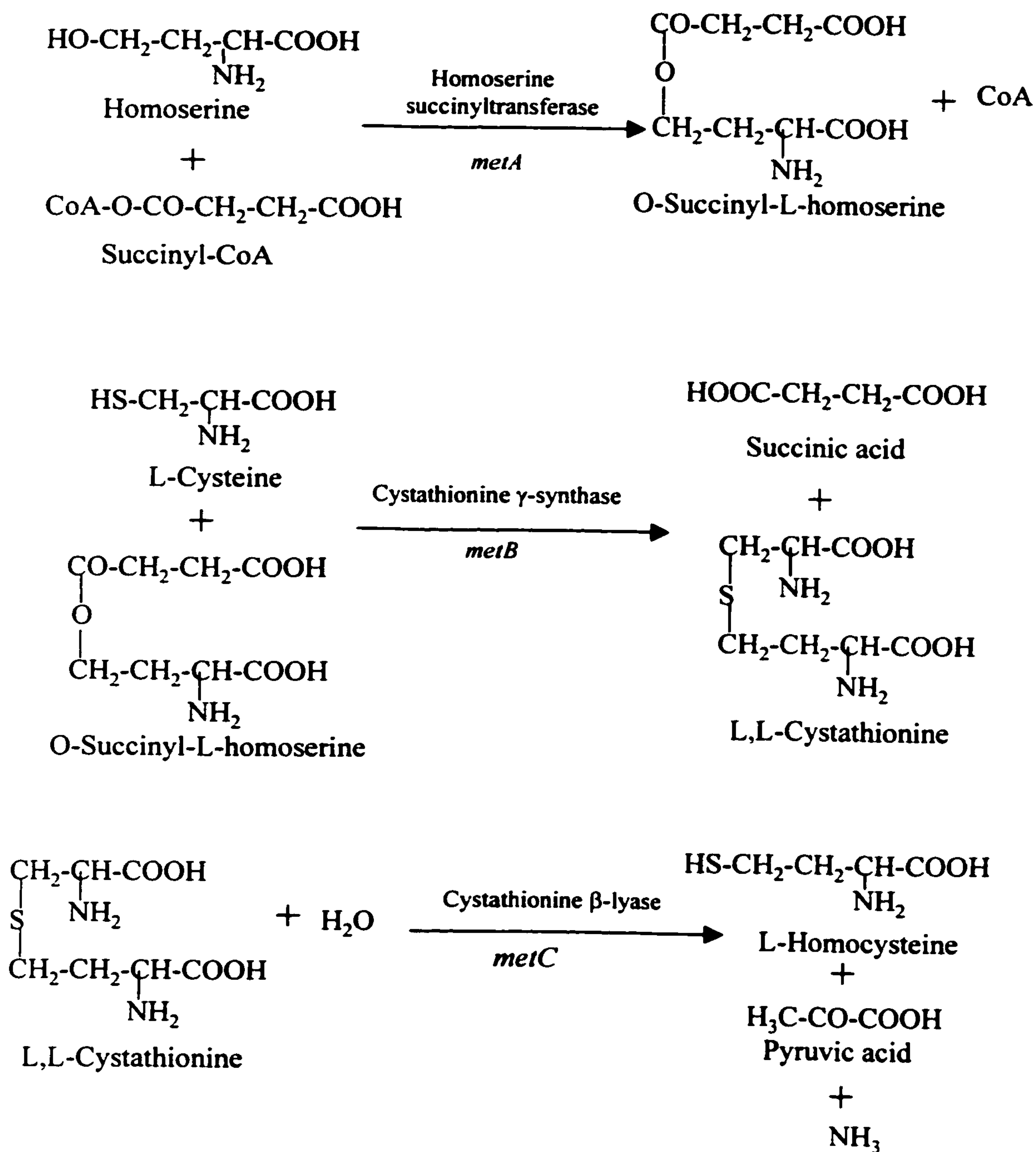


Fig. 6. Homocysteine synthesis in the pathway for L-methionine biosynthesis in *E. coli* and *S. typhimurium*

at homoserine. Branching is achieved by converting homoserine to an O-substituted derivative. In enteric bacteria the enzyme catalyzing the reaction is homoserine succinyltransferase, the product of *metA* (Kaplan & Flavin 1965; Duclos et al., 1989), and the product is O-succinylhomoserine. Cystathionine γ-synthase, the product of *metB*, catalyzes the condensation of O-succinylhomoserine with L-cysteine to form cystathionine. The same enzyme can catalyze in vitro sulfhydrylation of O-succinyl- L-homoserine by H_2S to produce homocysteine. This would provide an alternative pathway for methionine synthesis if the main route is impaired (Flavin & Slaughter 1967; Simon & Hong 1983). Cystathionine β-lyase (encoded by *metC*) cleaves cystathionine to form homocysteine, pyruvate and ammonia. Like other transsulfuration enzymes, cystathionine β-lyase is pyridoxal phosphate-dependent (Dwivedi et al., 1982).

The last step in methionine biosynthesis is the methylation of homocysteine, catalyzed by methionine synthase. The reaction transfers the methyl group of 5-methyl-tetrahydropteroylglutamate to the thiol group of homocysteine. In *E. coli* and *S. typhimurium* two genes, *metE* and *metH*, encode the enzyme (Greene, 1997). The cobalamin-dependent protein encoded by *metH* shows many similarities to the enzyme present in animals, and is used to recycle homocysteine formed when the methyl group is transferred from S-adenosylmethionine. The enzyme encoded by *metE* is cobalamin-independent (Whitfield et al., 1970).

The synthesis of S-adenosylmethionine is catalyzed by methionine adenosyltransferase, the product of *metK*. This enzyme uses methionine and ATP as co-substrates, and needs a divalent cation such as magnesium, manganese or cobalt (Markham

et al., 1980). The properties of *metK* mutants and temperature-sensitive *metK* mutants of *E. coli* point to the existence of another adenosylmethionine synthase gene, *metX* (Greene et al., 1973; Satishchandran et al., 1990). Whereas *metK* is expressed in cells grown on minimal medium (Knoch et al., 1989), the *metX* product is found only in cells grown on rich medium (Greene, 1997). S-Adenosylhomocysteine is formed when the methyl group of S-adenosylmethionine is transferred in a methylation reaction; it is hydrolysed to homocysteine before being recycled to methionine.

Homoserine succinyltransferase (the *metA* product) is feedback inhibited by methionine and S-adenosylmethionine. Most other genes in the *met* regulon are controlled at the level of expression by the *metJ* product. MetJ is a "universal" repressor, and acts on transcription of *metB, metF, metJ* and *metK*. Repression of these genes increases in the presence of S-adenosylmethionine (Shoeman et al., 1985). The octameric consensus sequence 5'-AGACGTCT-3' has been identified at their *metJ* repressor binding sites (Befaiza et al., 1986). *metH* is the only gene in the *met* regulon not controlled by the *metJ* system.

Besides release from *metJ* repression, *metA* and *metE* require stimulation by a *metR* product for full expression (Mares et al., 1992; Cai et al., 1989; Urbanowski et al., 1987; 1989a; 1989;). Homocysteine is required for stimulation of *metE* (Cai et al., 1989a; Urbanowski et al., 1989). Expression of *metR* is autoregulated, and is itself repressed by MetJ (Maxon et al. 1989; Urbanowski and Stauffer 1985). The *metR* product stimulates transcription by binding to specific sequences [TGAAN(T/A)NNTTCA] of regulated genes, such as *metA*, *metE* and *metH* but not *metC*. Expression of *metC* is regulated by the *metJ* system.

B. Biosynthesis of sulfur-containing amino acids in other bacteria

Biosynthesis of L-cysteine by sulfhydrylation of O-acetyl-L-serine with H_2S is a universal pathway in bacteria. Cysteine synthase (acetylserine sulfhydrylase) activity has been detected in *Paracoccus denitrificans* (Burnell & Whayley, 1977) and *Bacillus sphaericus* (Nagasawa & Yamada, 1987), as well as in the enterics. The genes have been cloned from *Bacillus subtilis* (Ganon et al., 1994), *Rhodobacter sphaeroides* 2.4.1 (O'Gara et al., 1997), *Flavobacterium* K3-15 (Muller et al., 1996), *Haemophilus influenzae* (Fleischmann et al., 1995) and a *Synechococcus* (cyanobacterium) (Nicholson et al., 1995). The deduced amino acid sequence from the gene of *Flavobacterium* shows a conserved motif of seven amino acids for a pyridoxal phosphate binding site (SIKDRIA). The Lys-42 (K) of this cysteine synthase is conserved in all known cysteine synthases. In *Synechococcus* DNA, two genes with significant sequence similarity to O-acetyl-L-serine sulfhydrylase (cysteine synthase) and serine acetyltransferase were clustered in an indigenous cyanobacterial plasmid (Nicholson et al., 1995).

Unlike *cysK*, *cysM* is not present in all bacteria. When *Thiobacillus denitrificans* and 14 species of phototrophic bacteria were assayed for cysteine synthase and S-sulfocysteine synthase, only *Chromatiaceae, Rhodospirillaceae* and *Thiobacillus* species tested positive for S-sulfocyteine synthase (Hensel & Truper 1976). Of two proteins with cysteine synthase activity purified from *Rhodospirillum tenue*, one (57 kDa) showed only cysteine synthase activity, and catalyzed the formation of L-cysteine from O-acetylserine and H_2S. The other (46 kDa) showed both cysteine synthase and S-sulfocysteine synthase activity, and also converted thiosulfate to S-sulfocysteine (Hensel & Truper 1983). The *cysM* recently cloned

from the micro-aerophilic Gram-negative bacterium *Campylobacter jejuni* (Garvis et al., 1997) was demonstrated to be functionally similar to the *cysM* of *E. coli* in its ability to complement an *E. coli* cysteine auxotroph.

Homoserine is a precursor of L-methionine in bacteria; the series of reactions involved are catalyzed by O-acylhomoserine acyltransferase (succinyltransferase or acetyltransferase), cystathionine γ-synthase, cystathionine β-lyase and methionine synthase. With some exceptions, the pathway in Gram-negative bacteria proceeds with O-succinylhomoserine as an intermediate, whereas in Gram-positive bacteria such as *Bacillus, Corynebacterium* and *Brevibacterium*, the intermediate is O-acetylhomoserine. In the Gram-positives, streptomycetes may be exceptional in that the cystathionine γ-synthase uses mainly O-succinylhomoserine (Kanzaki et al., 1986).

Homoserine acetyltransferase has been found in *Corynebacterium* (Kase & Nagayama 1974), *Brevibacterium* (Miyajima & Shiio 1973), *Bacillus subtilis* (Brush & Paulus 1971) and *Bacillus polymyxa* (Wyman & Paulus 1975; Wyman et al., 1975). It is inhibited by methionine and/or S-adenosylmethionine in *B. polymyxa*. *B. subtilis* and *Brevibacterium flavum* (Sugimoto & Shiio, 1980). The enzyme is also repressed in *B. flavum* and *Corynebacterium glutamicum* (Miyajima & Shiio 1973). O-Succinylhomoserine synthase and succinylhomoserine sulfhydrylase (homocysteine synthase) catalyzing sulfhydrylation of O-succinylhomoserine have been identified in *Pseudomonas aeruginosa* (Foglino et al., 1995).

The synthesis of L-homocysteine in *B. flavum* by direct sulfhydrylation is a special case in methionine metabolism. Homocysteine is synthesized by O-acetylhomoserine

sulfhydrylase from O-acetylhomoserine and H_2S, instead of via the cystathionine pathway (Ozaki & Shiio 1982). In contrast to the enzyme in *S. cerevisiae*, where O-acetylhomoserine sulfhydrylase (AHS) also shows high O-acetylserine sulfhydrylase (ASS) activity, the *B. flavum* enzyme has slight ASS activity in vitro. L-Cysteine is formed only by the mono-functional ASS. Another exception in methionine synthesis occurs in *P. aeruginosa*. This is the only organism synthesizing homocysteine mainly from O-succinylhomoserine by direct sulfhydrylation (Foglino et al., 1995). However, Gunther et al. (1979) concluded from the properties of cysteine mutants and the effect of cysteine synthesis inhibitors on the mutants that a reverse transsulfuration pathway was also present in *P. aeruginosa*.

C. Metabolism of sulfur-containing amino acids in streptomycetes

Streptomycetes are important as antibiotic producers; the focus of research has been on their secondary metabolism. In the absence of contrary evidence, they were assumed, like the enterobacteria, to lack the reverse transsulfuration pathway from methionine to cysteine. However, in the cephamycin C producer *Streptomyces clavuligerus*, where L-cysteine is a direct precursor of the antibiotic, incorporation of labeled methionine into cephamycin C (Whitney et al., 1972) indicates that reverse transsulfuration is functioning. Cystathionine γ-lyase is present in *Streptomyces lactamdurans* (Kern & Inamine 1981), and the enzyme is widely distributed in streptomycetes and other actinomycetes (Nagasawa et al., 1984). Cystathionine γ-lyase, which converts cystathionine to cysteine, α-ketobutyric acid and ammonia, was purified from *Streptomyces phaeochromogenes* and characterized (Nagasawa et al., 1984). The enzyme requires pyridoxal-5'-phosphate as a cofactor and consists of four

identical subunits.

Kitano et al. (1985) tested various mutants of *Streptomyces griseus* disturbed in sulfur metabolism for their growth requirements, and from the effects of supplementation with various sulfur compounds, proposed that thiosulfate is a direct intermediate in sulfate reduction and cysteine biosynthesis in *S. griseus* subsp. *cryophilus* C-19393 (Fig. 7). That thiosulfate acts as a direct precursor of L-cysteine in actinomycetes was supported by cloning and disruption of a gene for thiosulfate formation in *Saccharopolyspora erythraea* (formerly *S. erythraeus*) (Donadio et al., 1990). Nevertheless, the isolation of cystathionine γ-lyase from streptomycetes, and evidence that a sulfide oxidase is required for cephalosporin C production, does not fully establish the physiological role of the enzyme, nor of the transsulfuration pathway in streptomycetes.

5,10-Methylenetetrahydrofolate reductase has been cloned from *S. lividans* (Blanco et al., 1998) and shown to resemble the enzyme from enteric bacteria in catalyzing the formation of 5-methyltetrahydrofolate, the methyl donor for homocysteine methylation in the last step of methionine synthesis. The cloned gene resembled its homologue in *S. typhimurium*. Disruption of the gene resulted in methionine auxotrophy, indicating that, in streptomycetes as in enterobacteria, synthesis of the folate cofactor is essential for methionine biosynthesis.

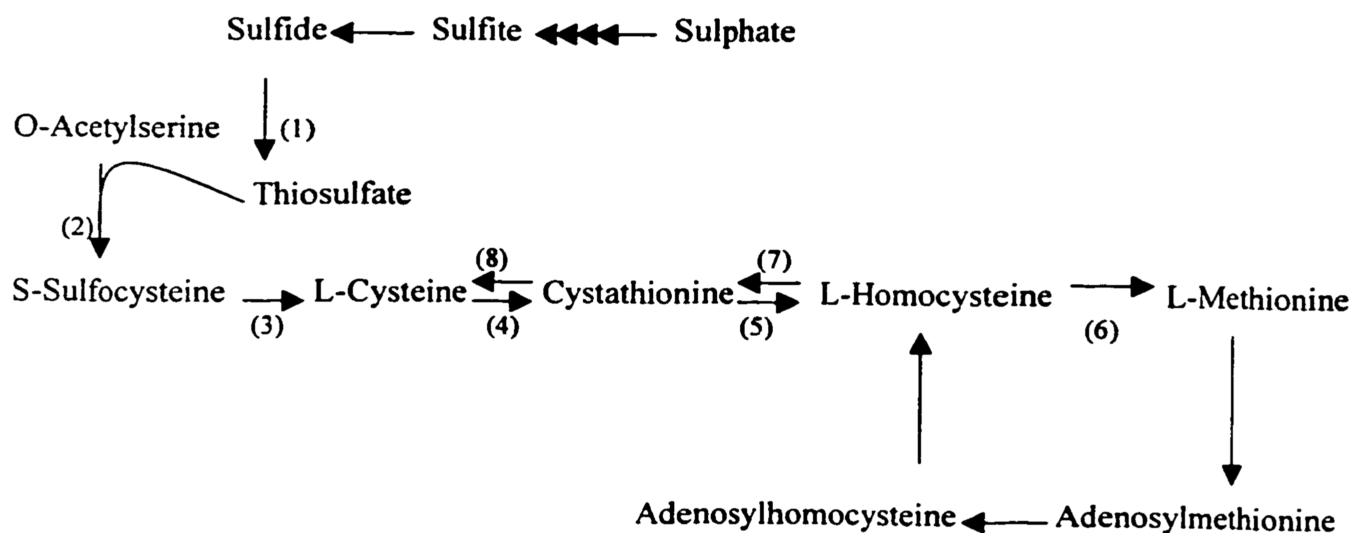


Fig. 7. Proposed synthesis of sulfur-containing amino acids in streptomycetes (modified from Donadio et al., 1990). (1) sulfide oxidase; (2) S-sulfocysteine synthase; (3) S-sulfocysteine reductase; (4) cystathionine γ-synthase; (5) cystathionine β-lyase; (6) methionine synthase; (7) cystathionine β-synthase; (8) cystathionine γ-lyase.

D. Biosynthesis of sulfur-containing amino acids in plants

L-Cysteine biosynthesis in plants is similar to that in bacteria: serine acetyltransferase generates O-acetylserine which, with sulfide becomes the substrate for a sulfhydrylation reaction catalyzed by O-acetylserine sulfhydrylase. In plants, three isoforms of cysteine synthase have been found in the three cell compartments where protein is synthesized: chloroplasts, mitochondria and cytosol (Yamaguchi et al., 1998; Takahashi & Saito 1996; Yamaguchi & Masada, 1995). Though the enzymes exhibit the same activity, they differ in primary structure. The cDNAs for cysteine synthases from different cell compartments have been cloned from many plants, including *Arabidopsis* (Hell et al., 1994; Barroso et al., 1995; Hesse & Altmann, 1995), spinach (Saito et al., 1994; Hell et al., 1993; Rolland et al., 1993) and watermelon (Noji et al., 1994). The deduced amino acid sequences of the genes derived from cDNAs show similarity to those of their bacterial counterparts, and they are able to complement *E. coli* auxotrophs lacking cysteine synthase. Thus they clearly are of the same evolutionary origin (Hell et al., 1994, Barroso et al., 1995; Noji et al., 1994). Serine acetyltransferase and O-acetylserine sulfhydrylase interact with each other to form cysteine synthase complexes in plants (Droux et al., 1998; Zhu et al., 1998; Bogdanova & Hell 1997; Saito et al., 1995). The formation of such complexes probably confers kinetic advantages in the sequential reactions from serine to cysteine.

E. Transsulfuration in Mammals

In eukaryotes, L-cysteine can be formed by a reverse transsulfuration reaction, with cystathionine as an intermediate. Cystathionine is then cleaved by γ-cystathionase to yield

L-cysteine. In animals, reverse transsulfuration begins with methionine and involves a series of reactions. Transfer of the adenosyl group from ATP to the sulfur atom of methionine gives S-adenosylmethionine, the major methyl group donor. Donation of the methyl group to a recipient leaves adenosyl-homocysteine,, which is then hydrolyzed to homocysteine. A simple condensation of L-serine and L-homocysteine, catalyzed by cystathionine β-synthase, forms cystathionine. Cleavage of cystathionine by cystathionine γ-lyase yields L-cysteine.

F. Biosynthesis of L-cysteine and L-methionine in yeasts and fungi

The routes for L-cysteine biosynthesis in yeasts and fungi are diverse. More than one pathway may exist in each species. The biological role of each pathway may differ in different species and depend on the metabolic conditions of the cell and its regulatory mechanisms. *Saccharomyces cerevisiae* has two routes for cysteine biosynthesis. One is analogous to the bacterial pathway in which L-cysteine is formed from L-serine via O-acetyl-L-serine, catalyzed by serine O-acetyltransferase and O-acetylserine sulfhydrylase (Langin et al., 1986). Cysteine is converted to methionine via cystathionine and homocysteine through the activities of cystathionine γ-synthase and cystathionine β-lyase. The O-acetylserine sulfhydrylase in *S. cerevisiae* is bifuntional: it can react not only with O-acetylserine to form L-cysteine, but also with O-acetylhomoserine to form L-homocysteine. But its bifunctional activities could be detected only in potassium phosphate buffer (Yamagata et al., 1974; Brzywczy & Paszewski, 1993). Cysteine can also be synthesized by reverse transsulfuration from homocysteine and serine with cystathionine as an intermediate. In *S. cerevisiae*, this is considered to be the main pathway of cysteine

synthesis (Ono et al., 1984; Cherest & Surdin-Kerjan 1992), mediated by cystathionine β-synthase and cystathionine γ-lyase. Homocysteine can be formed by two alternative pathways: direct sulfhydrylation of acetylhomoserine by H_2S, and transsulfuration from O-acetylhomoserine and cysteine. The direct sulfhydrylation of homoserine is catalyzed by homocysteine synthase (O-acetylhomoserine sulfhydrylase), and is considered to be the main pathway for homocysteine biosynthesis. Because serine acetyltransferase is not found in all strains of *S. cerevisiae*, and mutants defective in cystathione γ-lyase and cystathionine β-synthase can accumulate large amounts of cystathionine and homocysteine, respectively, it is reasonable to believe that L-cysteine is formed mainly by the transsulfuration pathway via homocysteine and cystathionine (Brzyczy & Paszewski, 1993; Cherest & Surdin-Kerjan 1992; Ono et al., 1984). The cloning of a cystathionine γ-lyase-like gene from *S. cerevisiae* and the recovery of cysteine auxotrophs by disrupting this gene support such a conclusion (Barton et al., 1993). The other route for homocysteine formation is catalyzed by cystathionine γ-synthase and cystathionine β-lyase from O-acetylhomoserine and cysteine (Savin & Flavin 1975; Yamagata et al., 1975). A monofunctional O-acetylserine sulfhydrylase catalyzing the in vitro synthesis of L-cysteine from O-acetylserine and H_2S was detected in *S. cerevisiae*, but its physiological role in L-cysteine synthesis has not been established (Yamagata 1980; Yamagata et al., 1982).

L-Methionine biosynthesis in *S. cerevisiae* starts from L-homoserine. This is converted to O-acetylhomoserine by homoserine acetyltransferase, and the O-acetylhomoserine is then converted to homocysteine by the bifunctional sulfhydrylase. In *S. cerevisiae* succinyl-CoA does not replace acetyl-CoA as an acyl donor (Yamagata

1987). As described above, there are two alternative pathways from O-acetylhomoserine to homocysteine. Because a low level of cystathionine γ-synthase activity was detected, and in *S. cerevisiae* cystathionine cannot be used as a methionine precursor, the cystathionine pathway is not obligatory. Nevertheless, the ability of a mutant impaired in O-acetylhomoserine sulfhydrylase to grow on cysteine, indicates that cysteine can be converted to methionine via transsulfuration (Kerjan et al., 1986). These somewhat contradictory results bearing on the function of the cystathionine pathway may reflect a difference between various isolates of *S. cerevisiae,* possibly associated with poor permeation of cystathionine into the organism, as has been encountered in *P. aeruginosa* (Foglino et al., 1995). Cystathionine γ-synthase activity is also exhibited by purified cystathionine γ-lyase if O-succinylhomoserine is used as a substrate (Ono et al., 1993). Mutants deficient in both L-serine acetyltransferase and cystathionine γ-lyase require cysteine, indicating that L-cysteine is synthesized through two alternative pathways (Ono et al., 1984, Ono et al., 1988). The metabolism of sulfur-containing amino acids in *S. cerevisiae* is summarized in Fig. 8.

Like other enzymes in transsulfuration, the bi-functional (OAH-OAS) sulfhydrylase of *S. cerevisiae* requires a pyridoxal phosphate cofactor. The activity of this enzyme is regulated through inhibition by methionine and/or S-adenosylmethionine (de Robichon-Szulmajster & Cherest 1967). Transcription of the gene for this enzyme is repressed by L-methionine, and translation of the enzyme is repressed by S-adenosylmethionine (; Sangsoda et al., 1985; Surdin-Kerjan & de Robichon-Szulmajster 1975; Yamagata, 1987). L-Cysteine plays a negative regulatory role by lowering the cellular

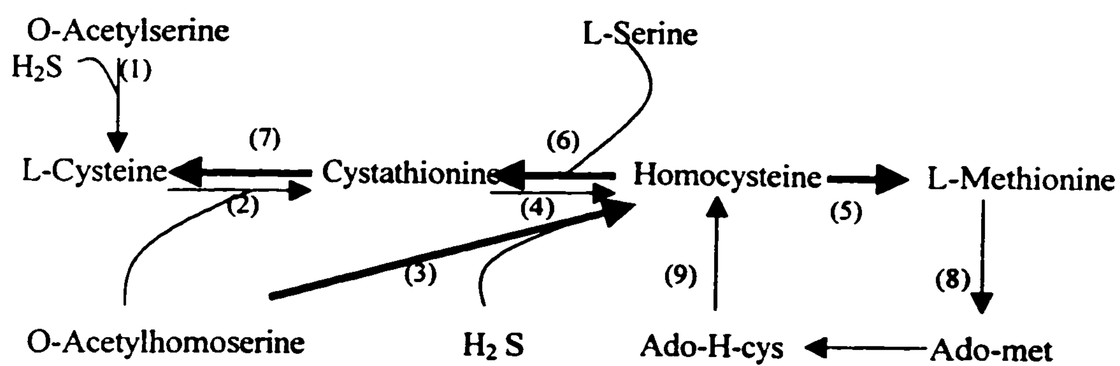


Fig. 8. Biosynthesis of sulfur-containing amino acids in *S. cerevisiae*. Thick arrows represent dominant routes. Enzymes: (1) O-acetylserine sulfhydrylase; (2), cystathionine γ-synthase; (3) O-acetylhomoserine sulfhydrylase; (4) cystathionine β-lyase; (5) methionine synthase; (6) cystathionine β-synthase; (7) cystathionine γ-lyase; (8) S-adenosylmethionine synthase; (9) S-adenosylhomocysteine hydrolase.

concentration of OAS, which is believed to be an inducer of OAH-OAS sulfhydrylase (Ono et al., 1996).

In *Kluyveromyces lactis*, homocysteine synthase contributes markedly to homocysteine synthesis even though it is not indispensable (Brzywczy & Paszewski 1993). Nevertheless, in *Neurospora crassa*, homocysteine is synthesized solely through the transsulfuration pathway via cystathionine (Ferr 1971; Kerr & Flavin 1970), while homocysteine synthase acts as an alternative enzyme for homocysteine synthesis (Piotrowska et al., 1980). In *Aspergillus nidulans*, two alternative pathways for L-cysteine synthesis have been demonstrated (Paszewski & Grabski 1973; Pieniazek et al., 1974; Paszewski & Grabski 1975). The O-acetylserine sulfhydrylation pathway plays the main role in cysteine biosynthesis. The reverse transsulfuration pathway from homocysteine, (containing the three enzymes, O-acetylhomoserine sulfhydrylase, cystathionine β-synthase and cystathionine γ-lyase) plays an alternative role in cysteine biosynthesis. In this filamentous fungus, three genes from three cell compartments, *cysB*, *cysC* and *cysE*, encoding cysteine synthases have been identified (Cybis et al., 1988). *cysB*, a mitochondrial gene, was cloned by complementation (Topczewski et al., 1997). In *A. nidulans* the pathway for methionine synthesis from cysteine and O-acetylhomoserine via cystathionine and homocysteine is indispensible, because impairment of either enzyme, cystathionine γ-synthase or cystathionine β-lyase, results in auxotrophs. OAH sulfhydrylase forms an alternative pathway for homocysteine and cysteine synthesis (Paszewski & Grabski 1974; 1975; Paszewski 1993).

In *Schizosaccharomyces pombe*, L-cysteine is synthesized solely by sulfhydrylation

of O-acetylserine. Neither cystathionine β-synthase nor cystathionine γ-lyase of the reverse transsulfuration pathway was found in the fission yeast (Paszewski 1993; Brzywczy & Paszewski 1994). Homocysteine is biosynthesized by OAH sulfhydrylase from OAH. The OAH sulfhydrylase also reacts with L-homoserine and O-succinylhomoserine (Yamagata 1984). This yeast is the only eukaryotic microorganism possessing an O-succinylhomoserine sulfhydrylase and reacting with the three states of homoserine. The enzyme is very unstable, but can be stabilized with 25% (w/w) sucrose or glycerol.

In *Saccharomycopsis lipolytica*, the cysteine requirement of mutants defective in both serine acetyltransferase and cystathionine γ-lyase suggests that cysteine can be synthesized by two pathways (Morzycka & Paszewski 1979). Some of the homocysteine needed can be synthesized from cysteine via cystathionine (Morzycka & Paszewski 1982), but the sulfhydrylation pathway from acetylhomoserine is dominant. Mutants with elevated OAH sulfhydrylase can overproduce methionine, indicating that this enzyme contributes to methionine synthesis (Morzycka et al., 1976). Two cysteine synthases were purified from this organism, a low molecular weight (74 kDa) monofunctional O-acetylserine sulfhydrylase, and high molecular weight (220 kDa) bifunctional sulfhydrylase. The proportion of OAS to OAH sulfhydrylase activity (5%) was much lower than in *S. cerevisiae* (16%). In contrast to the situation in *S. cerevisiae*, the monofunctional enzyme seemed to function in cysteine synthesis.

In *C. acremonium*, monofunctional cysteine synthase and the closely related, less specific, bifunctional cysteine-homocysteine synthase are separated (Dobeli & Nuesch 1980). The cysteine moiety in cephalosporins is derived preferentially from methionine via reverse

transsulfuration (Caltrider & Niss 1966). Cystathionine γ-lyase is essential for cephalosporin production (Treichler et al., 1978). In *Penicillium chrysogenum*, no OAS sulfhydrylase was detected during the penicillin-producing phase, implying that cysteine is synthesized from cystathionine and homocysteine.

MATERIALS AND METHODS

I. Organisms and Vectors

Bacterial strains and vectors are listed in Table 1.

II. Chemicals and Biochemicals

Bacto-agar, Bacto-peptone, tryptone and yeast extract were purchased from Difco Laboratories, Detroit, MI. Cystathionine, O-acetyl-L-serine, O-acetyl-DL-serine, O-succinyl-L-homoserine, L-cysteine, DL-homocysteine, polyethylene glycol (PEG) 1000 for protoplast transformation, PEG8000 for phage precipitation, and ampicillin, were from Sigma Chemical Company, St. Louis, MO. DL-Serine, L-methionine and glycine were from B.D.H. Inc, Toronto, Ont. Chemicals for preparing polyacrylamide gels were from Bio-Rad, Richmond, CA. Positively charged nylon membrane used for hybridization experiments, and the QIAEX II kit for isolating DNA from electrophoresis gels were purchase from QIAGEN Inc, Mississauga, Ont. *Taq* DNA polymerase, lysozyme, RNase and DNase I were from Boehringer Mannheim Canada, Montreal, Que. Some restriction endonucleases, T_7 Sequencing™ kit, SureClone™ ligation kit, Ready·To·Go™ DNA labeling beads (-dCTP), M13mp18 RF DNA, lambda phage DNA were purchased from Pharmacia Biotech Inc, Piscataway, NJ. Some restriction endonucleases were from New England Biolabs Inc, Missisauga, Ont. Other restriction endonucleases and the EXOIII/S1 deletion kit were from MBI Fermentas Inc, Flamborough, Ont. T4 DNA ligase, thermosensitive alkaline phosphatase (TsAP) and the random primer DNA labeling system were from Gibco/BRL,

Table 1. Strains and vectors

Strains	Genotype/phenotype	Source/Reference
	Streptomyces venezuelae	
ISP5230	wild-type	Stuttard, 1982
VS263	*cys-28*	Doull et al., 1986
CHA	ISP5230 (pJV215)	This study
CHB	ISP5230(pJV217)	This study
CHCp1	ISP5230(pJV218)	This study
CHCp2	ISP5230(pJV219)	This study
CHC1	CHCp1(CBS disruptant)	This study
CHC2	CHCp2(CBS disruptant)	This study
CCHCp1	VS263(pJV218)	This study
CCHCp2	VS263(pJV219)	This study
CCHC1	CCHCp1(CBS disruptant)	This study
CCHC2	CCHCp2(CBS disruptant)	This study
CHFA	ISP5230(pJV222)	This study
CHFB	CHFA(disruptant of ORF2)	This study
CHG5	ISP5230(pJV231)	This study
	Streptomyces lividans	
TK23	SLP1$^-$ SPL2$^-$ spc	JII[a]
UC8882	UC8390(pUC1169)	Chung, 1987
CH101-117	TK23(*cys* mutants)	This study
CH218	CH114(cys+ pJV204)	This study

Table 1 continued on page 39

Table 1 continued

Strain/Plasmid	Genotype/Description	Source/Reference
	Escherichia coli	
DH5α	F'/*endA*1 *hsdR*17 (rK- mK+) *supE*44 *thi*-1 *recA*1 *gyrA*(Nalr) *relA*1(*lac(ZYA-argF*) U169 *deoR* (80d*lac* (*lacZ*ΔM15)	BRL
ET12567	*dam*⁻ *dcm*⁻ *hsdM*⁻	MacNeil et al., 1992
LE392	F-, *hsdR*514, *supE44*, *supF*58, *lacY*1 or Δ(*lacIZY*)6, *galK*2, *gal*T22, *metB*1, *trpR*55 λ⁻	Maniatis, et al., 1982
TG1	Δ(*lac-proAB*), *supE*, *thi*, *hsd* Δ5 F'(*traD*36, *proAB*+, *lacI*ᴿ, *lacZ* ΔM5)	Carter et al., 1985
	Plasmids	
pHJL400	*tsr*, *amp*, *LacZ*	Larson & Hershberger 1986
pIJ702	*tsr*, *mel*+	Katz et al., 1983
pBluescript II SK+ (Usually abbreviated to pSK+)	phagemid amp, LacZ	Stratagene
pUC18	*amp*, *LacZ*	Pharmacia
pUC1169	pMT660::Tn*4560*	Chung 1987
pJV228	fusion plasmid of pUC1169(*Bam*HI)	This study
pJV230	*Vsp*I-*Mlu*I fragment(Tn*4560*) of pJV228 cloned in pUC18(*Sma*I)	This study
pJV207	9,2 kb fragment of ISP5230 DNA containing ORFs 1 & 2 cloned in pSK+	This study
pJV208/209	4.0-kb Pst I fragment of pJV207 subcloned in pSK+ in two orientations	This study
pJV210/211	3.0-kb *Pst* I fragment of pJV207 subcloned in pSK+ in two orientations	This study

Table 1 continued on page 40

Table 1 continued

pJV213	pJV208 with *apr* inserted at *Sal* I site of insert	This study
pJV214	0.4-kb *Pst* I fragment of pJV207 cloned in pSK+	This study
pJV215/216	4.0-kb *Pst*I fragment of pJV207 subcloned in pHJL400	This study
pJV217	pJV216 with 0.8-kb *Bgl*II fragment replaced by 1.5-kb *Nco*I fragment of pUC120A containing *apr*	This study
pJV218/219	pJV213 insert subcloned in pHJL400	This study
pJV220/221	4.0-kb *Xho*I-*Bgl*II fragment of pJV207 cloned in pHJL400	This study
pJV222	pJV220 with the 1.5-kb *Nco*I fragment of pUC120A inserted in the *Nco*I site of ORF2	This study
pUC120A	*Nco*I cassette containing *apr*	A.S. Paradkar
pR4	*Bam*HI-*Pst*I fragment of PR3 containing *apr* subcloned in pSK+	J.Y. He
pJV223	*Pst*I-*Eco*RI fragment of pR4 cloned in *Xho*I site of pSK+	This study
pJV225	cassette containing *apr* flanked on each side by MCS	This study
pJV226	pJV225 with *apr* fragment replaced by *vph* fragment (*Pst* I) of pUC1169	This study
pJV227	*vph* gene cloned in pHJL400 (*Pst* I)	This study
pJV224	pSK+ containing 0.6-kb chromosomal DNA fragment from *S. venezuelae* flanked on each side by MCS	This study This study

Table 1 continued on page 41

Table 1 continued

pJV204	8.0-kb *S. venezuelae* genomic DNA cloned in pIJ702 by 'complementing' *S. lividans* mutant CH114 host	This study
pJV201	pSK+ containing 4.0-kb *Sac*I fragment hybridizing with *bca*	This study
pJV202	pHJL400 containing 4.0-kb *Sac*I fragment hybridizing with *bca*	This study
pJV203	pJV202 disrupted with *apr* (*Nco*I)	This study
	Phage	
VCSM13	km, derivative of M13K07	Stratagene
ZX1/2	'*cysK*' fragment of pUK4 cloned in M13mp18 and M13mp19	This study
ZX201-212	recombinant GEM11 phage hybridizing with cloned *cys* gene fragment amplified by PCR	This study

a JII = John Innes Institute, Norwich, UK

Gaithersburg, MD. ID-ZYME™ DNA polymerase was from ID Labs Biotechnology, London, Ont. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), dithiothreitol (DTT) isopropyl-β-D-thiogalactopyranoside (IPTG), were from Diagnostic Chemicals Ltd., Charlottetown PEI. Thiostrepton was from the Squibb Institute for Medical Research, Princeton, NJ. The bicinchoninic acid (BCA) reagent used for quantitative measurement of protein, and the OPA reagent used for precolumn derivatization in HPLC analysis of amino acids were purchased from Pierce Chemical Co., Rockford, IL All other chemicals were from commercial sources, and were of Reagent grade.

III. Media

Unless otherwise noted, media were autoclaved at 121 °C for 20 min. The pH was adjusted with 10% NaOH. Solutions of heat-labile materials were sterilized by filtration through cellulose acetate membranes with 0.22 μm pores.

LB broth for cultures of *E. coli* (Sambrook et al., 1989):

Bacto-tryptone		10 g
Yeast extract		5 g
Sodium chloride		10 g
Distilled water	to	1000 ml
pH		7.0

LB agar for routine maintenance of *E. coli* cultures:

LB broth with 1.5% agar.

2xYT for *E. coli* cultures used to prepare single-stranded DNA, and for *E. coli* cultures used for transformations (Sambrook et al., 1989):

Bacto-Tryptone		16 g
Bacto-yeast extract		10 g
NaCl		5 g
Distilled water	to	1000 ml
pH		7.0

TBG for lambda phage propagation (Sambrook et al., 1989):

Bacto-Tryptone		1.2 g
Yeast extract		0.2 g
Glycerol		0.4 ml
Water	to	90 ml

After autoclaving, the solution was supplemented with 1.8 ml of 20% (w/v) glucose and 10 ml of a solution containing 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4.

MYM for routine use in culturing *Streptomyces venezuelae* (Stuttard 1982):

Maltose		4.0 g
Yeast extract		4.0 g
Malt extract		10.0 g
Water to		1000 ml
pH	to	7.3

MYM Agar for surface cultures of *S. venezuelae*:

As for MYM with addition of 20 g of agar per 1000 ml.

K1 Agar for routine maintenance of *Streptomyces lividans* (Aidoo, 1989):

Maltose	10.0 g
Yeast extract	5.0 g
Casamino acid	0.2 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
$FeSO_4.7H_2O$	0.01 g

Agar		15.0 g
Water to		1000 ml

TO agar for sporulation of *Streptomyces*:

Tomato paste		20 g
Pablum		20 g
Agar		20 g
Water	to	1000 ml
pH		6.8

Toy agar for fast sporulation of *Streptomyces*:

Tomato paste		20 g
Pablum		20 g
Yeast extract		2.5 g
Agar		20 g
Water	to	1000 ml
pH		6.8

MM for testing auxotrophs (Hopwood et al., 1985):

Maltose		10.0 g
$MgCl_2 \cdot 6H_2O$		0.2 g
$FeSO_4 \cdot 7H_2O$		0.01 g
Asparagine		0.5 g
Agar		15 g
Water	to	1000 ml
pH		7.2-7.4

MMY for enzyme assays with *S. venezuelae*:

Glucose		10.0 g (autoclaved separately)
$MgCl_2 \cdot 6H_2O$		0.2 g
$FeSO_4 \cdot 7H_2O$		0.01 g
Asparagine		0.5 g
Yeast extract		1 g
Agar		15 g
Water	to	1000 ml
pH		7.2-7.4

YEME medium (Hopwood et al., 1985) for cultures used to prepare protoplasts and to isolate DNA from *Streptomyces*:

Yeast extract		3.0 g
Malt extract		3.0 g
Bacto-peptone		5.0 g
Glucose		10.0 g
Sucrose		340 g
Distilled water	to	1000 ml

The medium was adjusted to pH 7.5 with NaOH before autoclaving. After autoclaving, 2 ml of 2.5 M $MgCl_2 \cdot 6H_2O$ was added. For protoplast preparation, 25 ml of 20% glycine was added. For plasmid isolation, the medium was supplemented with 25 μg/ml of thiostrepton or other appropriate antibiotics. For *S. venezuelae*, 10.3% sucrose was used to promote sedimentation of the mycelium when centrifuged.

J-medium for protoplast preparation and DNA isolation from strains growing poorly in YEME:

Tryptic soy broth		30 g
Yeast extract		10.0 g
Sucrose		103 g
$MgCl_2 \cdot 6H_2O$		10.12 g
Water	to	1000 ml
pH		7.0

For protoplast preparation, the medium was dispensed as 19.6 ml aliquots. Before use, 0.4 ml of 0.5 M $CaCl_2$ was added to each aliquot.

GNY for growing vegetative inoculum:

Glycerol	20 ml

Nutrient broth	8.0 g
Yeast extract	3.0 g
K_2HPO_4	5.0 g
Water to	1000 ml
pH to	7.0

Soft Nutrient Agar for protoplast transformation of *Streptomyces*:

it was used as an overlay containing antibiotic (s) for selection of transformants after 12-14 h incubation of protoplasts on regeneration medium.

Nutrient broth powder		8.0 g
Agar		3.0 g
Distilled water	to	1000 ml

R2YE (R5) for regeneration of *S. lividans* protoplasts:

Sucrose		103 g
K_2SO_4		0.25 g
$MgSO_4.7H_2O$		10.1 g
Glucose		10 g
Casamino acid		0.1 g
Yeast extract		5.0 g
TES buffer		5.73 g
Water	to	1000 ml
Agar		16 g

At the time of use, the following were added to each 1000-ml portion:

KH_2PO_4 (0.5%)	10.0 ml
$CaCl_2 \cdot 2H_2O$) (5 M)	4.0 ml
L-proline (20%)	15 ml
NaOH (1 M)	10.0 ml
1× trace element C	2 ml

The plates were kept at room temperature before use, and dried in a sterile air stream (laminar-flow hood) until about 20% weight was lost (ca 2 h).

R5N for regeneration of *S. venezuelae* protoplasts:

R2YE Medium was modified by replacing the 10.3% sucrose and 1.0% glucose with

0.3 M NaCl and 1.0% maltose.

TSBG Medium for vegetative inoculum cultures for Cm production

Trypticase Soy Broth		30 g
Glucose		10 g
Distilled water	to	1000 ml

GI Medium for Cm production:

Glucose		30 g (autoclaved separately)
$MgSO_4{\cdot}7H_2O$		0.2 g
KH_2PO_4		4.5 g
K2HPO4		10.5 g
L-isoleucine		7.5 g
Salt solution (1% NaCl, 1% $CaCl_2$)		9.0 ml
$FeSO_4{\cdot}7H_2O$ (0.2%)		4.5 ml
Trace element solution A		4.5 ml
Distilled water	to	1000 ml

IV. Stock Solutions and Buffers

X-gal: 20 mg/ml in dimethylformamide.

IPTG: 200 mg/ml in water and sterilized by membrane filtration.

Thiostrepton: 50 mg/ml in dimethyl sulfoxide (DMSO).

Other antibiotics were dissolved in water and sterilized by filtration.

TE Buffer for DNA maintenance: containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0) (Sambrook et al., 1989).

20 x SSC for DNA hybridization:

NaCl (175.3 g) and sodium acetate (88.2 g) were dissolved in 800 ml of distilled

water. Several drops of 10 N NaOH were added to adjust pH to 7.0. The volume was adjusted to 1000 ml with water.

Solution I for *E. coli* plasmid DNA isolation (Sambrook et al., 1989):

Glucose	50 mM
Tris-HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM

Solution II for *E. coli* plasmid DNA isolation (Sambrook et al., 1989):

NaOH	0.2 N
SDS	1%

Solution III for *E. coli* plasmid DNA isolation (Sambrook et al., 1989):

Potassium acetate (5 M)	60 ml
Glacial Acetic acid	11.5 ml
Distilled water	28.5 ml

TEPD buffer for enzyme assay (this study):

Tris-HCl (pH 8.0)	100 mM
EDTA (pH 8.0)	1 mM
Pyridoxal phosphate	0.1 mM
Dithiothreitol (DTT)	0.1 mM

PEPD buffer for enzyme assay: same as TEPD except that Tris buffer was replaced with 100 mM phosphate buffer (pH 7.6).

TM buffer for NTG mutagenesis:

Tris	0.05 M
Maleic acid	0.05 M
pH	8.0

Stop dye for sequencing reaction:

10 mM NaOH, 99% deionized formamide,
0.1% bromophenol blue, 0.1% xylene cyanol green.

Protoplasting buffer for rapid screening of plasmids in *E. coli*:

Tris-HCl (pH 8.0)	30 mM,
EDTA	50 mM
NaCl	50 mM,
Sucrose (w/v)	20%
Lysozyme	1 mg/ml,
RNase	50 μg/ml

Preloading buffer for rapid screening of plasmids in *E. coli*:

TBE	0.5 X
SDS	2% (w/v)
Sucrose	5% (w/v)
Bromophenol Blue	0.04% (w/v)
Xylene cyanol green	0.04% (w/v)

EZ lysis buffer for rapid preparation of plasmid DNA from *E. coli*

Tris-HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM
Sucrose	15% (w/v)
Lysozyme	2 mg/ml
RNase	0.2 mg/ml
BSA	0.1 mg/ml

The buffer was stored at -20 °C.

Lysozyme buffer for DNA isolation from streptomycetes:

Sucrose	0.3 M
Tris-HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	25 mM

Immediately before use, lysozyme was added to a final concentration of 2 mg/ml.

RNase solution for DNA purification.

RNase I "A" was dissolved at a concentration of 5 mg/ml in water or in a solution

containing 10 mM Tris-HCl and 15 mM NaCl. The solution was heated in a boiling water bath for 15 min and cooled slowly to room temperature. RNase solution was dispensed as 1-ml aliquots and stored at -20 °C.

L-buffer for protoplast preparation (Hopwood et al., 1985):

Sucrose (10.3%)**	100 ml
TES buffer (5.73% pH 7.2)	10 ml
K_2SO_4 (2.5%)	1 ml
Trace element solution C	0.2 ml
KH_2PO_4 (0.5%)	1 ml
$MgCl_2 \cdot 6H_2O$ (2.5 M)	0.1 ml
$CaCl_2$ (0.25 M)	1 ml

** Replace with 0.3 M NaCl for *S. venezuelae*

Lysozyme was added to a final concentration of 2 mg/ml and the L-buffer solution was sterilized by filtration.

P-buffer (Hopwood et al., 1985):

Sucrose	103 g (0.3 M NaCl for *S. venezuelae*)
K_2SO_4	0.25 g
$MgSO_4 \cdot 7H_2O$	2.02 g
Trace element solution C	2 ml
Distilled water to	800 ml

The solution was dispensed in 40-ml aliquots and autoclaved. Before use, the following sterile solutions were added:

KH_2PO_4 (0.5%)	0.5 ml
$CaCl_2$ (0.25 M)	5 ml
TES buffer (5.73%, pH 7.2)	5 ml

T-buffer for protoplast transformation (Hopwood et al., 1985):

Sucrose (10.3% w/v)	25 ml (0.3 M NaCl for *S. venezuelae*)
Trace element solution C	0.2 ml
K_2SO_4 (2.5% w/v)	1 ml
Distilled water	75 ml

Sterile solutions of each component were mixed and kept as a stock solution at room temperature. Just before use, 9.3 ml of the stock solution was supplemented with 0.2 ml of $CaCl_2$ (5 M) and 0.5 ml of Tris-maleic acid (1 M, pH 8.0). Three parts of supplemented solution were mixed with one part (by weight) of sterile PEG1000.

SM buffer for phage elution (Sambrook et al., 1989):

Tris-HCl (1 M, pH 7.5)		20 ml
$MgSO_4$ (1 M)		1 ml
NaCl (5 M)		20 ml
Gelatine		1 g (melted in 10 ml of water)
Water	to	1000 ml

Trace element solution A for general use in preparing media and buffers:

H_3BO_3		1.6 mg
$(NH_4)_6Mo_7O_{24}\cdot4H_2O$		10 mg
$CuSO_4\cdot5H_2O$		10 mg
$MnCl_2\cdot4H_2O$		10 mg
$ZnCl_2$		40 mg
$FeCl_3\cdot6H_2O$		200 mg
Water	to	1000 ml

Trace element solution C for sulfur-limited media and buffer:

$ZnCl_2$		40 mg
$FeCl_3\cdot6H_2O$		120 mg
$CuCl_2\cdot2H_2O$		10 mg
$(NH_4)_6Mo_7O_{24}\cdot4H_2O$		10 mg
$MnCl_2\cdot4H_2O$		10 mg
$Na_2B_4O_7\cdot10H_2O$		10 mg
Water	to	1000 ml

Gel-stock solution for polyacrylamide gel preparation:

Distilled water	134 ml
5 X TBE	50 ml
Liqui-gel solution	62.5 ml
Ultrapure urea	210 g
Water to	500 ml

The solution was filtered and degassed under vacuum for 30 min. LiQui-Gel (ICN, Cleveland, OH) solution is a proprietory stabilized 40% (w/v) aqueous solution of acrylamide and bis-acrylamide (19:1).

V. Maintenance of Stock Cultures

A. Bacterial stocks

a. *E. coli*

E. coli stock strains were prepared as overnight cultures on LB agar (with 100 mg/ml ampicillin for strains harboring plasmids). The cells were collected from the plates, suspended in 20% glycerol and stored at -20°C (short-term) or –70 °C for long-term storage.

b. *Micrococcus luteus*

The procedure was the same as for *E. coli* but GNY medium was used.

c. Streptomycetes

Streptomycetes were maintained temporarily as plate cultures. For *S. venezuelae* the medium was MYM agar (supplemented with antibiotics for strains with plasmids). For *S.*

lividans, K1 agar was used, except that TO or TOY agar were used for strains that sporulated poorly on MYM or K1 medium. For long-term maintenance, spores were harvested, washed with sterile water, resuspended in 20% (v/v) glycerol and stored at -70°C.

B. Phage stock

a. Lambda phage

Phage from single plaques were eluted into 1 ml SM buffer with a drop of chloroform added, and stored at 4 °C. For long term maintenance, 7% of DMSO was used instead of chloroform and storage was at -70°C.

For maintenance of genomic DNA libraries in phage vectors, 3×10^5 recombinant phage particles were used to infect *E. coli* strain LE392. Well-isolated plaques were formed in TB top agar by incubating at 37 °C for 10-12 h. The phage particles were eluted into SM buffer and the eluate was centrifuged at 3000 x g for 10 min to remove cell debris. The supernatant containing phage particles was transferred to a new glass tube and DMSO was added to a final concentration of 7% (v/v).

b. Helper phage VCSM13

A culture of *E. coli* TG1 was grown in TBG medium to an OD_{600} of 0.8. Phage VCSM13 was diluted 10-fold with TBG and a portion (0.1 ml) of the diluted phage was mixed with 0.1 ml of the freshly made TG1 culture and incubated at 37 °C for 20 min. The culture was mixed with 3 ml of soft agar which was melted and cooled to 42 °C. The soft agar was poured on top of LB agar to obtain well isolated plaques on a lawn of TG1. A single

plaque was picked and dispersed in 2 ml of TBG medium containing kanamycin (70 μg/ml) in a 10-ml culture tube. The culture was incubated at 37 °C for 12-16 h with shaking. A 1.5 ml sample of the culture was transferred to a sterile microcentrifuge tube and centrifuged at 12,000 ×g for 2 min. The supernatant was stored in a new microfuge tube at 4 °C. The bacteriophage stock was titred by plaque formation on *E. coli* TG1.

VI. Culture Conditions

A. *E. coli*

a. Cultures for single stranded DNA preparation

An *E. coli* DH5α colony containing a phagemid, and 5-10 μl of helper phage VSCM13 at 5×10^8 to 1×10^9 pfu/ml, were used to inoculate 2 ml of 2 × YT medium supplemented with 100 μg/ml ampicillin. The culture was incubated at 37 °C with shaking for 1.5 h. Kanamycin was added to a final concentration of 75 μg/ml and incubation was continued for 16-20 h. At this time, the culture was centrifuged and the supernatant was used for single-stranded DNA isolation.

b. Cultures for plasmid DNA isolation

A single colony was inoculated in 2 ml of LB medium containing 100 μg/ml of ampicillin in a 10-ml test tube and incubated at 37 °C overnight with constant shaking at 220 rpm. Cells were harvested by centrifugation. For large scale plasmid DNA isolation, *E. coli* strains were cultured in 50 ml LB medium supplemented with 100 μg/ml of ampicillin. Cells were harvested after incubation at 37 °C overnight with constant shaking at 220 rpm.

B. Streptomycetes

a. Cultures for genomic DNA isolation

For genomic DNA isolation, streptomycetes were grown in YEME with 10.3% (w/v) sucrose and 0.5% (w/v) of $MgCl_2 \cdot 6H_2O$ at 30 °C for 24 h with shaking at 220 rpm. For *S. lividans*, a large number of spores was used to obtain dispersed growth. For strains of *S. lividans* that sporulates poorly on agar plates, mycelium was used to inoculate 10 ml of J-medium in a 125-ml Erlenmeyer flask. After incubation at 30 °C for 48 h on a rotary shaker at 220 rpm, the mycelium pellets were collected and homogenized with a 30-ml Glas-Col homogenizer. The homogenate was used to inoculate 20 ml of YEME medium in a 250-ml Erlenmeyer flask. The mycelium was collected after overnight incubation at 30 °C.

b. Cultures for plasmid isolation

Culture conditions for routine plasmid isolation were the same as those for genomic DNA isolation except that the medium contained 10 μg/ml thiostrepton.

c. Cultures for chloramphenicol production

An *S. venezuelae* spore suspension (50 μl) was used to inoculate 10 ml of TSBG medium in a 125-ml Erlenmeyer flask. After the culture had been shaken at 30 °C for 24 h, a 1-ml portion was used to inoculate 25 ml of GI medium in a 250-ml Erlenmeyer flask. The GI culture was incubated at 30 °C for 4-7 days with shaking. At intervals, 5 ml samples were removed aseptically to assay Cm by HPLC.

d. Cultures for enzyme assays

An *S. venezuelae* spore suspension (100 μl) was used to inoculate 50 ml of MYM or MMY medium in a 500-ml Erlenmeyer flask. After the culture had been incubated at 30 °C for 24 h with shaking, the mycelium was harvested by centrifugation at 4 °C, and washed twice with TEPD or PEPD buffer.

VII. Isolation of *cys* Mutants from *Streptomyces*

Spores of *Streptomyces* were collected, washed with water and then resuspended in TM buffer (0.05 M Tris and 0.05 M maleic acid adjusted to pH 8.0 with NaOH) at a concentration of $\sim 10^7$ cfu/ml. N-Methyl-N'-nitro-N- nitrosoguanidine (NTG, MNNG) was added to the suspension to a final concentration of 2 mg/ml and the mixture was incubated at 30 °C for 2 h. The NTG-treated spores were washed three times with TM buffer and suitable dilutions were spread on plates containing MM agar supplemented with 100 μg/ml L-cysteine to obtain single colonies. After incubation at 30 °C for 3 days, the colonies were patched in duplicate, one set on MM agar and the other on MM agar supplemented with L-cysteine. The colonies that grew only on MM with L-cysteine were saved for further examination.

VIII. Assays and Analyses

A. Chloramphenicol assays

a. Bioassay of Cm production

To screen for Cm production mutants after transposition mutagenesis, spores were

collected with sharp-tip toothpicks from 30 colonies and used to point-inoculate MYM agar in 15-cm diameter Petri plates. After the colonies had been incubated at 30 °C for 12-14 h, the agar was carefully overlaid with 7 ml of soft GNY agar seeded with 1.0% (v/v) of a *Micrococcus luteus* culture grown overnight in TSBG medium. The overlaid plates were incubated overnight and examined for inhibition zones. Colonies of interest were re-examined by the same procedure, and finally assayed by HPLC.

b. Assay of Cm and related metabolites by HPLC

A 5-ml portion of Cm production culture was removed aseptically and filtered through Whatman No. 5 paper. The filtrate was extracted with 5 ml of ethyl acetate, and 4 ml of the clarified ethyl acetate was transferred to a vial. The solvent was evaporated under an air jet, and the residue was redissolved in 0.5 ml of 25% methanol for HPLC analysis. Samples (20 μl) were injected on to a 4.6 × 45-mm reverse phase C-18 column, and a programmed methanol-water gradient was pumped through the column. Elution of Cm was detected by measuring absorbance at 273 nm. The concentration of methanol in the gradient was increased from 0% to 60 % in 5 min, then to 100% in 3 min, and kept at 100% for an additional 3 min. The gradient was programmed by a System Gold solvent module 126. Absorbance was monitored with a System Gold detector (module 166, Beckman, Palo Alto, Ca).

B. Chemical Syntheses

a. 1,2-Diamino-4,5-dimethoxybenzene (DDB)

4,5-Dinitroveratrol was synthesized from veratrole by the method described by Ehrlich and Bogert (1947). To 27.8 ml of conc. nitric acid, 10 g (9.23 ml) of veratrole was added dropwise at 0 °C during at least 80 min. At the end of this step, the solution became very thick and mechanical stirring was needed. After stirring for another 5 min, 15 ml of conc. sulfuric acid was added dropwise within 1 h at 3-5 °C. The mixture was stirred for another 15 min at the same temperature and then at room temperature for 30 min. The mixture was gradually warmed up to 54-56 °C in 5 min and kept for 10 min. The temperature was then raised to 58-60 °C over 5 min, and held there for 10 min. The mixture was allowed to cool to room temperature, and then poured slowly into 100 ml of ice-cold water; more water was then added to a final volume of 350 ml. After 30 min occasional stirring, the yellow sandy crystals were recovered by filtration and washed with water until free of acid. The crystals were dried overnight and re-crystallized from ethanol to give 13.7 g of fine yellow needles. They were identified as 4,5-dinitroveratrole by nuclear magnetic resonance (NMR) spectroscopy (see Appendix V, Fig. AppdxV-1).

4,5-dinitroveratrole was reduced to 1,2-diamino-4,5-dimethoxybenzene (DDB) by the method described by Ohmori and colleagues (1991). Sodium hydrosulphite ($Na_2S_2O_4$; 80 g) and sodium hydroxide pellets (10 g) were dissolved in 500 ml of water in a three-necked flask with a reflux condenser, a dropping funnel and a nitrogen gas inlet. Dinitroveratrole (11.4 g) dissolved in a mixture of ethanol (300 ml) and dioxane (100 ml) was added dropwise over 40 min to the well stirred reaction mixture sparged with nitrogen

and kept at 70 °C. After the reaction had proceeded for 70 min, the mixture was cooled to room temperature and filtered. The filtrate was concentrated to about 100 ml under reduced pressure and extracted three times with 100 ml portions of chloroform. The chloroform extracts were combined, back-extracted twice with 100 ml of 3 M HCl, and evaporated to dryness under reduced pressure. The residue (2 g) was crystallized from ethanol and confirmed to be 1,2-diamino-4,5-dimethoxybenzene by NMR spectroscopy (see Appendix V, Fig. AppdxV-2).

b. O-Acetyl-DL-homoserine

The procedure for synthesis of O-acetyl-DL-homoserine was modified from that described by Nagai and Flavin (1967). Homoserine was acetylated with acetic acid in a strong acid (perchloric acid) that prevented the formation of N-acetyl-DL-homoserine. The main side-product of the reaction was homoserine lactone, which was easily separated from desired product.

To a 250-ml round-bottom flask, 10 ml of glacial acetic acid and 4.05 ml of acetic anhydride were added. These reagents were combined under stirring with 715 mg of DL-homoserine dissolved in 5 ml of glacial acetic acid and added dropwise over 5 min. The flask was then stoppered and the reaction was allowed to continue for 90 min at ambient temperature before 0.11 ml of water was added to decompose the excess acetic anhydride. The perchloric acid was neutralized by adding 0.4 ml of diacetylamine. The pH was monitored with pH paper, and enough extra diacetylamine was added to adjust the pH to 3-5. Mixing 150 ml of diethyl ether with the reaction solution caused a precipitate to appear, and

the mixture was then kept on ice. Two hours later, 75 ml of ether was added and the mixture was kept at 0 °C overnight. Finally it was chilled to -20 °C for 1 h and filtered. The precipitate recovered by filtration was air-dried, redissolved in 5 ml of water and freed from insoluble materials by filtration. After dilution with 10 volumes of ethanol, and cooling at 0 °C overnight the solution deposited white crystals of O-acetyl-DL-homoserine (701 mg). These were recovered by filtration, washed with ether and air-dried. Their identity was confirmed by NMR (Appendix V, Fig. AppdxV-3).

O-Acetyl-L-homoserine was prepared by the foregoing procedure except that L-homoserine was used as the starting material.

C. Analyses

a. Specific measurement of cysteine

The specific colorimetric method for cysteine described by Gaitonde (1967) was modified as follows:

1. Preparation of the acid ninhydrin reagent

Ninhydrin (2.5 g) was dissolved in a mixture of 60 ml of acetic acid and 40 ml of conc. HCl (w/v). At 4 °C, the reagent is stable for two weeks.

2. Procedure

To a 10-ml test tube with 0.5 ml of the cysteine-containing solution, 0.5 ml of glacial acetic acid and 0.5 ml of the ninhydrin reagent were added and mixed in thoroughly. The mixture was heated in boiling water for exactly 90 s and cooled on ice for 2 min. The

contents of each tube were mixed with 2 ml of 95% ethanol, and the absorbance was measured at 560 nm. The concentration of cysteine in the sample was calculated from a calibration curve prepared with known concentrations of pure cysteine (Fig. 9). For samples containing cell extract, the heating time was crucial. The colour contributed by free cysteine was fully developed in a reaction time of 90 s. Heating for a longer time resulted in the formation of cysteine by decomposition of proteins present in the reaction mixture. When the reaction time was limited to 90 s, proteins in the sample did not affect the result. The assay results were not affected by the presence of O-acetyl-L-serine, homocysteine, cystathionine or methionine.

b. Specific assays for cystathionine β-lyase and cystathionine γ-lyase activity.

Cystathionine is cleaved by cystathionine β- and γ-lyases to form pyruvic acid and α-ketobutyric acid, respectively. By reaction with 1,2-diamino-4,5-dimethoxybenzene (DDB), pyruvic acid is converted to 3-methyl-2-hydroxy-6,7-dimethoxyquinoxaline (MHDQ), and α-ketobutyric acid can be converted to 3-ethyl-2-hydroxy-6,7-dimethoxyquinoxaline (EHDQ). MHDQ and EMDQ can be separated on a reverse-phase column by HPLC, and their elution detected by monitoring the absorbance of the column effluent at 360 nm (Ohmori et al., 1992).

1. Assay procedure

The sample for assay, as a solution (0.5 ml) in 1 M HCl was mixed with 0.5 ml of 0.1 M DDB reagent in 1 M HCl. The reaction mixture was incubated at 40 °C for 30 min, and

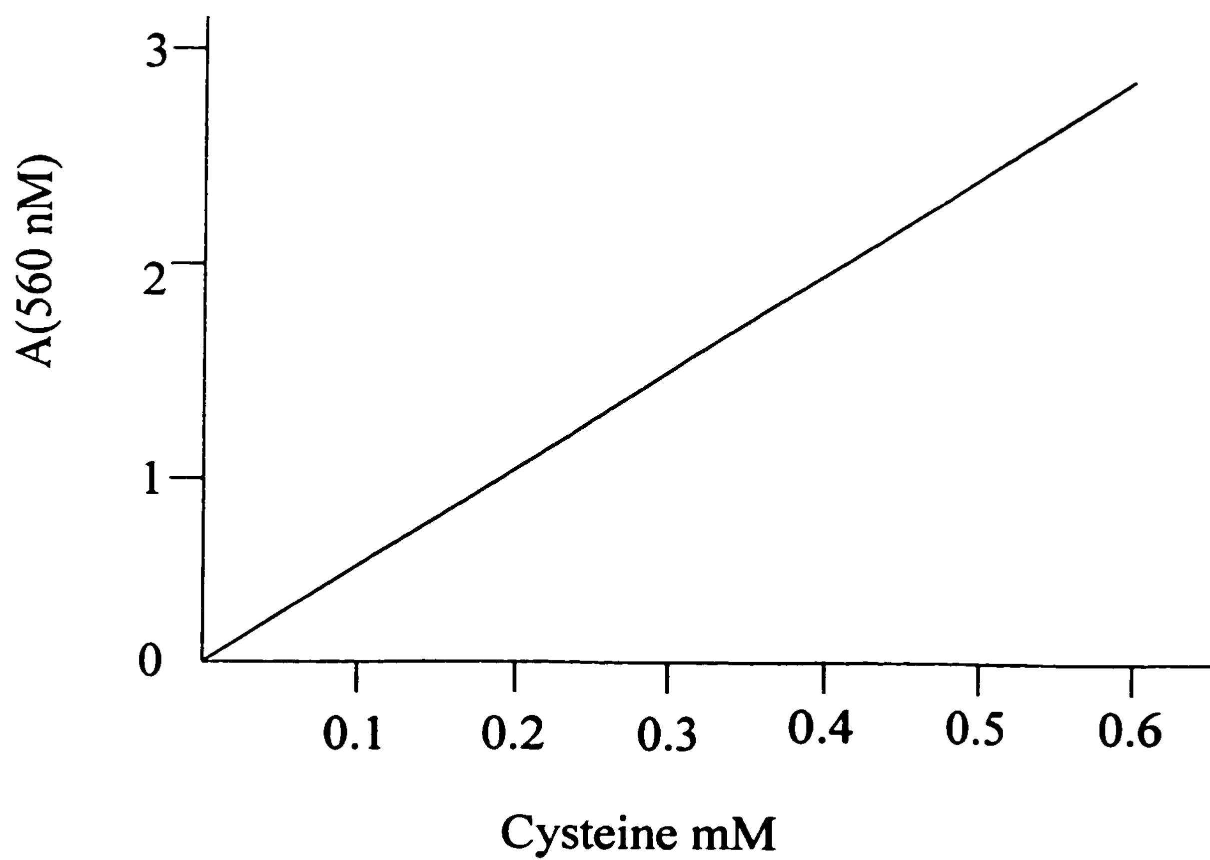


Fig. 9. Calibration curve for specific cysteine assay

was then extracted with 3 ml of ethyl acetate. The two layers were separated by centrifugation and 2 ml of the top layer was transferred to a glass vial. The solvent was evaporated with an air jet, and the residue was redissolved in 100 μl of the HPLC mobile phase A. A 20 μl portion of the solution was injected on to the HPLC column.

2. Measurement of α-ketobutyric acid and pyruvic acid by HPLC

EHDQ and MHDQ were analyzed by HPLC with a 4.6 × 45-mm reverse phase C-18 silica column, using a programmed gradient of mobile phases A and B. Phase A was a 90:10 (v/v) mixture of 10 mM KH_2PO_4 (adjusted with phosphoric acid to pH 2.1) and acetonitrile. Phase B contained the same solvents as phase A but had a higher proportion of acetonitrile (30%, v/v). Elution of EHDQ and MHDQ was detected by measuring absorbance at 360 nm. The proportion of phase A in the gradient was decreased from 100 % to 0 % over 6 min, held at 0% for 2 min, returned from 0% to 100% over 1 min, and then kept at 100% for 1 min. The flow rate was maintained throughout at 1 ml/min. The gradient was programmed by a System Gold solvent module 126. Absorbance was monitored with a System Gold detector module 166 (Beckman, Palo Alto, Ca). Solutions of α-ketobutyric acid and pyruvic acid of known concentrations were analyzed by the same procedure described above and used as standards to calibrate samples.

c. Measurement of amino acids by HPLC

Each sample (neutral pH, 40 μl) was mixed with 40 μl of *o*-phthaldialdehyde reagent (OPA reagent, Pierce Chemical Co., Rockford, IL). To the reaction mixture, after 1 min, 120

μl of 0.1 M sodium acetate (pH 6.2) was added. Samples (200 μl) were injected on to a 4.6 × 45-mm Beckman ultrasphere ODS column, and a programmed methanol-solvent A gradient was pumped through the column. The derivatives of amino acids eluted were detected with a fluorescence detector (Beckman) (excitation 305-395 nm, emission 420-650 nm). Solvent A contained 100 ml of a 95:5 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 increased 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 and increased to 100% in 0.5 min.

d. Measurement of protein in cell extracts

The BCA (bicinchoninic acid) protein assay system (Pierce Chemical Co) was used to measure total protein in cell extracts and in enzyme assay mixtures. Reagent A (50 volumes) was mixed with 1 volume of Reagent B. Each 100 μl of sample was added to 2 ml of the mixed reagent and vortexed briefly. The reaction mixture was incubated at 37 °C for 30 min. The absorption was read at 562 nm.

The protein concentration in a test sample was estimated from a calibration curve.

D. Enzyme assays

a. Preparation of cell extracts

An *S. venezuelae* spore suspension (100 μl) was used to inoculate 20 ml of MYM medium in a 250-ml Erlenmeyer flask. The culture was grown at 27 °C overnight, then used

(1 ml) to inoculate 50 ml of MYM or MMY medium in a 500-ml Erlenmeyer flask. Each *cys* mutant was grown in MMY medium alone and supplemented individually with methionine, cysteine and cystathionine at a concentration of 50 μg/ml. Cultures were incubated at 27 °C for 24 h on a rotary shaker at 220 rpm. The mycelium from each culture was harvested by centrifugation (10,000 × g) at 4 °C for 10 min and washed with TEPD buffer. For homocysteine synthase assays, PEPD buffer was used. The harvested mycelium was disrupted by ultrasonic vibration with a Branson Sonifier (Model 210) for 6 × 15 s at 4 °C. The homogenized solution was centrifuged (12,000 × g) for 20 min at 4 °C. The supernatant was transferred to a new tube and used as cell extract. For the enzyme assays, where both MMY and MYM media were used, cell extracts of all strains were adjusted with TEPD or PEPD buffer to the same total protein level for comparison.

b. Assay of cysteine synthase activity

The term cysteine synthase is used here to mean the enzyme activity that catalyzes the formation of L-cysteine from O-acetyl-L-serine and Na_2S. The cysteine formed in the reaction was selectively measured by the specific assay procedure for cysteine. Cell extract (250 μl) prepared in TEPD buffer was used in a 500 μl reaction mixture, which contained 30 mM O-acetyl-L-serine and 20 mM Na_2S prepared in TEPD buffer. The mixture was incubated at 30 °C for 30 min before 50 μl of 50% (w/v) trichloroacetic acid was added to terminate the reaction. The precipitate was removed by centrifugation and the supernatant was used for L-cysteine determination by the specific colorimetric method described above.

Sulfocysteine synthase was assayed by a similar procedure except that sodium sulfide

was replaced with sodium thiosulfate.

c. Assay of cystathionine β- and γ-synthases

Cystathionine can be formed from L-homocysteine and L-serine by cystathionine β-synthase, or from O-acyl-L-homoserine (succinyl- or acetyl-) and L-cysteine by cystathionine γ-synthase. Cystathionine formed was measured by HPLC. The reaction mixture (500 µl) contained 250 µl of cell extract in PEPD buffer. Other reagents were also dissolved in PEPD buffer. For both cystathionine β-synthase and cystathionine γ-synthase assays, the final concentration of each substrate was 20 mM. The reaction was terminated after 30 min at 30 °C by freezing (–20 °C), and the cystathionine formed was measured by HPLC.

d. Assay of cystathionine γ- and β-lyases

Cystathionine was cleaved into α-ketobutyric acid, cysteine and ammonia by cystathionine γ-lyase, or to pyruvic acid, homocysteine and ammonia by cystathionine β-lyase. The cysteine formed by cystathionine γ-lyase could be measured by the specific colorimetric method for cysteine described above. The α-ketobutyric acid and pyruvic acid formed by the enzymes was determined by HPLC.

The reaction mixture (500 µl) for the lyase assay consisted of 250 µl of cell extract, 200 µl of cystathionine (10 mM in 0.01 M HCl) and 50 µl of TEPD buffer. The reaction was carried out at 30 °C for 30 min and was terminated by adding 50 µl of 50% (w/v) trichloroacetic acid if assayed by the colorimetric method, or by adding 500 µl of 2 M HCl

if assayed by HPLC. The supernatant obtained after centrifugation was used to measure L-cysteine or α-keto acids.

e. Assay of homocysteine synthase

Homocysteine synthase catalyzes the direct sulfhydrylation of O-acetylhomoserine or O-succinylhomoserine to form homocysteine. The resulting homocysteine reacts with the aryl disulfide [5,5'-dithiobis-(2-nitrobenzoic acid), DTNB] to form a yellow compound that can be measured photometrically at 412 nm (Flavin & Slaughter, 1971). Na_2S and H_2S can react with DTNB and must be removed by degassing (under vacuum or by bubbling with nitrogen) at acidic pH. Because the yellow substance is formed only when the pH is above 5.5, excess acid in enzyme reaction mixtures must then be neutralized. It is also necessary to exclude dithiothreitol and mercaptoethanol from buffers. In addition, the procedure cannot distinguish between L-homocysteine and L-cysteine.

The enzyme reaction mixture in PEPD buffer (250 μl) consisted of 20 mM O-acetyl-L-homoserine (or O-succinyl-L-homoserine) and 20 mM Na_2S. To this 250 μl of cell extract was added and the reaction was allowed to proceed at 30 °C for 30 min before termination by mixing with 0.5 ml of 1 M HCl. After centrifugation at 12,500 rpm for 5 min, the supernatant was transferred to a glass vial, degassed under vacuum for 20 min and bubbled with nitrogen for 2 min to remove the H_2S formed. The pH was adjusted to 7 by adding 1 M Tris-HCl (pH 8.0). To 1 ml of the treated mixture, 0.1 ml of DTNB solution (4 mM in 0.1 M potassium phosphate buffer, pH 7.0) was added. The yellow compound formed was measured photometrically at 412 nm after 4 min. The amount of homocysteine

formed in the reaction was calculated from a calibration curve prepared with measured amounts of homocysteine.

To confirm the results of this enzyme assay, the homocysteine formed was also measured by HPLC.

IX. DNA Manipulation

A. DNA Preparation

a. Plasmid DNA isolation

1. From *E. coli*

The alkali lysis procedure (Sambrook et al., 1989) was routinely used for plasmid DNA isolation from *E. coli*. The cells from 1.5 ml of a culture were pelleted in a microcentrifuge tube at 12,000 × g and resuspended in 100 μl of ice-cold Solution I by vortexing. When the suspension had been cooled on ice for 20 min, 200 μl of Solution II was added to lyse the cells. After mixing and further cooling, 150 μl of Solution III were added and the contents of the tube, again mixed and cooled well, were subjected to centrifugation at 12,000 × g for 5 min. The supernatant, after transfer to a new tube, was extracted with an equal volume of phenol/chloroform. To the aqueous layer, 1/10 volume of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol were added and mixed in well. The mixture was centrifuged and the pellet was washed with 70% ethanol, dried under vacuum, dissolved in 50 μl of TE buffer and kept at -20 °C. To remove RNA, RNase was added to a final concentration of 10 μg/ml. After incubation at room temperature for 1 h, RNase was removed by extraction with phenol/chloroform and then with chloroform. The

DNA was recovered by ethanol precipitation. For large scale plasmid DNA isolation, 50 ml of culture was used and the procedure was scaled up proportionately.

2. Isolation of plasmid DNA from streptomycetes

For routine plasmid isolation from streptomycetes, the alkaline lysis method just described for *E. coli* plasmids was modified by replacing Solution I with P-buffer containing 2 mg/ml of lysozyme (Solution I*), and incubating the cells with it at 37 °C for 30 min instead of cooling on ice. Other steps were the same as in the *E. coli* method.

For large scale isolation of plasmid DNA from streptomycetes, the mycelium from 500 ml of culture was resuspended in 50 ml of lysozyme solution and incubated at 37 °C for 30-40 min. To the cell suspension 25 ml of SDS/NaOH (2% SDS, 0.3 M NaOH) was added and mixed in thoroughly. The mixture was heated at 70 °C for 15 min and allowed to cool at room temperature for 30 min. The lysate was extracted with 8 ml of acidic phenol/chloroform. The aqueous phase was transferred to a centrifuge tube and mixed with 0.1 volume of 3 M sodium acetate (pH 5.2) and 1 volume of isopropanol to precipitate DNA. Plasmid DNA was pelleted by centrifugation, washed with 70% ethanol, dissolved in 1 ml of TE buffer, and examined by agarose gel electrophoresis.

b. Isolation of genomic DNA from streptomycetes

Genomic DNA of streptomycetes was routinely isolated by the method described by Hopwood et al. (1985). Mycelium from a 10 ml culture was collected by centrifugation and washed twice with 10.3% sucrose and then resuspended in 1 ml of Lysozyme buffer. The

mycelium suspension was incubated at 37 °C for 30 min with periodic swirling. EDTA (0.5 M, 0.24 ml) and 26 µl of 2 mg/ml proteinase K were added and mixed in gently by swirling. After 0.14 ml of 10% (w/v) SDS had been added, the contents were incubated at 37 °C for 2 h. The mixture was then extracted with 1.2 ml of neutral phenol/chloroform by shaking thoroughly several times for 5 min. The emulsion was centrifuged at 10,000 × g for 10 min. The clear upper aqueous layer was carefully transferred with a wide-mouth pipette to a new centrifuge tube and re-extracted with 1.2 ml of neutral phenol/chloroform. The aqueous phase was transferred to a sterile beaker and mixed with 0.1 volume of 3 M sodium acetate. Two volumes of ethanol were layered on top of the aqueous solution and the DNA was spooled on a glass hook by gently stirring around the surface between the two layers. The DNA spooled on the glass hook was washed in 70% ethanol for 5 min, air dried for 10 min and then dissolved in 1 ml of TE buffer. Higher quality genomic DNA was obtained if 1 volume of ethanol was used in the ethanol precipitation step.

This procedure was scaled up for large-scale genomic DNA isolation.

c. Isolation of single-stranded DNA templates for sequencing

To 1.2 ml of the supernatant from an *E. coli* culture grown in 2X YT medium for single-stranded DNA isolation, 200 µl of 20% (w/v) PEG8000 in 2.5 M NaCl was added. The mixture was inverted several times and put on ice for 30 min. The phage particles were pelleted by centrifugation at 12,000 × g for 5 min at 4 °C. The supernatant was decanted. After another brief centrifugation, the residual supernatant was withdrawn with a pipette. The pellet was dissolved in 100 µl of TE buffer, extracted twice with phenol/chloroform and

once with chloroform. Single-stranded DNA was precipitated from the aqueous solution with ethanol and resuspended in 20 µl TE.

d. Isolation of lambda phage DNA

A phage suspension of known titre was incubated with 100 µl of *E. coli* strain LE392 at 37 °C for 30 min; then TB top agarose (3 ml) supplemented with 10 mM $MgCl_2$ and 0.2% maltose was added. The mixture was used to overlay LB agar in a 9-cm plate. After the top agarose had hardened, the plate was inverted and incubated overnight at 37 °C.

Phage lysate was recovered from the agar surface, and mixed with 8 ml of λ-diluent (SM without gelatine). The plate was kept at 4 °C overnight, then shaken for 1 h and the phage eluate was collected in a 12 ml centrifuge tube; the plate was washed again with 4 ml of λ-diluent, and the phage eluate was centrifuged at 4000 × g for 10 min. The supernatant was transferred to a new centrifuge tube and RNase and DNase were added to a final concentration of 10 µg/ml. After incubation at 37 °C for 30 min, equal volumes of 20% PEG8000 and 2 M NaCl in λ-diluent were added, and the mixture was kept on ice for 1 h. Phages were pelleted by centrifugation at 10,000 × g for 15 min and the supernatant solution was drained off by inverting the tube on a paper towel. The pellet was dissolved in 1.2 ml of TE buffer to which SDS and proteinase K had been added to a final concentration of 0.5% and 5 µg/ml respectively. The mixture was incubated at 68 °C for 1 h and extracted with phenol/chloroform and then with chloroform. DNA was precipitated from the aqueous phase by adding 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol. The DNA pellet was washed with 70% ethanol, dried and dissolved in 100 µl of TE.

For large scale lambda DNA isolation, a modification of the procedure described by Sambrook et al. (1989) was used. Phage stock was mixed with *E. coli* LE392 grown at 37 °C overnight in 50 ml of TB medium supplemented with 0.5 ml of 1 M $MgSO_4$ and 0.5 ml of 20% maltose. The mixture was used to inoculate 100 ml of LB medium containing 10 mM $MgSO_4$, and the culture was incubated at 37 °C until complete lysis had occurred. To the phage lysate, NaCl was added and dissolved by swirling to give a final concentration of 1 M. After 1 h on ice the mixture was centrifuged (10,000 × g for 10 min) to remove cell debris. To the supernatant, PEG8000 was added to a final concentration of 10 % and dissolved by slowly stirring. The mixture was kept on ice for 2 h and centrifuged at 12,000 × g to pellet the particles. The pellet was resuspended in 5 ml of SM buffer containing DNase and RNase at concentrations of 10 µg/ml. The suspension was incubated at room temperature for 30 min and then extracted with 5 ml of chloroform. To the aqueous layer, EDTA, SDS and proteinase K were added to final concentrations of 20 mM, 5 % (w/v) and 50 µg/ml, respectively. The mixture was incubated at 56 °C for 1 h and then extracted once with 5 ml of neutral phenol/chloroform and once with chloroform. After 0.1 volume of 3 M sodium acetate had been mixed with the aqueous solution, 2 volumes of ethanol were added to precipitate DNA. Lambda DNA was pelleted by centrifugation and dissolved in TE buffer.

B. Rapid screening of plasmids in *E. coli*

The method developed by Sekar (1987) was used. Cells were suspended in 10 µl of protoplasting buffer in a microtitre plate. The plate was kept at room temperature for 15-30 min to lyse the cells. The wells were loaded with 3 µl of preloading buffer and the cell

lysates were added. Samples were electrophoresed in 0.5x TBE buffer with 0.05% of SDS, first at 45 v for 15 min and then at 100 v for 45-60 min. The gel was stained in 0.1% ethidium bromide and the plasmids were visualized under UV light.

C. Rapid preparation of *E. coli* plasmid DNA for enzyme digestion

A single colony was used to inoculate 2 ml LB medium with 100 µg/ml ampicillin and the culture was incubated overnight at 37 °C. A sample of the culture (0.5 ml) was transferred to a 1.5-ml microfuge tube. After centrifugation for 20 s at high speed, supernatant was decanted and drained off. The cell pellet was resuspended in 100 µl of EZ buffer. After 2-3 min at room temperature, the cell suspension was put in a boiling water bath for 60 s and then on ice for 2-3 min. The lysate was centrifuged at high speed for 15 min at room temperature and the supernatant was transferred to a fresh tube. The supernatant (10-20 µl) was used for restriction enzyme digestion in a total volume of 40 µl.

D. DNA elution from agarose gels

DNA fragments were extracted from agarose gels using the QIAEX II Gel Extraction Kit following the protocol described by Qiagen.

E. Restriction enzyme digestion and ligation of DNA fragments

DNA (0.1-1 µg) in 1-2 µl was mixed with water, 10 x buffer and 0.5 µl restriction enzyme to a final volume of 20 µl. The concentration of the buffer and the temperature used were as recommended by the supplier. Digestion was normally completed in 2 h and

was terminated by adding 4 μl of stop buffer before examination by agarose gel electrophoresis. For ligation, the restriction enzyme was destroyed by heating or removed by phenol extraction and ethanol precipitation. Linearized vector was normally dephosphorylated by thermosensitive alkaline phosphatase (TsAP) (BRL) before ligation. The dephosphorylation conditions were as recommended by the supplier. T4 DNA ligase (BRL) was routinely used for DNA ligation. The molecular ratio of insert and vector was adjusted to 2:1 and the ligation conditions were as recommended by BRL.

F. Electrophoresis of DNA

a. Agarose gel electrophoresis

Agarose gels (1%) in 1x TAE (40 mM Tris-acetate, 1 mM EDTA) buffer were routinely used for electrophoresis of DNA. Electrophoresis was normally carried out at room temperature at 50-100 v. For elution of DNA from an agarose gel, 0.6% agarose was used. After electrophoresis the gel was immersed in 1 μg/ml ethidium bromide for 30 min, viewed under UV light and recorded with the Gel Doc 1000 (Bio-Rad).

b. Polyacrylamide gel electrophoresis for DNA sequencing

To a 50-ml beaker, 10 ml of gel-stock solution, 50 μl of freshly made 25% (w/v) ammonium persulphate, and 10 μl of TEMED were added. The solution was mixed gently by swirling and poured across the entire length of a sealing strip and filter paper. The bottom edge of the pre-assembled 21 x 40 cm integral plate/chamber (IPC) unit of the electrophoresis apparatus (SEQUI-GEN®, Bio-Rad) was pressed against the paper until the

bottom of the IPC was sealed by solidified gel. In another beaker, 40 ml of gel-stock solution was gently mixed with 50 µl of the ammonium persulphate and 50 µl of TEMED. The mixture was slowly poured into the IPC chamber from a corner. The flat side of a sharkstooth comb was inserted into the 0.5 cm space between the plates. The plates were clamped together and wrapped with Saran Wrap until the gel hardened (2-4 h).

The IPC unit was placed in the lower buffer chamber with 400 ml of 0.5 x TBE buffer (45 mM Tris-borate, 1 mM EDTA), and the upper buffer chamber was also filled with 0.5x TBE buffer. The comb was carefully removed and the top surface of the gel was rinsed with 0.5x TBE buffer to remove unpolymerized gel-stock solution. The gel was warmed to 55 °C by supplying power (Model 3000 xi, Bio-Rad) at 1900 volts. The power was then turned off and the gel surface was rinsed again with the buffer. The comb was inserted with the teeth just touching the gel surface. The freshly denatured sequencing reaction solutions were loaded into the wells formed between the teeth and the surface of the gel. During electrophoresis for 2-4 h the temperature was monitored and controlled between 50-55 °C by adjusting the voltage.

The glass plates were separated and the gel on one of the plates was submerged in fixing solution (10% acetic acid, 10% methanol) for 15 min. The gel was then transferred to a sheet of Whatman 3MM paper and dried at 80 °C under vacuum in a gel drier (model 538, Bio-Rad). The gel was exposed in an intensifying cassette to a 18 × 43 cm BioMax MR film (EASTMAN KODAK Company, Rochester, NY) for 12-72 h and DNA sequences were read from the radioactive bands on the film. The gel also was sometimes scanned with GS-250 Molecular Imager (Bio-Rad) at a resolution of 100 µm.

G. Amplification and cloning of chromosome DNA fragments by PCR

a. PCR conditions

The reaction mixture (total volume 100 µl) consisted of:

dNTPs	26 µl	4 × 1.25 mmols
Primer ch3	1 µl	268 pmol
Primer ch4	1µl	182 pmol
Chromosomal DNA		100 ng
10 x buffer		10 µl
Water	to	100 µl

The mixture was divided into 20-µl aliquots in thin-walled 0.5-ml microcentrifuge tubes; to each tube a small drop (20 µl) of mineral oil was then added. The tubes were centrifuged briefly and kept at -20 °C for up to 24 h. Just before use, each aliquot was supplemented with 1U of *Taq* DNA polymerase, mixed by tapping, and placed in a thermal cycler (model PTC-100, MJ Research Inc., Watertown, Mass). When the temperature had increased to 80 °C, temperature cycling was begun. For the first 7 cycles the thermal cycler was programmed to give 96 °C for 1 min, 67 °C for 1 min and 72 °C for 1 min. For the next 30 cycles the denaturing and annealing times were reduced from 1 min to 45 s (The extension time was 1 min, the same as in the first 7 cycles).

b. Cloning the PCR products

PCR fragments were cloned with the SureClone™ Kit (Pharmacia Biotech), using the prescribed protocol without alteration. The amplified *S. venezuelae* DNA fragment was

inserted into the *Sma*I site of pUC18 to give plasmid pJV205. A 0.55-kb *Eco*RI-*Hin*dIII fragment of DNA from pJV205 was subcloned in the vectors M13mp18 and M13mp19 for sequencing.

H. Transformation

a. *E. coli*

1. Preparation of competent cells

Competent cells of *E. coli* were prepared as described by Hopwood et al. (1985). An overnight culture (0.1 ml) from a single colony of *E. coli* was used to inoculate 10 ml of LB medium containing 20 mM $MgCl_2$. After shaking at 37 °C for 2.5-3.0 h (OD_{600} = 0.5-0.8), the culture was chilled on ice for 15 min and centrifuged at 4 °C. The cells were resuspended in 10 ml of pre-chilled 0.1 M $CaCl_2$ and kept on ice for 20 min. The cells were pelleted at 4 °C and resuspended in 1 ml of 0.1M $CaCl_2$. The cell suspension was dispensed as 0.1 ml aliquots in pre-chilled microfuge tubes, incubated on ice for 4 h and then used for transformation. For long-term storage, an equal volume of 50% ice-cold glycerol was mixed with individual aliquots of the competent cells and kept at –70 °C.

2. Transformation procedure

Ice-cold competent cells (100 µl) were mixed with 0.1-0.2 µg of supercoiled plasmid DNA in 1-10 µl TE buffer. For ligated open circular plasmid DNA, more DNA (1-2 µg) was needed. The mixture was left on ice for 40 min and then heat shocked at 42 °C for 90 s. The heat shocked cells were cooled on ice for 2-3 min, mixed with 0.5 ml of 2 ×YT medium and

then incubated at 37 °C for 90 min. Up to 500 µl of the cell suspension was plated on LB agar supplemented with 100 µg/ml ampicillin or other relevant antibiotics. The plate was incubated inverted overnight and the colonies of transformants were screened.

3. Identification of recombinant plasmids

The recombinants were identified by α-complementation. X-gal (40 µl of 20 mg/ml) was mixed with 4 µl of IPTG (200 mg/ml) and spread on the surface of a LB agar supplemented with 100 µg/ml of ampicillin. The transformants were spread on the plate and incubated at 37 °C overnight. Those with recombinant plasmid were identified as white colonies.

b. Streptomycetes

1. Protoplast preparation

Mycelium from a 25-ml culture was washed twice with a 10.3% (w/v) sucrose solution. The washed mycelium was resuspended in 5 ml of filter-sterilized L-buffer containing 2 mg/ml lysozyme. The protoplasting mixture was incubated at 37 °C for 60-90 min with gentle shaking every 15 min and examined under a microscope until 90% of the mycelium were protoplasts. For *S. venezuelae*, a higher concentration (5 mg/ml) of lysozyme was required to achieve this yield of protoplasts. The protoplast suspension was mixed with 5 ml of P-buffer by pipetting, and filtered through sterile cotton in a 10-ml syringe to remove unprotoplasted mycelia. The protoplasts were pelleted by centrifugation at 1000 g for 10 min, washed twice with P-buffer, resuspended in 0.5 ml of P-buffer and divided into 100 µl

aliquots for transformation.

2. Transformation and regeneration of protoplasts

Up to 10 µl of DNA in TE buffer containing 1-2 µg DNA was mixed with 100 µl of freshly made protoplasts by tapping the tube several times. Immediately, 100 µl of T-buffer with PEG was added to the protoplast suspension. After 2 min at room temperature, the sample was mixed with 1.5 ml of P-buffer and centrifuged at 1000 g for 10 min. The supernatant was discarded and the protoplasts were resuspended in 1 ml of P-buffer. The protoplasts were plated on regeneration medium. After incubation at 30 °C for 14-18 h, the plate was overlaid with 2.5 ml of soft nutrient agar containing thiostrepton to give a final plate concentration of 10 µg/ml. Incubation was continued for 4-5 days until colonies appeared.

I. Construction of a genomic DNA library from *S. venezuelae*

a. Partial digestion of chromosomal DNA with *Sau*3A1

Genomic DNA (50-100 µg) was digested at 37 °C with 3U of *Sau*3A I in a 100-µl reaction mixture. Portions (10 µl) were removed every 5 min and mixed with 2 µl of loading buffer. The extent of the digestion was examined by agarose gel electrophoresis to determine the reaction time that gave the maximum yield of products of the required sizes. For construction of a library in lambda vector (GEM-11), the required size of digestion products was 9-23 kb, whereas for the plasmid vector (pSK+), 5-15 kb fragments were suitable. To isolate suitable fragments the sample was digested for the optimal time and terminated by

adding EDTA to a final concentration of 20 mM.

b. Fractionation of DNA fragments by sucrose gradient centrifugation

Sucrose solutions (10% and 40%) for gradient centrifugation were prepared in a buffer containing 1 M NaCl, 20 mM Tris-HCl (pH 8.0) and 5 mM EDTA (pH 8.0). To prepare the gradient, 5 ml of 10% sucrose solution was carefully layered on top of 5 ml of 40% sucrose solution in a 14 × 89 mm polyallomer centrifuge tube (Beckman) and capped. The tube was slowly rotated to a horizontal position in a Beckman SW41 rotor at 26,000 rpm for 3-4 h, then slowly rotated back to stop. Up to 250 µl of *Sau*3A1 digested DNA (up to 200 µg) was loaded on top of the sucrose gradient and centrifuged at 26,000 rpm for 20-24 h at 15 °C and slowly rotated back to stop. The centrifuge tube was carefully transferred to a tube holder and the bottom of the tube was punched with a needle. Fractions (0.5 ml) were collected in 20 1.5-ml microfuge tubes. The DNA in each tube was precipitated with an equal volume of isopropanol and resuspended in 50 µl TE buffer. The samples were examined by agarose gel electrophoresis. The required fractions were pooled, further purified and then dissolved in 50 µl of TE buffer.

c. Construction of a DNA library in phagemid pSK+

A sample (10 µg) of pSK+ DNA was digested with *Bam*HI and dephosphorylated with TsAP. The vector DNA was then purified by phenol/chloroform extraction. After ethanol precipitation, it was resuspended in 50 µl of TE. In a ligation reaction of 100 µl, 20 µl (2 µg) of vector DNA was mixed with 10 µl of *S. venezuelae* genomic DNA fractionated

by sucrose gradient centrifugation. Ligation was carried out with 5 µl of T_4 DNA ligase (BRL) for 3 h at room temperature. The ligation mixture was used to transform competent cells of *E. coli* strain DH5α.

d. Construction of a genomic library in lambda vector GEM-11

Genomic DNA partially digested with *Sau*3A1 (with -GATC termini) and *Xho*I digested lambda arms (with -TCGA termini) were partially filled-in by incubation with Klenow fragment in the presence of only A and G. After ligation of the genomic DNA to the lambda arms, the recombinant phages were packaged with a packaging system (Promega) and infected with *E. coli* LE392. Phage particles were amplified by the procedure described above.

J. DNA Sequencing

a. Generation of nested, overlapping deletions

DNA fragments were cloned in phagemid vector pBluescript SK+. The recombinant phagemid DNA with its insert (5-10 µg) was linearized by digestion with two endonucleases that cut appropriately within the MCS, one close to the sequencing primer side (e.g. *Kpn* I or *Sac* I) to generate ExoIII-resistant 3'-protruding ends, the other on the insert side to give 3'-recessed or blunt ends (ExoIII-accessible). The linearized DNA was treated with ExoIII/S1 Deletion kit (MBI) to generate nested, overlapping deletions. The S1 nuclease mixture (200 µl) was prepared by adding 27 µl of 7.4 x S1 buffer and 3 µl of S1 nuclease (50 u) to 170 µl of water in a 1.5-ml microfuge tube. An aliquot of 37.5 µl (7.5 x 5) was

transferred to 5 individual microfuge tubes labeled '0-5', '6-10', '11-15', '16-20', and '20-25' and kept on ice. In an another tube, double-digested DNA (5-10 μg) dissolved in 50 μl of 1 x ExoIII buffer was incubated at 30 °C. After 2 μl of the ExoIII mixture was removed and mixed with the '0-5' S1 mixture, 2.5 μl of ExoIII (500 U) was added to make an ExoIII mixture. A portion (2 μl) of the ExoIII digest was transferred to an S1 mixture every minute. The first five transfers were pooled as the '0-5' S1 mixture, the second five as the '6-10' S1 mixture, and so on. After the ExoIII reaction was complete, all the S1 reaction mixtures were incubated at room temperature for 30 min. S1 stop buffer (5 μl) was added to each, and the S1 nuclease was inactivated by heating at 70 °C for 10 min. The DNA was examined by gel electrophoresis. The DNA from the deletion reactions was self-ligated with T4 DNA ligase reagent. Ligation mix (100 μl) was prepared by adding 10 μl of 10 x ligation buffer, 20 μl of PEG400 (50 % solution), 1 μl of T4 DNA ligase and 69 μl of deionized water. The ligation mixture was divided into 20 μl portions and mixed with each deletion sample (3.5 μl). The ligation reaction was carried out at room temperature for 1 h. The recircularized phagemid DNA was used to transform *E. coli* DH5α competent cells. The transformants were screened and aligned for size, and single-stranded DNA was prepared for sequencing.

b. DNA sequencing reaction

A T_7Sequencing™ kit from Pharmacia Biotech was used for the DNA sequencing reaction. For single-stranded DNA templates, the annealing mixture consisted of 10 μl of DNA template (1-2 μg of DNA), 2 μl of 1:5 diluted universal primer and 2 μl of annealing

buffer. The mixture was incubated at 60-65 °C for 10 min and then slowly cooled to room temperature over 30 min. To denature double-stranded DNA template, a solution containing 1-2 μg DNA was mixed with 8 μl of 2 M NaOH and water to a final volume of 40 μl. The mixture was vortexed and centrifuged briefly and incubated at room temperature for 10 min. The denatured DNA was precipitated by adding 7 μl of 3 M sodium acetate (pH 4.8), 4 μl of water and 120 μl of 100% ethanol. After 20 min at –70 °C, the DNA was pelleted by centrifugation for 10 min and washed with ice-cold 70% ethanol. After recentrifugation, the ethanol was drained off and the pellet was dried under vacuum without spinning. The denatured DNA was redissolved in 10 μl of water before the annealing step. For double-stranded DNA templates with a high G+C content, the annealing mixture consisted of 7 μl of denatured template, 2 μl of primer (5-10 pmols), 2 μl of annealing buffer and 3 μl of DMSO. The mixture was incubated at 37 °C for 20 min and then at room temperature for 20 min. For the labeling reaction, 14-15 μl of annealed template/primer mixture was mixed with 3 μl of Labeling Mix, 1 μl of [α^{32}P] dCTP and 2 μl of 1:5 diluted T_7 DNA polymerase. After the labeling mixture had been incubated at room temperature for 5 min, 4.5 μl was transferred to each of the four pre-warmed sequencing mixes. These termination reaction mixtures were incubated at 37 °C for 5 min before 5 μl of stop buffer was added to each. The DNA in the sequencing reactions was denatured at 85 °C for 5 min and cooled on ice before fractionation by polyacrylamide gel electrophoresis.

K. DNA Hybridization

a. Southern blot and hybridization

1. Transfer of DNA from agarose gels to nylon membranes

After electrophoresis, the agarose gel was stained with ethidium bromide and photographed. The gel was soaked in 0.25 M HCl for 15-20 min with gentle agitation and rinsed with distilled water. The gel was then treated twice for 20 min with denaturing solution (1.5 M NaCl, 0.5 M NaOH) and rinsed with distilled water. The gel was neutralized with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH7.2 and 1 mM EDTA) for 20 min and rinsed with distilled water. The DNA was transferred to a nylon membrane with a vacuum transfer system (Model VAC-1000, American Bionetics, Hayward, CA) in 10 x SSC (for 1 h. The nylon membrane was air dried for 10 min, sandwiched between two sheets of Whatman 3 mm paper and then baked at 85 °C for 2 h.

2. Radioactivity labeling of DNA probe

DNA probes were labeled with a random primer labeling kit from BRL. DNA (up to 50 ng) was denatured in boiling water for 5 min and immediately cooled on ice for 2-3 min. The following components were included to the labeling reaction:

Denatured template DNA (50 ng)	5 µl
dATP, dGTP, dTTP (0.25 mM each)	3 x 2 µl
Random primer buffer	15 µl
[α-^{32}P] dCTP (3000 Ci/mmol; 10Ci/µl)	5 µl
Water	19 µl
Klenow fragment	1 µl

The mixture was incubated at room temperature for 1-2 h and mixed with 5 µl of stop

buffer. The probe was purified through a Sephadex G50 column and denatured at 95 °C for 5 min before use.

3. Hybridization

The nylon membrane with DNA immobilized on it was soaked in 2 x SSC and rotated in a hybridization bottle containing 30 ml of 2 x SSC for 30 min. The SSC buffer was then replaced with 30 ml of prewarmed prehybridization solution (6 x SSC, 5 x Denhardt's reagent, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA). After the membrane had been washed in the prehybridization solution at 68 °C for 1-2 h, denatured probe was added to the solution. Hybridization was performed at 65 °C overnight. The membrane was first washed in a large volume (300-500 ml) of 2 x SSC with 0.1% SDS for 10 min at room temperature, and then washed in 50 ml of 0.1 x SSC with 0.1% SDS at 65 °C for 2 h with rotation. The filter was washed again in 0.1 x SSC with 0.1% SDS at 65 °C for 30 min. The filter was wrapped in Saran Wrap and exposed to a GS-250 phosphor screen for 3-24 h. The screen was then scanned at a resolution of 200 μ with a GS-250 Molecular Imager (Bio-Rad) and the image was documented and edited with the Bio-Rad Imager Analysis System (Bio-Rad Laboratories, Molecular Bioscience Group, Hercules, CA).

b. Colony and plaque hybridization

Single colonies or plaques were prepared on a LB agar plate. The plate was chilled for 30-60 min at 4 °C. A nylon filter (2-3 mm smaller in diameter than the plate) marked in three or more asymmetric locations was placed on the surface of the plate with the marked side down. The plate was then marked at positions corresponding to the marks on the filter.

After the filter was completely wet, it was carefully peeled off the plate with blunt-ended forceps and placed with the marked side up on a sheet of Whatman 3 MM filter paper saturated with 10% SDS for 3 min and then transferred to another filter paper with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 min. For plaque hybridization, the filter was transferred to filter paper saturated with denaturing solution. The filter was then transferred to a filter paper saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH7.4, 1 mM EDTA) for 5 min, and then to a filter paper saturated with 2 x SSC. The filter membrane was air dried for 30 min, sandwiched with two sheets of Whatman 3 MM filter paper and baked at 85 °C for 2 h.

L. Analysis of DNA sequences

To compare the sequences obtained to known DNA or protein sequences, the GenBank database was searched. The potential open reading frames (ORFs) and restriction enzyme sites as well as the secondary structures of oligonucleotides were analyzed with the Gene Runner program (Hastings Software, Inc). The Codonpreference, Fragment Assembly and Pileup programs of the GCG Wisconsin Package were used for sequence fragment assembly and to detect potential ORFs, as well as for codon usage and bias analysis and sequence alignments. Similarities between the derived sequence and database sequences were determined by using Clustal W and BLAST to search the NCBI database.

RESULTS

SECTION 1. GENETIC MARKERS RELATED TO CHLORAMPHENICOL BIOSYNTHESIS GENES

At the start of the work the immediate goal was to facilitate cloning of Cm biosynthesis genes in the genetically mapped *cml* cluster by defining a marker near *pdxH* in the *S. venezuelae* chromosome so that the direction of chromosome walking could be readily determined. Initially, the most promising marker was a 4.0-kb *Sac*I fragment from a recombinant lambda phage carrying DNA cloned during earlier chromosome walking. This fragment had been shown to hybridize weakly with a cloned bromoperoxidase catalase gene (*bca*) of *S. venezuelae* (K. A. Aidoo, personal communication). Since *bca* was believed at that time to direct halogenation, and to correspond to the genetically mapped chlorination gene *cml-2*, the 4.0-kb fragment was presumed to lie in the *cml* cluster. Supporting this presumption was its location approximately 30 kb from *pdxH* (see Fig. 3).

I. Sequence Analysis of the 4.0-kb *SacI* Fragment Hybridizing with *bca*

When the 4.0-kb *Sac*I fragment was sequenced (data not shown), sequence analysis indicated that it did not contain an ORF resembling *bca*. Instead, the deduced amino acid sequence of the 4.0-kb *Sac*I fragment showed similarities to D-alanyl-D-alanine dipeptidase, lipid carrier proteins and ATP/GTP binding proteins of microorganisms (Table 2). A BlastX search of the sequenced region of the *S. coelicolor* A3(2) genome located a matching sequence in cosmid unk1 (L10), flanked by the *argA*, *argB*, and *argC* genes (Redenbach et

Table 2. Result of a BlastX search of the GenBank database for protein sequences matching deduced amino acid sequence of the 4.0-kb *Sac*I fragment of *S. venezuelae* genomic DNA.

Sequences producing significant alignments:	Score (bits)	E Value
gb|AAD25390.1|AF120672_2 (AF120672) D-alanyl-D-alanine dipeptid...	99	6e-36
emb|CAA19989| (AL031124) putative ATP-GTP binding protein [Stre...	140	6e-32
emb|CAA19985| (AL031124) putative ATP/GTP binding protein [Stre...	139	8e-32
emb|CAA15747| (AL009198) hypothetical protein Rv3362c [Mycobact...	131	1e-29
emb|CAB08880| (Z95554) hypothetical protein Rv1627c [Mycobacter...	87	1e-27
gb|AAD19836| (AF060799) D-alanyl-D-alanine dipeptidase [Amycola...	52	2e-20
gi|2984130 (AE000759) putative protein [Aquifex aeolicus]	83	8e-15
gi|4104708 (AF039028) D-ala-D-ala dipeptidase; VanXst [Streptom...	46	1e-11
sp|Q47749|VANX_ENTFA D-ALANYL-D-ALANINE DIPEPTIDASE (D-,D-DIPEP...	44	6e-11
sp|Q06241|VANX_ENTFC D-ALANYL-D-ALANINE DIPEPTIDASE (D-,D-DIPEP...	47	8e-10

al., 1996). This region is separated by the insertion sequence, IS117-A, from the "*pdxH*" gene cloned by D. A. Aidoo (personal communication), which is located in the overlapping cosmid I35 of the *S. coelicolor* A3(2) genomic DNA sequence. These results imply that *bca* is not close to "*pdxH*", and in this respect they are consistent with those from chromosome walking (D. A. Aidoo, personal communication); since *bca* evidently does not correspond to *cml-2*, the data are also consistent with those from genetic mapping obtained earlier (Vats et al., 1987). Sequence alignment with *bca* located a 150-bp region with 52.3% identity to the *bca* nucleotide sequence, but no hybridization signal was obtained when the 4.0-kb fragment was reprobed with *bca* under slightly more stringent conditions. Since it was, therefore, uncertain whether the DNA fragment contained a halogenation gene, and since by then *bca* had been disrupted and shown to be unnecessary for Cm production (Facey et al., 1996), further investigation of the 4.0-kb *Sac* I fragment was discontinued.

II. Characterization of the *cys-28* Mutation and Attempts to Clone *cys-28* by Complementation.

Based on genetic mapping data (Doull et al., 1986; Vats et al., 1987), the *cml* gene cluster is flanked by *pdxH* and *cys-28*. As the next candidate for a marker that could be used in conjunction with *pdxH* to establish directionality in the *cml* cluster, *cys-28* was, therefore, a logical choice. The first step was to characterize the growth requirements of strain VS263, the mutant of *S. venezuelae* ISP5230 carrying the *cys-28* mutation.

A. Phenotype of VS263 (*cys-28* mutation)

Examination of the literature on the cysteine biosynthetic pathway indicated that sulfate, sulfite, sulfide and thiosulfate ions can contribute to the sulfur atom in cysteine, whereas O-acetylserine, homoserine (H-ser), homocysteine (H-cys),) and cystathionine are each able to contribute the carbon skeleton of the amino acid (Fig.10). When the growth of *S. venezuelae* VS263 was tested on minimum agar supplemented with individual substances potentially able to meet the nutritional requirement for sulfur, the results indicated that not only cysteine but methionine, homocysteine and cystathionine (underlined in Fig. 10) restored growth of the mutant comparable to that of the wild type. Thiosulfate restored poor growth, but sulfite, sulfide, L-serine, O-acetylserine, homoserine and O-succinylhomoserine did not support growth of the mutant. On this evidence, it was concluded that the metabolic step from O-acetylserine to cysteine is blocked in VS263. The reaction is catalyzed by O-acetylserine sulfhydrylase, which in enteric bacteria (e.g., *E. coli* and *S. typhimurium*) may be encoded as isoenzymes by either of two genes, *cysK* or *cysM*. The ability of thiosulfate to partially meet the growth requirement suggested that sulfocysteine synthase (the product of *cysM* in *E. coli* and *S. typhimurium*), if present in *S. venezuelae,* remains intact in VS263, though perhaps at a low level. This pointed to a mutation in the *cysK*-encoded acetylserine sulfhydrylase (simply called cysteine synthase) as the cause of the *cys-28* phenotype.

B. Isolation of *cys* mutants from *S. lividans*.

Because of the low efficiency with which protoplasts of *S. venezuelae* were

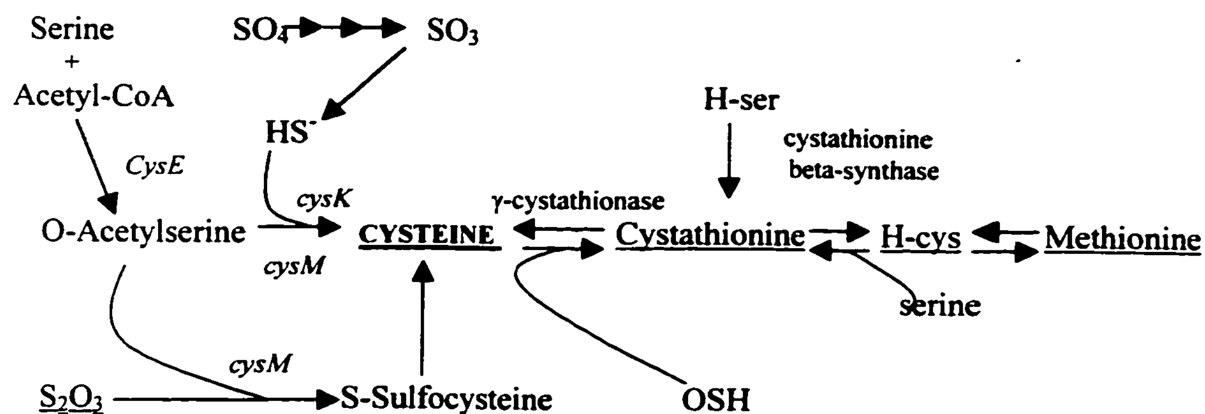


Fig. 10. Tentative metabolic pathways for sulfur-containing amino acids in streptomycetes, showing supplement tested for complementation of the *cys-28* mutant (strain VS263). Only the substances underlined restored the growth of VS263 on minimal medium. H-ser, homoserine; H-cys, homocysteine; OSH, O-succinylhomoserine.

transformed compared to the efficiencies obtained with *S. lividans* and some other streptomycetes, *cys* mutants were generated from *S. lividans* by mutagenic treatment so that their usefulness as cloning hosts could be assessed. About 8,000 colonies from NTG-treated spores were screened by replica-plating on diagnostic media to detect those with a cysteine requirement for growth. Seventeen such mutants were obtained (Table 3). Three of them (CH107, CH113 and CH114) had growth requirements similar to those of VS263. Since no cross-feeding among the mutants or with VS263 was detected, all of the strains were considered to have the same phenotype, and likely also the same mutation as VS263. Two of the strains, CH107 (*cys-107* mutation) and CH114 (*cys-114* mutation) were chosen as hosts for complementation to prototrophy by transformation with a vector containing genomic DNA fragments from *S. venezuelae*.

Table 3 indicated that in mutant CH115, the reduction of $SO_4^=$ to $SO_3^=$ was blocked because both Na_2S and Na_2SO_3 can meet its growth requirement. In CH107 and CH113, the mutation was likely at the reduction of Na_2SO_3 to generate sulfide. The position of thiosulfate in the assimilation of sulfur was confusing, because in some mutants such as CH103, CH117 and CH113, either thiosulfate or sulfide could meet the growth requirement. For mutants CH114 and VS263, only thiosulfate, but not sulfide could restore growth on minimal medium. In strain CH105, on the other hand, sulfide but not thiosulfate could meet the growth requirement of the mutant. It was noteworthy that the growth requirement of most *cys* mutants was met by methionine and homocysteine. The results of growth requirements of the *cys* mutants indicated that reverse transsulfuration occurs in *S. venezuelae* and *S. lividans*. Based on these results and the metabolic pathways proposed by Kitano et al. (1985)

Table 3. Growth requirements of *S. venezuelae* VS263 and *S. lividans cys* mutants of *S. lividans*

Supplement	Mutants													
	VS263	CH101	CH103	CH105	CH107	CH109	CH110	CH111	CH112	CH113	CH114	CH115	CH116	CH117
Cysteine	++	++	++	++	++	+++	++	–	+++	++	++	++	+++	+++
Methionine	+	+	±	±	++	++	++	–	+++	++	+	++	++	++
L-serine	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Acetyl-serine	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H-cysteine	+	–	–	±	+	++	+	–	++	+	+	+	++	++
cystathionine	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
OSH[a]	–	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
$Na_2S_2O_3$	+	–	±	–	+	–	–	–	–	±	+	–	–	–
Na_2S	–	±	±	±	+	–	–	–	–	±	–	+	–	–
Na_2SO_3	–	–	–	–	–	–	–	–	–	–	–	+	–	–

[a] O-succinylhomoserine. +++ : growth as well as wild-typed strain; ++: good growth; +: obvious growth; ±: poor growth; –: no growth; NA: not available in this experiment.

and Lydiate et al. (1988), the metabolism of sulfur-containing amino acids in streptomycetes was proposed to be as shown in Fig. 10.

C. Cloning an *S. venezuelae* DNA fragment "complementing" *S. lividans* mutant CH114

Mutant CH114 was chosen as a host in which to clone the disfunctional gene in VS263 by complementation because it showed the same growth requirements as VS263. Partial *Sau*3A1 digests of *S. venezuelae* genomic DNA were shotgun–cloned in the *Bam*HI site of pIJ702, and the vector library was used to transform *S. lividans* CH114. One prototrophic thiostrepton-resistant transformant CH118 was obtained from 500 thiostrepton-resistant transformants screened. Plasmid DNA was isolated from this strain and purified as pJV204. An 8.0-kb *S. venezuelae* fragment was identified as an insert of pJV204 (Fig. 11). To confirm its ability to complement the host mutation, pJV204 plasmid DNA was then used to transform mutants VS263 and CH114, and 10 to 20 thiostrepton-resistant colonies (retransformants) were obtained from each host. However, none of these colonies could grow on minimal medium. To explain this result, the characteristics of pJV204 obtained from the "retransformants" of VS263 and CH114 were examined further. The "retransformants" were confirmed to harbor plasmid pJV204 based on ability to reisolate the plasmid from them. It was concluded that prototrophic growth of CH118 was due to reversion of the *cys-114* mutation in CH114, independent of transformation; thus plasmid pJV204 contributed only to the thiostrepton resistance of the transformant. The 8.0-kb fragment of *S. venezuelae* DNA cloned in pJV204 was later probed with the 4.0-kb *Pst* I fragment containing the cloned cystathionine β-synthase gene of *S. venezuelae* (see Section 2, II C), but no

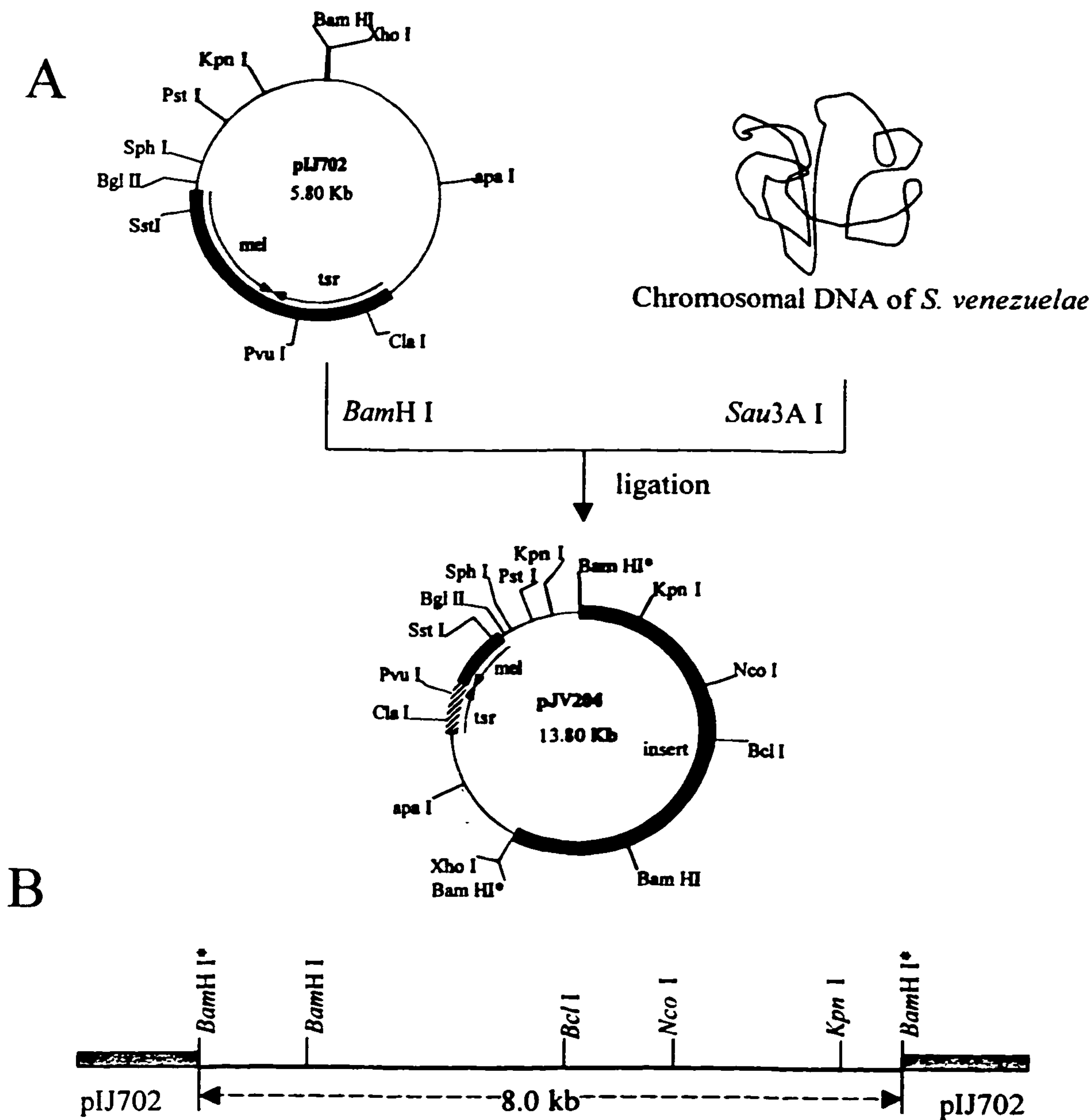


Fig. 11: A, Procedure used to clone in pIJ702 an *S. venezuelae* chromosomal DNA fragment "complementing" the *cys* mutation in *S. lividans* CH114; B, A restriction map of the cloned fragment. The sites marked with asterisks are the junctions of the *Bam*HI and *Sau*3AI segments from the vector and insert, respectively.

hybridization was detected. The prototrophy and resistance of CH118 were the due to the chance circumstance in which pJV204 had transformed a strain carrying a revertant of *cys-114* instead of the CH114 mutant itself. To test the feasibility of using the complementation strategy to clone *cys* genes, the reversion frequency of CH114 was investigated. Spores of CH114 were collected from a K1 plate and prepared as a uniform suspension of individual spores by glass bead separation and cotton filtration. After suitable dilution, measured volumes of the diluted spore suspension were spread in duplicate on plates of K1 and MM medium. The colonies that appeared were counted, and the spontaneous reverse mutation frequency was calculated to be 2.38×10^{-4}. This was higher than anticipated and greatly reduced the likelihood of cloning the gene corresponding to *cys-28* since this depended on the number of transformants of the host obtained. The recombinant plasmid complementing the *cys* mutant would be isolated only if a large number of transformants with the wild phenotype were obtained.

III. Transposition of Tn*4560* in *S. venezuelae*

Transposition mutagenesis is potentially a direct and simple approach to isolating antibiotic biosynthesis genes. Insertion of a transposon into genes involved in antibiotic biosynthesis could not only alter or block production of the antibiotic, but also, because of the markers on the transposon, identify the site of the mutation. The disrupted gene and any nearby antibiotic biosynthesis genes should then be readily detected and cloned. Tn*4560* has been found to transpose into many locations in chromosomes of various *Streptomyces* spp.

(Chung, 1987; 1988; Chung & Crose, 1989; Ikeda et al., 1993; Schauer et al., 1991; Yagi, 1990). Tn*4560* has also been tested in *S. venezuelae* and transposition mutants have been obtained (Gong, 1991). Unfortunately no *cml* mutants could be detected directly in these *S. venezuelae* transposants because the host strain used was a *cml* mutant. To determine whether transposon mutagenesis could be used as an alternative approach to cloning a marked gene from the *cml* cluster, an attempt was made to isolate *cml* mutants by transposing Tn*4560* into wild-type strain, *S. venezuelae* ISP5230.

The plasmid pUC1169 used by Chung (1987) to introduce Tn*4560* into streptomycete host strains transformed *S. venezuelae* inefficiently, and was not readily eliminated (Gong, 1991). The possibility of using pHJL400 as a more suitable transposon delivery vehicle was therefore explored. An *E. coli/S. venezuelae* shuttle vector that facilitated vector construction and allowed convenient isolation of plasmid DNA containing Tn*4560* from *E. coli* was constructed by initially fusing a *Bam*HI fragment of pUC1169 with a *Bam*HI digest of pSK+ to yield pJV228 (Fig. 12). This intermediate construct was modified by retrieving a *Vsp*I-*Mlu*I fragment containing Tn*4560* from pUC1169 and inserting it into the *Bam*HI site of pHJL400 by blunt-end ligation to give pJV231. For reasons not determined, introduction of Tn*4560* into pHJL400 appeared to increase the copy number and stability of the plasmid in *S. venezuelae*. As a result, an abundant plasmid band was observed when DNA isolated from strains CHG5 and CHG7, transformed with pJV231, was examined by gel electrophoresis.

To facilitate transposition of Tn*4560* into the chromosome, *S. venezuelae* CHG5 was grown at a relatively high temperature on MYM agar without antibiotic for 4 days. The frequency of transposition and efficiency of plasmid curing were estimated by testing spores

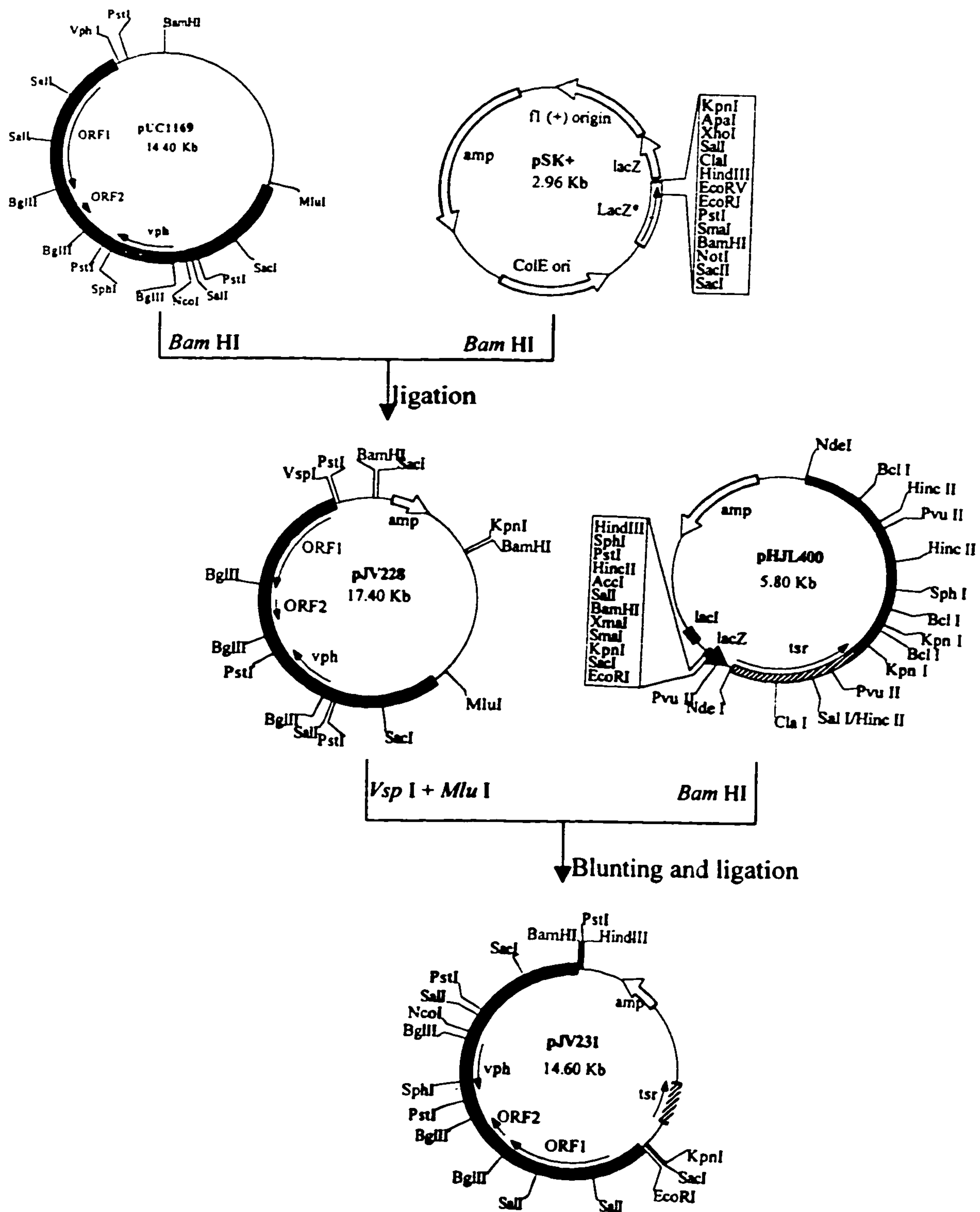


Fig. 12. Construction of pJV231 (pHJL400::Tn*4560*)

for resistance to viomycin and to viomycin + thiostrepton. From the results it was concluded that the vector was rapidly eliminated (98% efficiency) under the conditions used, and that Tn*4560* transposed into the genome of *S. venezuelae* at a frequency of 1.4×10^{-3}. One auxotrophic mutant was identified in 465 colonies screened (0.2%), which is a mutagenic frequency similar to that reported by others (Chung & Crose, 1989; Ikeda et al., 1993). About 3000 colonies were bioassayed for production of chloramphenicol, but no *cml* mutants were identified. This transposition frequency was considered too low to make Tn*4560* a suitable choice for transposition mutation of chloramphenicol biosynthesis genes. The decision against its use was strengthened by reports of its non-random insertion into *Streptomyces* chromosomes (Ikeda et al, 1993; Yagi, 1990; Gong, 1991).

I. Cloning a Cysteine Synthase-encoding Gene Fragment from *S. venezuelae* by PCR

A. Designing PCR primers

Another approach to clone the gene related to *cys-28* was to use PCR. Because feeding experiments indicated that the *cys-28* mutation blocked cysteine biosynthesis at the step between O-acetyl-L-serine and L-cysteine, the enzyme cysteine synthase (CS), corresponding to the *cysK* product in *E. coli,* was presumed to be involved. The deduced amino acid sequences of *cysK* of *E. coli* and of CSs from other bacteria were retrieved from the NCBI database and aligned using the Clustal V program (Higgins & Sharp, 1989, see Fig. 13). The amino acid sequence alignment showed that the CSs of different prokaryotic organisms are also similar in sequence to cystathionine β-synthases (CBSs) of eukaryotes, especially in the conserved regions of the CSs (data not shown). Conserved regions were chosen to provide primer sites for PCR. Two primers ch3 and ch4, 500-bp apart, were designed using the Gene Runner analysis program (Hastings Software, Inc., 1994), taking account of codon usage in *Streptomyces* (Bibb et al., 1984; Seno & Baltz, 1989; Wright & Bibb, 1992):

B. PCR conditions for amplification of the acetylserine sulfhydrylase gene of *S. venezuelae*

The optimum composition of the PCR reaction mixture, and the optimum thermocycling conditions for the PCR reaction were determined in trial experiments. The

A.

```
cysK-E.coli              MSKIFEDNSLTIGHTPLVRLNRIG---NGRILAKVESRNPSFSVKCRIGANMIWDAEKRG 57
cysK-H.influenzae        -MAIYADNSYSIGNTPLVRLKHFGH--NGNVVVKIEGRNPSYSVKCRIGANMVWQAEKDG 57
cysK-M.tuberculosis      -MSIAEDITQLIGRTPLVRLRRVTDGAVADIVAKLEFFNPANSVKDRIGVAMLQAAEQAG 59
cysK-B.subtilis          MVRVANSITELIGNTPIVKLNRLADENSADVYLKLEYMNPGSSVKDRIGLAMIEAAEKEG 60
                            :  . :  **.**:*:*.:.       . :  *:*  **. *** ***  *:  **: *

cysK-E.coli              VLKPGVELVEPTSGNTGIALAYVAAARGYKLTLTMPETMSIERRKLLKALGANLVLTEGA 117
cysK-H.influenzae        TLTKGKEIVDATSGNTGIALAYVAAARGYKITLTMPETMSLERKRLLCGLGVNLVLTEGA 117
cysK-M.tuberculosis      LIKPDTIILEPTSGNTGIALAMVCAARGYRCVLTMPETMSLERRMLLRAYGAELILTPGA 119
cysK-B.subtilis          KLKAGNTIIEPTSGNTGIGLAMVAAAKGLKAILVMPDTMSMERRNLLRAYGAELVLTPGA 120
                          :. .   :::.*******.** *.**:* :  *.**:***:**: **  . *.:*:** **

cysK-E.coli              KGMKGAIQKAEEIVASNPEKYLLLQQFSNPANPEIHEKTTGPEIWEDT-DGQVDVFIAGV 176
cysK-H.influenzae        KGMKGAIAKAEEIVASDPSRYVMLKQFENPANPQIHRETTGPEIWKDT-DGKVDVVVAGV 176
cysK-M.tuberculosis      DGMSGAIAKAEELAKTDQR-YFVPQQFENPANPAIHRVTTAEEVWRDT-DGKVDIVVAGV 177
cysK-B.subtilis          EGMKGAIKKAEELAEKHG--YFVPQQFNNPSNPEIHRQTTGKEIVEQFGDDQLDAFVAGI 178
                         .**.*** ****:. ..    *.: :**.**:** **. **. *: .:  *.::* .:**:

cysK-E.coli              GTGGTLTGVSRYIKGTKGKTDLISVAVEPTDSPVIAQALAGEEIKPGPHKIQGIGAGFIP 236
cysK-H.influenzae        GTGGSITGISRAIKLDFGK-QITSVAVEPVESPVISQTLAGEEVKPGPHKIQGIGAGFIP 235
cysK-M.tuberculosis      GTGGTITGVAQVIKERKPS--ARFVAVEPAASPVLSG-G-----QKGPHPIQGIGAGFVP 229
cysK-B.subtilis          GTGGTITGAGEVLKEAYPS--IKIYAVEPSDSPVLSG-G-----KPGPHKIQGIGAGFVP 230
                         ****::** .. :*     .        ****  ***::          : *** ********:*

cysK-E.coli              ANLDLKLVDKVIGITNEEAISTARRLMEEEGILAGISSGAAVAAALKLQEDESFTNKNIV 296
cysK-H.influenzae        KNLDLSIIDRVETVDSDTALATARRLMAEEGILAGISSGAAVAAADRLAKLPEFADKLIV 295
cysK-M.tuberculosis      PVLDQDLVDEIITVGNEDALNVARRLAREEGLLVGISSGAATVAALQVARRPENAGKLIV 289
cysK-B.subtilis          DILNTEVYDEIFPVKNEEAFEYARRAAREEGILGGISSGAAIYAALQVAKK-LGKGKKVL 289
                           *: .: *.:  : .: *:  ***    ***:* *******  ** :: .      .* ::

cysK-E.coli              VILPSSGERYLSTALFADLFTEKELQQ 323
cysK-H.influenzae        VILPSASERYLSTALFEGIEG------ 316
cysK-M.tuberculosis      VVLPDFGERYLSTPLFADVAD------ 310
cysK-B.subtilis          AIIPSNGERYLSTPLYQFD-------- 308
                         .::*. .******.*:
```

B.

```
ch3:  5'-GAG ACC ATC GGC AAC ACC CC-3'
ch4:  5'-GT GAT CGT GCC GCC GGT GCC-3'
```

Figure 13: A. Alignment of *cysK* product sequences. Asterisks indicate amino acids identical in all sequences; colons indicate highly conserved amino acids; periods indicate amino acids with similar properties; B. Sequences of the forward and reverse primers used for amplification of *S. venezuelae* chromosomal DNA by PCR.

parameters chosen are those given in "Methods" (Section G)

The amplified product obtained under optimized conditions gave a single band of about 500-bp when examined by agarose gel electrophoresis; the size was consistent with the distance between the two primers.

C. Cloning and sequencing of the PCR product

The SureClone ligation kit (Pharmacia) was used to ligate the amplified fragment into the *SmaI* site of pUC18 to give plasmid pJV205. A 0.55-kb *EcoR* I – *Hind* III DNA fragment was cut from pJV205 and subcloned in M13mp18 and M13mp19 for sequencing. The two primers ch3 and ch4 were each located at the expected positions in the sequence of the 0.5-kb DNA fragment.

A BlastX search with the sequence of the chromosomal fragment in pJV205 indicated a strong similarity to CS sequences in the GenBank database (Table 4). Although cystathionine β-synthases (CBSs) of eukaryotes also showed a high sequence similarity to the PCR product, the *cysK* of *Mycobacterium* leprae (sequence ID, 699279) gave the highest score. Moreover at that time it was thought that CBS was not present in prokaryotic organisms. So although the possibility that the insert in pJV205 might originate from a CBS gene of *S. venezuelae* was not excluded, it was judged less likely. As a working hypothesis, the cloned DNA fragment of *S. venezuelae* was presumed to be the acetylserine sulfhydrylase (often called cysteine synthase, see Table 4) gene component of a CS complex.

Table 4. Proteins in the GenBank database ranked in a BlastX search for sequences resembling the deduced sequence of the PCR product.

Sequences producing High-scoring Segment Pairs		Reading Frame	High Score	Smallest Sum Probability P(N)	N
gi|699279	(U15183) cysteine synthase [Mycob...	+3	135	1.8e-14	2
gnl|PID|e290741	(Z83860) unknown [Mycobacterium t...	+3	138	4.8e-14	2
pir||C42790	cystathionine beta-synthase (EC 4...	+3	111	1.8e-13	3
pir||B42790	cystathionine beta-synthase (EC 4...	+3	111	2.7e-13	3
pir||JX0145	hemoprotein H-450 precursor - rat...	+3	111	2.7e-13	3
gi|206600	(M88346) cystathionine beta-synth...	+3	111	2.7e-13	3
sp|P32232|CBS RAT	CYSTATHIONINE BETA-SYNTHASE (SERI...	+3	111	2.9e-13	3
gnl|PID|e259018	(Z78415) C17G1.7 [Caenorhabditis ...	+3	137	8.8e-12	1
sp|P35520|CBS HUMAN	CYSTATHIONINE BETE-SYNTHASE (SERI...	+3	103	2.1e-11	3
pir|A55760	cystathionine beta-synthase (EC 4...	+3	103	2.1e-11	3
SP|P37887|CYSK BACS	CYSTEINE SYNTHASE (O-ACETYLSERINE...	+3	132	4.1e-11	1
gnl|PID|e284817	(Z83236) K10H10.d [Caenorhabditis...	+3	132	4.3e-11	1
sp|P46794|CBS DICDI	CYSTATHIONINE BETE-SYNTHASE (SERI...	+3	127	2.5e-10	1
gi|1651736	(D90899) cysteine synthase [Synec...	+3	118	3.8e-09	1
pir||S55321	srpG protein - Synechococcus sp. ...	+3	118	3.8e-09	1
gi|393279	(L14578) cystathionine bets-synth...	+3	102	4.0e-09	2
pir|B55760	cystathionine beta-synthase (EC 4...	+3	102	4.0e-09	2
gi|929593	(Z50729) cysteine synthase [Flavo...	+3	77	8.2e-09	2
sp|P50867|CYSK EMEN	CYSTEINE SYNTHASE (O-ACETYLSERINE...	+3	115	1.0e-08	1
sp|P53206|CYSK YEAS	PUTATIVE CYSTEINE SYHTHASE (O-ACE...	+3	113	2.0e-08	1
gi|804950	(X84097) cysteine synthase [Arabi...	+3	111	3.5e-08	1
gi|415317	(D16496) cystathionine beta-synth...	+3	94	4.8e-08	2
gi|416161	(D16502) cystathionine beta-synth...	+3	94	4.8e-08	2
sp|P32582|CBS YEAST	CYSTATHIONINE BETA-SYNTHASE (SERI...	+3	94	4.8e-08	2
gi|1067074	(Z49131) ZC373.1 [Caenorhabditis ...	+3	90	5.4e-08	2
sp|P38076|CYSK WHEA	CYSTEINE SYNTHASE (O-ACETYLSERINE...	+3	108	9.3e-08	1
pir||S46438	cysteine synthase - watermelon /g...	+3	108	9.3e-08	1
sp|P48028|CYSM PSES	CYSTEINE SYNTHASE B (O-ACETYLSERI...	+3	106	1.1e-07	1
pir||A41863	probable O-acetylserine (thiol)-1...	+3	106	1.3e-07	1
pir|S49587	cysteine synthase (EC 4.2.99.8) c...	+3	106	1.9e-07	1

II. Cloning and Sequencing of a Cysteine Synthase-like Gene of *S. venezuelae*

A genomic library of *S. venezuelae* ISP5230 was constructed in the phagemid pSK+ and screened by colony hybridization using the insert from pJV205 as a probe. Several strongly hybridizing colonies were obtained and purified by single colony isolation. The plasmid DNA isolated from different clones and digested with *Sac*I showed the same pattern of bands by gel electrophoresis, suggesting that the clones were siblings that had arisen as progeny of a few successful transformants (or even from a single parent) during prolonged incubation. For further studies pJV207, containing a 9.2-kb insert of *S. venezuelae* genomic DNA, was used (Fig. 14).

In a parallel experiment a genomic library of *S. venezuelae* ISP5230 prepared by S. Facey in lambda GEM-11 was screened by plaque hybridization with the same probe used to screen the phagemid library. This also yielded several positively hybridizing clones (ZX201 - ZX212). Samples of DNA from plasmid pJV207, phage ZX209 and ZX202 were digested with *Sac*I, electrophoresed on agarose gels and probed by Southern hybridization with the 550-bp DNA fragment from pJV205. While the phage DNA samples gave different combinations of *Sac*I bands (Fig. 15A), all of them contained fragments of 0.35 and 1.6 kb that hybridized with the probe (Fig. 15B). This indicated that DNA from the three clones overlapped in the area amplified by PCR. Subsequent studies were limited to pJV207.

A restriction map of the 9.2-kb fragment of *S. venezuelae* DNA cloned in pJV207 was prepared (see Fig. 14). Hybridization probing indicated that a 4.0-kb *Pst*I fragment of

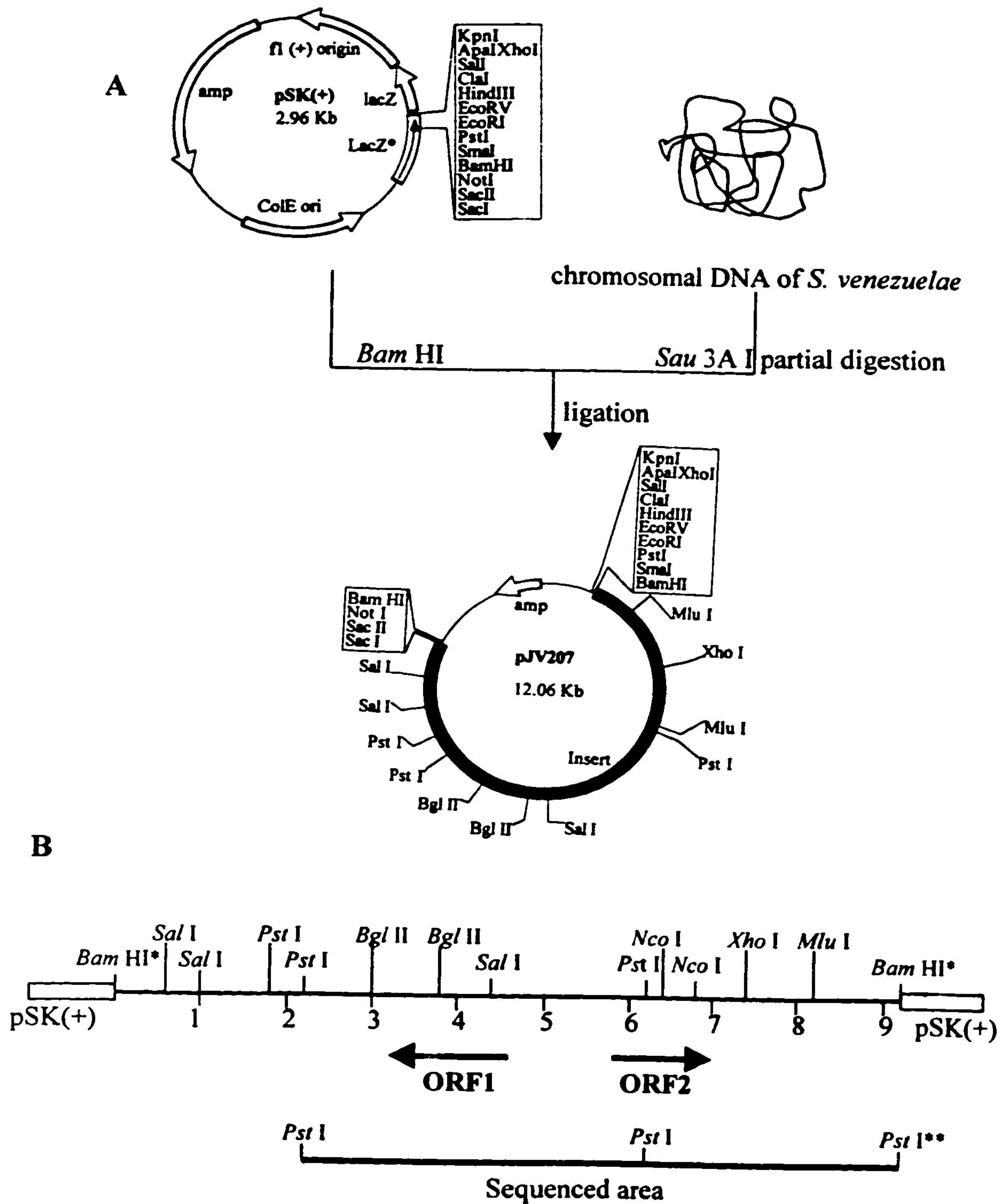
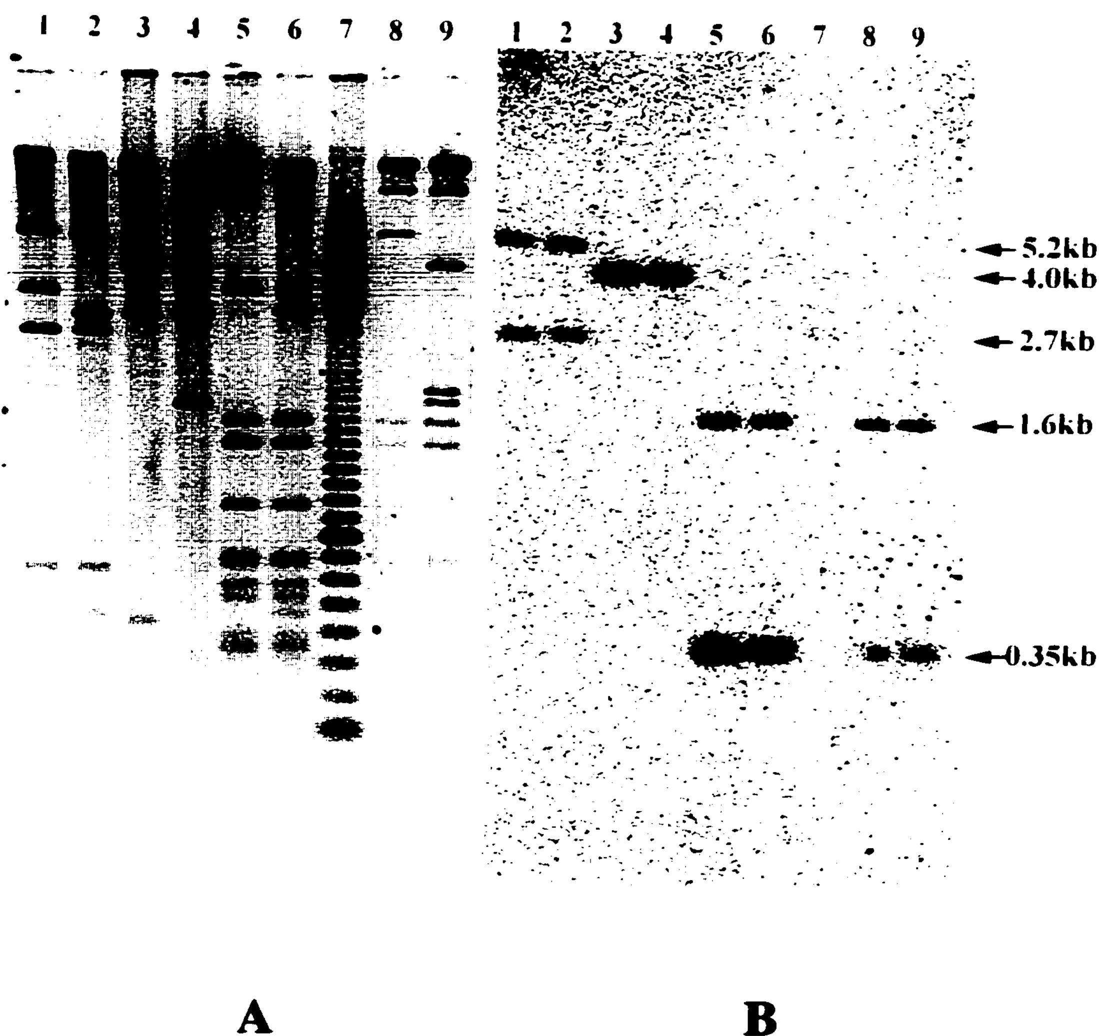


Fig. 14. A, Cloning in pSK+ of a fragment of *S. venezuelae* genomic DNA (pJV207) hybridizing with the PCR-amplified insert from pJV205; B, restriction map of 9.2 kb fragment. *: the hybrid *Sau*3A1/*Bam*HI sites, **: *Pst*I site in vector MCS.



A B

Fig. 15: A, Agarose gel electrophoresis; B, Southern hybridization of pJV207 and phage clones ZX209 and ZX202 digested with various enzymes. The cloned PCR product was used as the probe. Lanes 1-6: pJV207 digested with *Sal* I; *Sal* I+*Bam*H I; *Pst* I; *Pst* I+*Bam*H I; *Sac* I and *Sac* I+*Bam*H I, respectively. Lanes 8 and lane 9: the Sac I digested phage clones ZX209 and ZX202, respectively. Lane 7: a 100 bp ladder.

pJV207 contained all of the probe sequence. Subcloning the *Pst* I fragment of pJV207 in pSK+ generated pJV208 and pJV209, which contained the entire amplified fragment in opposite orientations. Also, pJV210 and pJV211 contained the 3.0-kb *Pst*I fragment flanking the 4.0-kb *Pst* I fragment in opposite orientations. The 0.5-kb *Pst* I fragment of pJV207 was subcloned to give pJV214. The 5.0-kb *Pst* I fragment of pJV207, containing the vector pSK+ and a 2.0-kb insert, was circularized by self-ligation, resulting in plasmid pJV212.

III. Nucleotide Sequence of the 7.0-kb DNA Fragment Containing ORF1 and ORF2

Nested overlapping deletions of the 4.0-kb *Pst* I fragment of DNA cloned in pJV208 and pJV209 were created using an ExoIII/S1 deletion kit, and both strands of the insert were completely sequenced. The adjacent 3.0-kb *Pst*I fragment was also sequenced by the same method. The sequenced fragments were assembled into two contigs (Appendix II, Fig. 34) using the Fragment Assembly program of the GCG software package. *Pst*I sites were present at both ends of each contig. Therefore, to assemble the *Pst*I fragments correctly, the contigs were searched with BlastX to identify the coding regions of an acetyl-CoA acetyltransferase-like gene located on one end of each fragment. This allowed the contigs to be assembled into a continuous 7-kb sequence designated cys7.0 (Fig. 16). When the cys7.0 sequence was analyzed for ORFs with the Gene Runner program, it was apparent that only ORF2 spanned the two contigs. It encoded a product with marked similarity to the acetyl-CoA thiolases (acetyltransferases) of different organisms.

Fig.16. Sequence of the 7.0-kb *S. venezuelae* chromosomal DNA fragment (cys7.0) containing cystathionine β-synthase (ORF1) and acetoacetyl-CoA thiolase (acetyltransferase) (ORF2). Selected restriction enzyme sites are underlined; putative RBSs are marked with *; the putative terminator downstream of ORF2 is marked with thick arrows; the hatched arrows between the two strands show the hairpin loops; arrowheads mark the initiation sites and indicate the direction of ORFs.

```
Pst I   .         .         .         .         .         .
CTGCAGGGCGACGACTGACCCGTCACGACTCGGGGGAGGGGCCCGTCCGGCATCGCGCCG 60
GACGTCCCGCTGCTGACTGGGCAGTGCTGAGCCCCCTCCCCGGGCAGGCCGTAGCGCGGC

        .         .         .         .         .         .
GGCGGGCCCTTCCGCGTTCCCGGAGGGCTCAGAGCATTCTGCGGGCCGCCCGGCGCAGGT 120
CCGCCCGGGAAGGCGCAAGGGCCTCCCGAGTCTCGTAAGACGCCCGGCGGGCCGCGTCCA

        .         .         .         .         .         .
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GCGTGAGACGTCCTAGCGGAAGCGCACCGGCATCCGCTCGAGCACGCGGGGCGCCTCGGT

        .         .         .         .         .         .
GCTCACCCGGTCCTCGAAGCGGAGGAGGGAGGGGCCCTCTTCGACGGTTCTCATCCATTC 240
CGAGTGGGCCAGGAGCTTCGCCTCCTCCCTCCCCGGGAGAAGCTGCCAAGAGTAGGTAAG

        .         .         .         .         .         .
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CCGCTAGAGTGCTGGCCACGCCGCGCCCTACGCCCGGTCGTACAACGCTACCCAGAGAAG

        .         .         . Mlu I   .         .         .
GGAGAAAGTCTGGGACATCGGCGCCTCCGGACGCGTCGTACCGTGTGCTCCTCGTGCTCC 360
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        .         .         .         .         .         .
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        .         .         .         .         .         .
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        .         .         .         .         .         .
CGATCTGGCCGAAATCCTGCGAACGGCCCCTGCCGGGTCCCCTGCGGGGGAGCTGGCGGA 720
GCTAGACCGGCTTTAGGACGCTTGCCGGGGACGGCCCAGGGGACGCCCCCTCGACCGCCT

        .         .         .         .         .         .
GGCCGTGGGTCTGGTCCGTGCGGCGGCGGAGCGCTCGGGGCTGTAGCTGGGCCCCGTCGG 780
CCGGCACCCAGACCAGGCACGCCGCCGCCTCGCGAGCCCCGACATCGACCCGGGGCAGCC
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Fig. 16 continued on page 110

Fig. 16 continued

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      .         .         .         .         .         .
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      .         .         .         .         .         .
ACGTTCTCCTCGTCGGACGCGCCGTACGAGAAGGTGAGGGCGAAGGCGCCGGAGACGCCC 960
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      .         .         .         .         .         .
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      .         .         .         .         .         .
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      .         .         .         .         .         .
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      .Nco I    .         .   Xho I .         .         .
GCCTCCGCCTCCATGGCCTCCAGGGCCATCGCCTCGAGGCCGACGAAGTCCGCCTGGCGC 1380
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      .         .         .         .         .         .
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      .         .         .         .         .         .
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Fig. 16 continued on page 111

Fig. 16 continued

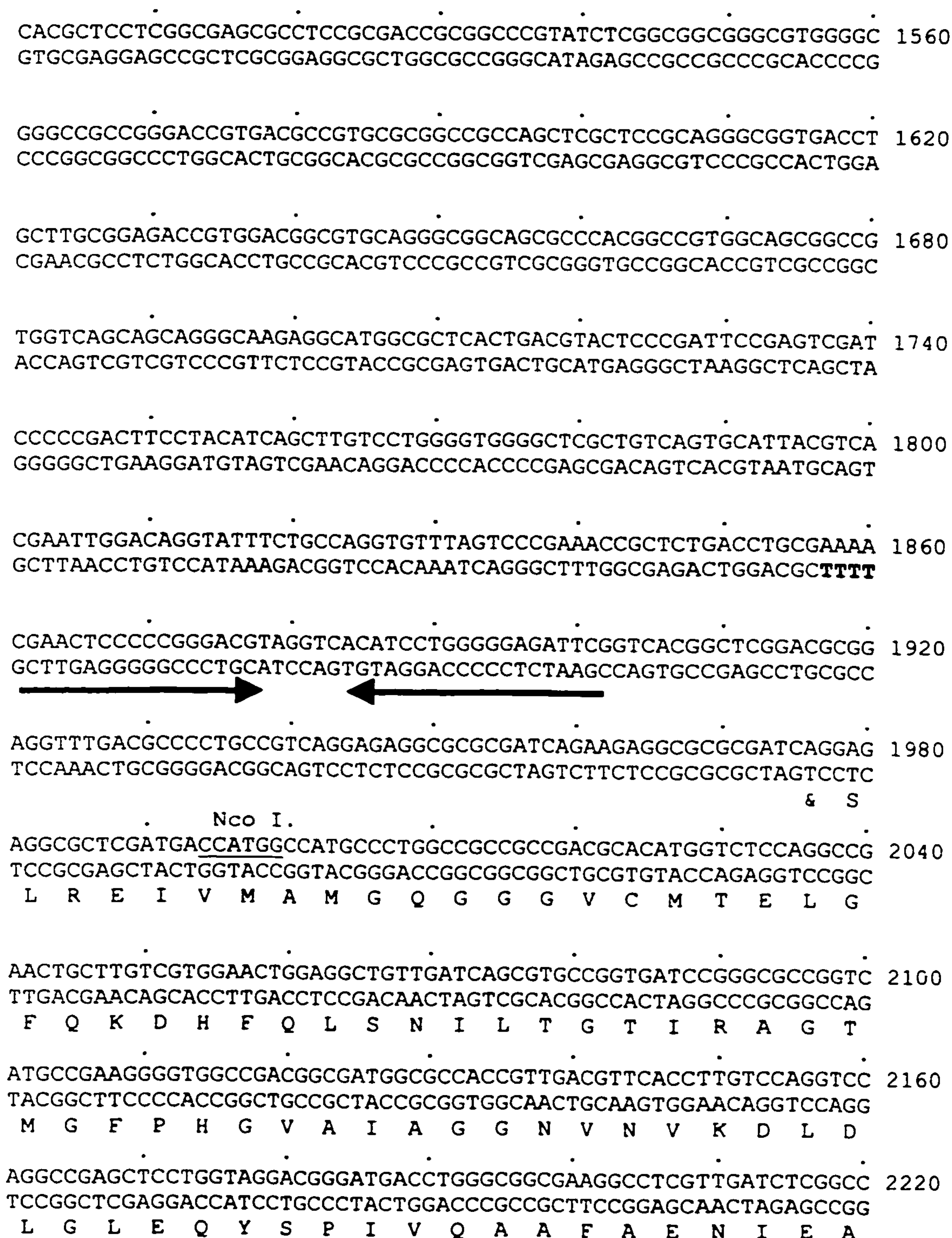

Fig. 16 continued on page 112

Fig. 16 continued

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 P  G  L  G  M  I  E  P  S  L  G  S  V  G  T  S  V  I  R  A

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 L  P  T  L  G  L  E  R  A  K  T  D  S  M  I  V  L  A  A  A

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 K  L  G  S  V  G  E  L  T  V  G  A  R  P  G  D  D  T  S  V

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 V  T  G  D  P  L  T  V  P  T  I  E  R  E  W  F  G  N  K  I

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 A  Q  E  A  L  N  Q  S  R  V  G  F  E  D  M  E  Q  R  T  I

                                   Nco I       Mlu I
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 K  V  F  R  S  V  M  E  V  G  A  S  I  F  V  D  G  E  G  A

                              Pst I
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 K  I  A  H  L  A  M  R  S  T  Q  L  S  S  S  C  Y  R  H  I
```

Fig. 16 continued on page 113

Fig. 16 continued

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CCCTGCTCGCCGCCCGGGAGGCCGCAGCCCAGCATCAGGTCGTCGATGTCGCGCGGGTCG 3060
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 G  Q  E  G  G  P  L  G  C  G  L  M  L  D  D  I  D  R  P  D

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                 <<<ORF2  RBS
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 V  A  E  P fM      ******

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Fig. 16 continued on page 114

Fig. 16 continued

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Fig. 16 continued on page 115

Fig. 16 continued

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 *****   *****     >>ORF1
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 T  G  V  G  L  A  I  V  A  Q  Q  K  G  Y  K  C  I  F  V  C
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 R  S  H  Y  E  T  T  G  P  E  L  W  E  Q  T  D  G  K  I  T
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```

Fig. 16 continued on page 116

Fig. 16 continued

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 G  G  S  G  R  P  Y  L  V  E  G  V  G  E  D  F  W  P  T  A
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         .         .         .         .         .         .

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 P  D  S  G  R  G  Y  M  S  K  I  F  S  D  E  W  M  A  G  H
GCCGGACTCCGGCCGCGGCTACATGTCGAAGATCTTCAGCGACGAGTGGATGGCCGGCCA 5400
                              Bgl II
CGGCCTGAGGCCGGCGCCGATGTACAGCTTCTAGAAGTCGCTGCTCACCTACCGGCCGGT
         .         .         .         .         .         .

 G  F  L  E  D  T  S  S  A  T  V  A  D  V  L  R  H  K  E  G
CGGCTTCCTGGAGGACACCTCCTCGGCCACCGTCGCGGACGTCCTGCGCCACAAGGAGGG 5460
GCCGAAGGACCTCCTGTGGAGGAGCCGGTGGCAGCGCCTGCAGGACGCGGTGTTCCTCCC
         .         .         .         .         .         .

 G  T  M  P  S  L  V  H  M  H  P  D  E  T  V  G  Q  A  I  E
CGGCACCATGCCCTCCCTGGTCCACATGCACCCGGACGAGACCGTCGGCCAGGCCATCGA 5520
GCCGTGGTACGGGAGGGACCAGGTGTACGTGGGCCTGCTCTGGCAGCCGGTCCGGTAGCT
         .         .         .         .         .         .

 V  L  R  E  Y  G  V  S  Q  M  P  I  V  K  P  G  A  G  H  P
GGTGCTCCGCGAGTACGGCGTCTCGCAGATGCCGATCGTGAAGCCGGGCGCGGGCCACCC 5580
CCACGAGGCGCTCATGCCGCAGAGCGTCTACGGCTAGCACTTCGGCCCGCGCCCGGTGGG
         .         .         .         .         .         .
```

Fig. 16 continued on page 117

Fig. 16 continued

```
 D  V  M  A  A  E  V  V  G  S  V  V  E  R  D  V  L  D  A  L
CGACGTGATGGCCGCCGAGGTCGTCGGATCGGTCGTCGAGCGTGACGTGCTCGACGCCCT 5640
GCTGCACTACCGGCGGCTCCAGCAGCCTAGCCAGCAGCTCGCACTGCACGAGCTGCGGGA
          .         .         .         .         .         .

 F  T  Q  R  A  S  L  E  D  S  L  E  S  H  M  S  A  P  L  P
CTTCACCCAGCGCGCCTCCCTGGAGGACTCGCTGGAGAGCCACATGAGCGCGCCGCTGCC 5700
GAAGTGGGTCGCGCGGAGGGACCTCCTGAGCGACCTCTCGGTGTACTCGCGCGGCGACGG
          .         .         .         .         .         .

 Q  V  G  S  G  E  P  V  A  A  L  M  S  V  L  G  D  A  A  D
GCAGGTCGGCTCGGGCGAGCCGGTCGCCGCCCTGATGTCCGTCCTCGGCGACGCGGCCGA 5760
CGTCCAGCCGAGCCCGCTCGGCCAGCGGCGGGACTACAGGCAGGAGCCGCTGCGCCGGCT
          .         .         .         .         .         .

 A  A  I  V  L  V  E  G  K  P  T  G  V  V  S  R  Q  D  L  L
CGCGGCGATCGTGCTGGTCGAGGGCAAGCCGACCGGTGTGGTGAGCCGTCAGGACCTGCT 5820
GCGCCGCTAGCACGACCAGCTCCCGTTCGGCTGGCCACACCACTCGGCAGTCCTGGACGA
          .         .         .         .         .         .

 A  F  L  A  N  G  G  A  K  *                               1
GGCCTTCCTGGCCAACGGCGGCGCCAAGTAGCACCCGGCGGCGCGGCGGCGGCGTTCGCG 5880
CCGGAAGGACCGGTTGCCGCCGCGGTTCATCGTGGGCCGCCGCGCCGCCGCCGCAAGCGC
          .         .         .         .         .         .

      2   .     1'    .   2'    .         .         .         .
CAATCGGTACGAGCGCGACACGTACCCGCAGCACCCGCTTAACACGGTCCGGCACAGTG 5940

GTTAGCCATGCTCGCGCTGTGCATGGGCGTCGTGGGCGAATTGTGCCCAGGCCGTGTCAC

          .         .         .         .         .         .
GTGGTTGTCGGCGTCACGGACCTCCGGAGCGGCTCCCGGACCTCCAGACGACGCCGCCGG 6000
CACCAACAGCCGCAGTGCCTGGAGGCCTCGCCGAGGGCCTGGAGGTCTGCTGCGGCGGCC

          .         .         .         .         .         .
ACGCGAGACCGGCCCTGACCCGGGCCCGTGTCCTCGCGGGGACCGCGTCGTCCCGCCCCC 6050
TGCGCTCTGGCCGGGACTGGGCCCGGGCACAGGAGCGCCCCTGGCGCAGCAGGGCGGGGG

          .         .         .         .         .         .
CGGTAACGTGCGCGGTCCCCGGCCGAACACCTCGTCACGACGACGGGCCCCTCGCCGAGG 6120
GCCATTGCACGCGCCAGGGGCCGGCTTGTGGAGCAGTGCTGCTGCCCGGGGAGCGGCTCC

          .         .         .         .         .         .
CGGGGTCCGGTCCAACGGCGAGGCCTCATGCGGGCGTTGGTGCCGGGCGCGGGCTGGCCC 6180
GCCCCAGGCCAGGTTGCCGCTCCGGAGTACGCCCGCAACCACGGCCCGCGCCCGACCGGG

          .         .         .   Bgl II  .         .
GTTCCCGGGGAGTCCCGGGCGGAGGTGGTGTAGGAAAGATCTCGTACGCCCCTTCGAACC 6240
CAAGGGCCCCTCAGGGCCCGCCTCCACCACATCCTTTCTAGAGCATGCGGGGAAGCTTGG
```

Fig. 16 continued on page 118

Fig. 16 continued

```
GGGAGTCATCCATGTCTGACGCCGACGACGTCCGCTCGGCCTGGTCCAGGATCACCCACT 6300
CCCTCAGTAGGTACAGACTGCGGCTGCTGCAGGCGAGCCGGACCAGGTCCTAGTGGGTGA

GGCTGGAGAAGCACGCGCCCGACGACCACGCCGCCCTGCGTCCCGGCGCCCCGGAGGCCG 6360
CCGACCTCTTCGTGCGCGGGCTGCTGGTGCGGCGGGACGCAGGGCCGCGGGGCCTCCGGC

ACCTCGCCGCCCTGGAGGACGGCCTCGGCTTCCCGCTGGACCCGCCCCTGCCGCGCGCTG 6420
TGGAGCGGCGGGACCTCCTGCCGGAGCCGAAGGGCGACCTGGGCGGGGACGGCGCGCGAC

CTCTCGGTCTCCGACGGCGTCGTGCCGCGCGTGGCGAGCGACGAGCCGGGGGCCTTCGTC 6480
GAGAGCCAGAGGCTGCCGCAGCACGGCGCGCACCGCTCGCTGCTCGGCCCCCGGAAGCAG

ATCGGCCACAGCCTCCTGGGCACCGGGCAGGTCCTGGAGGGGCAGCGCCACCTCGCCTCC 6540
TAGCCGGTGTCGGAGGACCCGTGGCCCGTCCAGGACCTCCCCGTCGCGGTGGAGCGGAGG

CGGGCGGCCGAGTTCGCCGAGGAGGGGTACGAGGACGAGGTCGGCAGGACGCACGCGCGC 6600
GCCCGCCGGCTCAAGCGGCTCCTCCCCATGCTCCTGCTCCAGCCGTCCTGCGTGCGCGCG

TGGGTTCCGTTCGCCCGGTCCGTCACCGGTGACCTGCTCTTCGTGGACCACCGGGAGGCC 6660
ACCCAAGGCAAGCGGGCCAGGCAGTGGCCACTGGACGAGAAGCACCTGGTGGCCCTCCGG

GGGTACGGGACCGTCGGGGAGCTCTCCTTCGGCGATCCGGACCACGAGCGCCCGGTGGCC 6720
CCCATGCCCTGGCAGCCCCTCGAGAGGAAGCCGCTAGGCCTGGTGCTCGCGGGCCACCGG

                                      Nco I
GGGCCTGGCGGTCATGCTCGACGACCTGGCCACCGCCATGGAGGCGCGGGTTCCGCTGTT 6780
CCCGGACCGCCAGTACGAGCTGCTGGACCGGTGGCGGTACCTCCGCGCCCAAGGCGACAA

CGTGGTGGGCCGCCGGCCCGTGGTCCACGAGGGCCGGATGCTGGAGTGGCCACGGCCTGA 6840
GCACCACCCGGCGGCCGGGCACCAGGTGCTCCCGGCCTACGACCTCACCGGTGCCGGACT

CCTGCTGTGCATATCCTTATACAGGAGATCCGCGTATGAATAGGTGAAGGGGCATGGCAT 6900
GGACGACACGTATAGGAATATGTCCTCTAGGCGCATACTTATCCACTTCCCCGTACCGTA

                       Pst I
GAGCGACGACCCGCCGGCCGGCTGCAGGCCCGGGG 6935
CTCGCTGCTGGGCGGCCGGCCGACGTCCGGGCCCC
```

Fig. 16.

IV. Sequence Analysis

A. Identification of Open Reading Frames

Several software programs were used to detect potential ORFs in the cys7.0 sequence. Streptomycete DNA has a high (68-72%) G+C content, and there is a strong bias favoring G or C at the third position of codons (Bibb et al., 1984). Two ORFs were detected when codon usage and third base G-C bias in the cys7.0 sequence were analyzed by the GCG Codonpreference program (Fig. 17). One was located from nt 4500 to 5900 in frame +2 (ORF1, Fig. 17 A); another was found on the other strand from nt 1900 to 3100 in frame –3 (ORF2, Fig. 17 B). Fig. 18 shows the distribution in the GenBank database of protein sequences similar to the deduced amino acid sequences of cys7.0, as revealed by the BlastX search, ORF1 and ORF2. Only two groups of relevant coding regions exhibited similarities to regions on the cloned DNA fragment. This result was consistent with the Codonpreference analysis.

A BlastX search confirmed that the ORF1 product had marked sequence similarity to CSs of both prokaryotes and eukaryotes, and to CBSs of eukaryotes (Appendix IV, Table AppdxIV-1. The deduced amino acid sequence of ORF1 showed 42% identity and 59% similarity to the CBS of *Homo sapiens*, and 42% identity (63% similarity) to the CS of *Clostridium perfringens*. ORF1 was thought more likely to encode CS than CBS, because CBS is commonly found in eukaryotes rather than bacteria. However, the strong sequence similarity to CBSs suggested that the real function of ORF1 would only be determined by functional analysis (e.g. complementation and/or disruption), Since ORF1 would, if it proved to encode a CBS, be the first such gene cloned from a prokaryotic

Fig. 17. CODONPREFERENCE analysis of (A) strand A and (B) strand B for codon bias, third base GC bias, rare codon usage and putative ORFs. The nucleotide number is plotted on the X axis. On the left side of the Y axis, the frequency of codon usage is based on the codon usage table of *S. venezuelae* genes (NCBI codon usage database). On the right side of the Y axis, the average GC usage at every third position in each codon is recorded. The upper curve shows the GC bias and the lower curve shows the codon preference. Possible ORFs are shown as boxes under the lower curve with short vertical lines that extend above the height of the boxes representing translation start codons and the short vertical lines that extend below the bottom of the boxes representing translation stop codons. Rare codons are displayed as short vertical lines just below the ORFs.

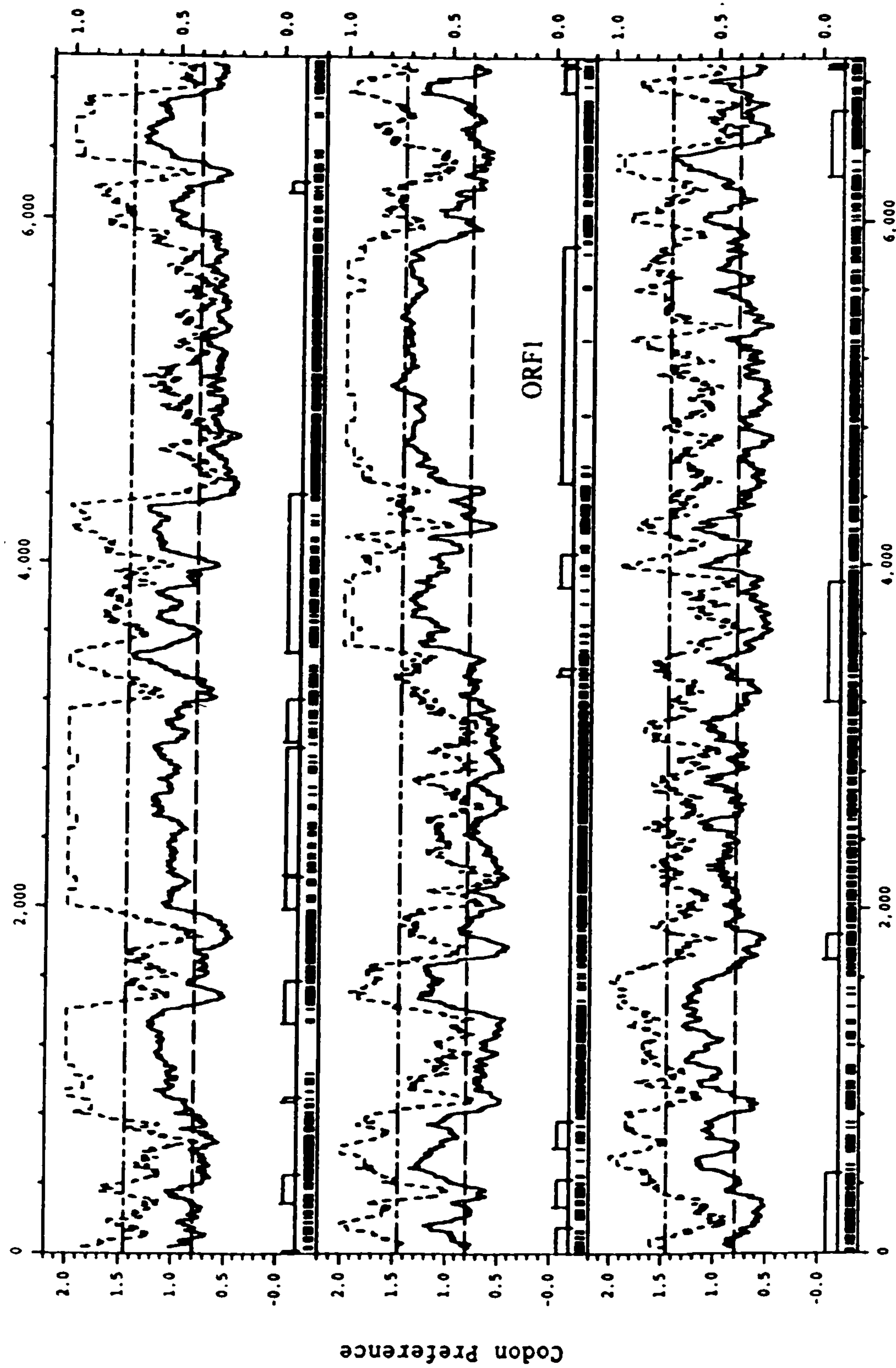

Fig. 17A

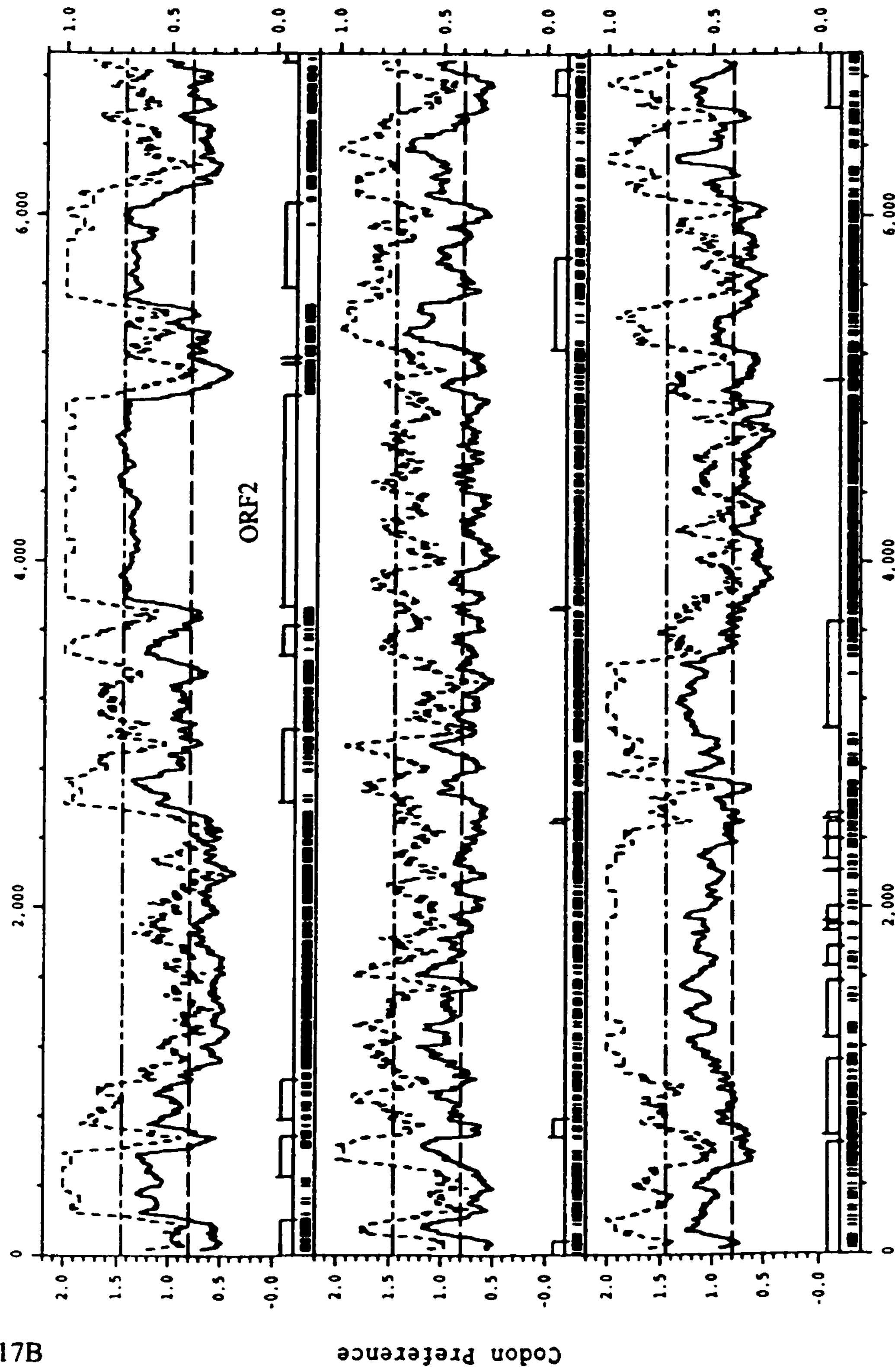

Fig. 17B

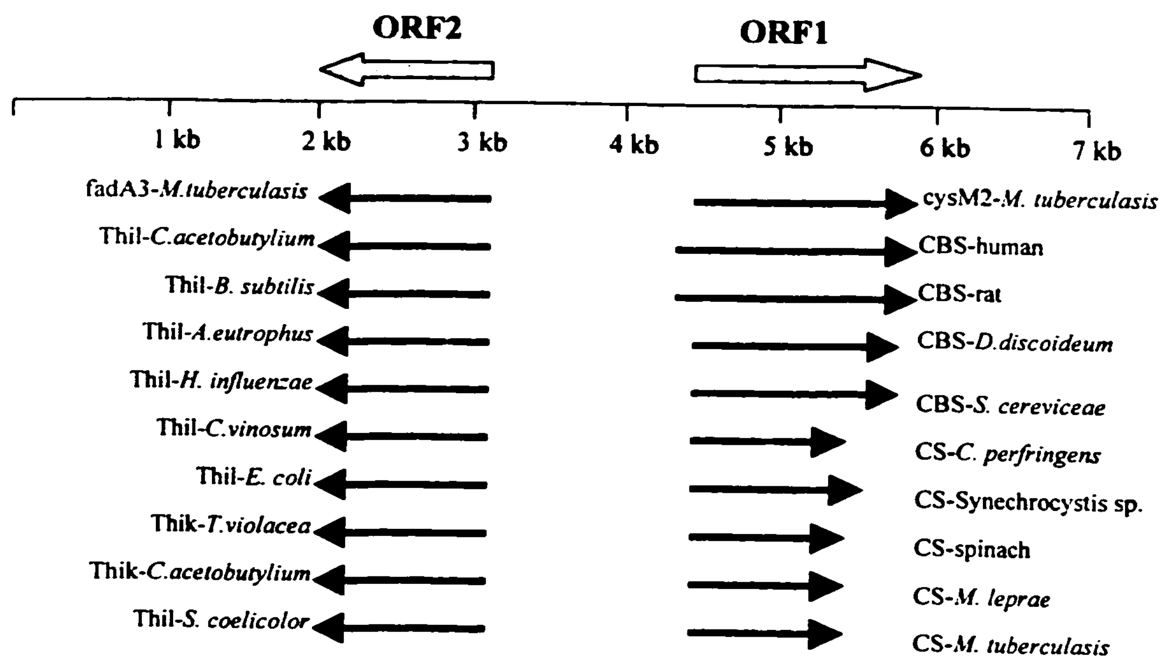

Fig. 18. GenBank database sequences with similarity to the deduced amino acid sequences of ORF1 and ORF2 identified in a BlastX search with the cys7.0 sequence. The unfilled arrows above the 7.0-kb reference line represent the directions, length, and locations of the two ORFs; the filled arrows below the 7.0-kb line indicate the direction, length and relative position of individual sequences detected in the BlastX search. Thil, acetyl-CoA acetyltransferase; thio: β-ketothiolase; CBS, cystathionine β-synthase; CS, cysteine synthase.

organism, and would supply direct evidence that sulfur amino acid metabolism in *Streptomyces* is more like that of fungi than of other bacteria. Gene disruption experiments were subsequently undertaken to obtain the information needed to resolve ORF1's identity.

A BlastP search for protein sequences with similarity to the deduced amino acid sequence of ORF2 identified in the GenBank database a number of acetyl-CoA C-acetyltransferases (thiolases) and acetyl-CoA acyltransferases (*thiL* and *thiK*) of bacteria (Appendix IV, Table AppdxIV-2) with marked similarity to the deduced amino acid sequence of ORF2. These enzymes participate in the oxidation of fatty acids (as a subunit of fatty acid oxidation multienzyme complexes). The ORF2 sequence showed 45% identity and 62% similarity to acetyl-CoA acetyltransferase of *Clostridium acetobutylicum*. Since ORF1 is of obvious interest, and ORF2 could potentially be involved in conversion of acetoacetyl-CoA to dichloroacetyl-CoA in the biosynthesis of Cm, the functions of both ORFs were investigated by complementation and disruption procedures.

B. Flanking sequences potentially associated with expression of ORF1 and ORF2

Regions upstream and downstream of ORF1 and ORF2 were examined for sequence elements possibly involved in translation, transcription and regulation.

a. Ribosome binding sites.

In *Streptomyces* genes, ribosome binding sequences are located 2-11 nt upstream of translational initiation codons. These sequences, to some degree, complement sequence at the 3' end of the 16S rRNA. For ORF1, no sequence complementary to the 3' end of the

16S rRNA of *S. lividans* (Bibb & Cohen, 1982) preceded the start codon (ATG) at position 4478. Six codons in front of ATG, a GTG is located in the same frame, and at 10 and 18 nt before the GTG codon, there are two copies of the sequence GGAGAT complementary to 3' end of 16S rRNA of *S. lividans* (Fig. 16). The GCG was considered to be the real start codon of ORF1 and the sequence GGAGAT was assigned as the putative RBS. Later amino acid sequence comparisons supported these assignments. In front of the ATG start codon of ORF2, a putative RBS (ATGGAG) was readily identified (Table 5).

b. Promoter sequences.

No transcriptional signals corresponding to the conserved −10 (tAggNT) and −35 (ttGacN) promoter sequences that have been associated with some streptomycete genes (Seno & Baltz, 1989) were detected in the DNA sequence preceding the putative RBSs of ORF1 and ORF2. Thus ORF1 and ORF2 appear to provide another example where transcriptional promotors of streptomycete genes are poorly conserved (Bibb & Cohen, 1982, Bibb et al., 1985, Seno & Baltz, 1989).

c. Terminators

A search with the Terminator program of the GCG package for sequences terminating transcription of the ORFs did not detect any putative terminator sequences following the stop codon of ORF1 on the same strand (strand A). However, several 6-nucleotide inverted repeats that could form hairpin loops were found 30 to 100 nt downstream of the stop codon

Table 5. Sequences of the putative ribosome binding sites for ORF1 and ORF2 compared with the sequence complementary to the 3'-end of *S. lividans* 16S rRNA.

DNA	Sequence
ORF1 RBS	5' GACCCGGAGAT 3'
S. lividans 16S rRNA	5' AGAAAGGAGGTGATC 3'
ORF2 RBS	5' CTCATGGAGCCG 3'
Conserved sequence	****

(marked with hatched arrows in Fig. 16). They may play a role in terminating translation of the gene. Further (80 nt) downstream of ORF2, a putative terminator in the form of complementary inverted repeats was present (marked with thick arrows in Fig. 16), followed by a run of 4 Ts. High A+T regions were located upstream and downstream of the coding regions of both ORF1 and ORF2. Several triple or tetra A-T runs were found only in these regions. As in *E. coli* (Rosenberg & Court, 1979), these A+T rich sequences probably contain transcriptional signals.

d. Codon usage and codon composition

Streptomycetes are noted for their high G+C content and the bias in their codon usage that favors G or C at the third codon position (Bibb et al., 1984, 1985). Where more than one codon can specify an amino acid, the one with C or G at the third position is normally used; C or G is also preferred over A or T at the first codon position. In Appendix IV, Tables AppdxIII-1 and AppdxIII-2 summarize codon usage and base composition in ORF1 and ORF2, and compare the values with the average values for *S. venezuelae* genes, as well as for genes used in the genus *Streptomyces*.

The overall G+C content of the 7.0-kb of DNA sequenced in this study was 72.5% mol%. Although the contents in ORF1 and ORF2 were lower than the overall value (71.9%), G and C dominated at the third codon positions, and the value was even higher (97.8% and 98.8% for ORF1 and ORF2 respectively) than the average value for *S. venezuelae* genes (95.2%). In ORFs1 and 2, C was substantially favoured (61.1% and 64.6%) over G (36.7% and 34.2%) in the third positions. As in other *Streptomyces* genes, G and C were also

preferred at the first codon positions of ORF1 (67.2%) and ORF2 (67.1%), but at this position G (45.6% and 41.5%) was favoured over C (21.6% and 25.6%).

e. Amino acid composition of gene products.

Amino acid selection in the two ORFs is given in Appendix III, Table AppdxIII-3. ORF1 contains high percentages of glycine (11.42%) and valine (12.28%), and low percentages of cysteine (0.86%) and tyrosine (0.86%). In ORF2, glycine (11.55%) and alanine (10.57%) were used more often, whereas tyrosine (0.74%) and tryptophan (0.74%) were rarely used.

C. Comparison of deduced amino acid sequence with protein sequences in databases

A BlastX search comparing the deduced amino acid sequences of ORFs1 and 2 of cys7.0 with the genome sequences of *S. coelicolor* and *M. tuberculosis* showed close similarities to two predicted genes, 1.4 kb apart, in cosmid E25 from *S. coelicolor* (82% identity and 87% similarity for ORF1; 89% identity and 91% similarity for ORF2). Two putative genes (*cysM2* and *fadA3*) of *M. tuberculosis* also showed strong similarity to the deduced amino acid sequences of ORF1 and ORF2, respectively. CysM2 has 68% identical amino acids (79% similar) to the ORF1 product; FadA3, a putative β-ketoacyl CoA thiolase shows 75% identity (84% similarity) to the ORF2 product. *cysM2* and *fadA3* are located 2.3-kb apart on the *M. tuberculosis* chromosome. Thus these pairs of genes are similarly organized on the chromosomes of *S. coelicolor*, *S. venezuelae* and *M. tuberculosis*. Blast search results of the ORF1 and ORF2 products are shown in Appendix IV, Tables AppdxIV-

1 and ApppdxIV-2).

The ORF1 product showed high sequence similarity to both CBSs and CSs of various organisms. Sequences matched with high scores in the Blast search were aligned with ClustalW (Fig 19). The N-terminal region of the translated amino acid sequence of ORF1 aligned very well with those of the CSs of *B. subtilis*, *Clostridium* and *cysM2* of *M. tuberculosis* if translation was initiated from the GTG at position 4460. Although CSs aligned well with CBS at their N-terminals, most were about 150 amino acids shorter than CBSs. In size the ORF1 product was similar to the CBSs (about 450 aa) and aligned well with human and yeast CBSs and with CysM2 (a putative CBS) of *M. tuberculosis* at its C-terminal end.

Altogether, 39 deduced amino acid sequences of *cysK* and *cysM*, other cysteine synthases (CSs) and cystathionine β-synthases (CBSs) of various bacteria, fungi, plants and animals were compared with the ORF1 product (CBS-*S. venezuelae*). The highly conserved area is shown in Fig. 20.

Fig. 19. Alignment of the deduced ORF1 sequence with CSs and CBSs. Asterisks represent identical amino acids in all sequences aligned; colons indicate highly conserved positions; periods represent amino acids with similar properties.

```
cys-C.perfringens          --------------------------------------------------
cbs-yeast                  --------------------------------------------------
cbs-human                  MPSETPQAEVGPTGCPHRSGPHSAKGSLEKGSPEDKEAKEPLWIRPDAPS 50
cbs-S.venezuelae           --------------------------------------------------
cysM2-M.tuberculosis       --------------------------------------------------
cysK-B.subtilis            --------------------------------------------------

cys-C.perfringens          -----------------------MKYYNDIRDLIGNTPILKLNNISTKEG 27
cbs-yeast                  ----------------MTKSEQQADSRHNVIDLVGNTPLIALKKLPKALG 34
cbs-human                  RCTWQLGRPASESPHHHTAPAKSPKILPDILKKIGDTPMVRINKIGKKFG 100
cbs-S.venezuelae           -----------------------VQFHDSMISLVGNTPLVKLNNVTAGIQ 27
cysM2-M.tuberculosis       -----------------------MRIAQHISELIGGTPLVRLNSVVPDGA 27
cysK-B.subtilis            ----------------------MVRVANSITELIGNTPIVKLNRLADENS 28
                                                        : . :*.**:: :: :

cys-C.perfringens          VN--IYAKIEGTSPGGSCKDRVGIYMVEKAEKEGKLKPG-STIIEATAGN 74
cbs-yeast                  IKPQIYAKLELYNPGGSIKDRIAKSMVEEAEASGRIHPSRSTLIEPTSGN 84
cbs-human                  LKCELLAKCEFFNAGGSVKDRISLRMIEDAERDGTLKPG-DTIIEPTSGN 149
cbs-S.venezuelae           AT--VLAKVEYFNPGGSVKDRIAVRMIEAAEQSGELKPG-GTIVEPTSGN 74
cysM2-M.tuberculosis       GT--VAAKVEYLNPGGSSKDRIAVKMIEAAEASGQLKPG-GTIVEPTSGN 74
cysK-B.subtilis            AD--VYLKLEYMNPGSSVKDRIGLAMIEAAEKEGKLKAG-NTIIEPTSGN 75
                               :  * *  ..*.* ***:.  *:* ** .* ::.. .*::*.*:**

cys-C.perfringens          TGIGIALAAINKGYKIIFIVPDKFSIEKQKIMKALGAEIINTP---KEEG 121
cbs-yeast                  TGIGLALIGAIKGYRTIITLPEKMSNEKVSVLKALGAEIIRTPTAAAWDS 134
cbs-human                  TGIGLALAAAVRGYRCIIVMPEKMSSEKVDVLRALGAEIVRTPTNARFDS 199
cbs-S.venezuelae           TGVGLAIVAQQKGYKCIFVCPDKVSLDKINVLRAYGAEVVVCPTAVDPEH 124
cysM2-M.tuberculosis       TGVGLALVAQRRGYKCVFVCPDKVSEDKRNVLIAYGAEVVVCPTAVPPHD 124
cysK-B.subtilis            TGIGLAMVAAAKGLKAILVMPDTMSMERRNLLRAYGAELVLTPGAEG--- 122
                           **:*:*: .  :* : ::  *:..* :: .:: * ***::  *

cys-C.perfringens          MEGAINLANSLLSEIPNSLSLNQFKNEANPLAHYETTGRELYDGL-DGE- 169
cbs-yeast                  PESHIGVAKKLEKEIPGAVILDQYNNMMNPEAHYFGTGREIQRQL-EDLN 183
cbs-human                  PESHVGVAWRLKNEIPNSHILDQYRNASNPLAHYDTTADEILQQC-DGK- 247
cbs-S.venezuelae           PDSYYNVSDRLVRETPGAWKPDQYSNPNNPRSHYETTGPELWEQT-DGK- 172
cysM2-M.tuberculosis       PASYYSVSDRLVRDIDGAWKPDQYANPEGPASHYVTTGPEIWADT-EGK- 172
cysK-B.subtilis            MKGAIKKAEELA-EKHGYFVPQQFNNPSNPEIHRQTTGKEIVEQFGDDQ- 170
                             .    :  *  :  .    :*: *  .*  *   *. *:     :.

cys-C.perfringens          ----IDYFVAGAGSGGTISGVLKFLKENIS-EVKGILADPVGS-IIGGGQ 213
cbs-yeast                  LFDNLRAVVAGAGTGGTISGISKYLKEQND-KIQIVGADPFGS-ILAQPE 231
cbs-human                  ----LDMLVASVGTGGTITGIARKLKEKCP-GCRIIGVDPEGS-ILAEPE 291
cbs-S.venezuelae           ----ITHFVAGVGTGGTISGTGNYLKEASGGSVKIIGADPEGS-VYSGGS 217
cysM2-M.tuberculosis       ----VTHFVAGIGTGGTITGAGRYLKEVSGGRVRIVGADPEGS-VYSGGA 217
cysK-B.subtilis            ----LDAFVAGIGTGGTITGAGEVLKEAYP-SIKIYAVEPSDSPVLSGGK 215
                               :  .**. *:****:*  . ***      :   .:* .* : .

cys-C.perfringens          CG------TYKIEGIGNNFIPETMDMSLVDDVIKVNDEEAFDAVKLLAKK 257
cbs-yeast                  NLNKTDITDYKVEGIGYDFVPQVLDRKLIDVWYKTDDKPSFKYARQLISN 281
cbs-human                  ELNQTEQTTYEVEGIGYDFIPTVLDRTVVDKWFKSNDEEAFTFARMLIAQ 341
cbs-S.venezuelae           GR------PYLVEGVGEDFWPTAYDRNVTDRIVAVSDKDSFQMTRRLAKE 261
cysM2-M.tuberculosis       GR------PYLVEGVGEDFWPAAYDPSVPDEIIAVSDSDSFDMTRRLARE 261
cysK-B.subtilis            PG------PHKIQGIGAGFVPDILNTEVYDEIFPVKNEEAFEYARRAARE 259
                                    : ::*:* .* *   :  : *     .:. :*  .:    :
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Fig. 19. Continued on page 132

Fig.19. Continued

```
cys-C.perfringens            EGLIVGSSSGAAFAAVLKLAEK----IEKGNIVTIFPDRGDRYFSTDLF- 302
cbs-yeast                    EGVLVGGSSGSTFTAVVKYCEDHPELTEDDVIVAIFPDSIRSYLTKFVDD 331
cbs-human                    EGLLCGGSAGSTVAVAVKAAQE---LQEGQRCVVILPDSVRNYMTKFLSD 388
cbs-S.venezuelae             EGLLVGGSCGMAVVAALEVARE---LGPDDVVVVLLPDSGRGYMSKIFSD 308
cysM2-M.tuberculosis         EAMLVGGSCGMAVVAALKVAEE---AGPDALIVVLLPDGGRGYMSKIFND 308
cysK-B.subtilis              EGILGGISSGAAIYAALQVAKK---LGKGKKVLAIIPSNGERYLSTPL-- 304
                             *.:: * *.* :. ..:: ...        :.::*.    *::. .

cys-C.perfringens            --------------------------------------------------
cbs-yeast11                  EWLKKNNLWDDDVLARFDSSKLEASTTKYADVFGNATVKDL-HLKPVVSV 380
cbs-human6                   RWMLQKG------FLK------EEDLTEKKPWWWHLRVQEL-GLSAPLTV 425
cbs-S.venezuelae1            EWMAGHG------FLED---------TSSATVADVLRHKEGGTMPSLVHM 343
cysM2-M.tuberculosis         AWMSSYG------FLRSR----LDGSTEQSTVGDVLRRKSG-ALPALVHT 347
cysK-B.subtilis              -----YQ------FD----------------------------------- 308

cys-C.perfringens            --------------------------------------------------
cbs-yeast                    KETAKVTDVIKILKDNGFDQLPVLTEDGKLSGLVTLSELLRKLSINNSN- 429
cbs-human                    LPTITCGHTIEILREKGFDQAPVVDEAGVILGMVTLGNMLSSLLAGKVQP 475
cbs-S.venezuelae             HPDETVGQAIEVLREYGVSQMPIVKP-GAGHPDVMAAEVVGSVVERDVLD 392
cysM2-M.tuberculosis         HPSETVRDAIGILREYGVSQMPVV---GA-EPPVMAGEVAGSVSERELLS 393
cysK-B.subtilis              --------------------------------------------------

cys-C.perfringens            --------------------------------------------------
cbs-yeast                    NDNTIKGKYLDFKKLNNFNDVSSYNENKSGKKKFIKFDENSKLSDLNRFF 479
cbs-human                    SDQVGKVIYKQFKQIRLTDTLGRLSHILEMDHFALVVHEQIQYHSTGKSS 525
cbs-S.venezuelae             ALFTQRASLEDSLESHMSAPLP---QVGSGEPVAALMSVLGDAADAAIVL 439
cysM2-M.tuberculosis         AVFEGRAKLADAVSAHMSPPLR---MIGAGELVSAAGKALRD-WDALMVV 439
cysK-B.subtilis              --------------------------------------------------

cys-C.perfringens            ----------------------------
cbs-yeast                    EKNSSAVITDGLKPIHIVTKMDLLSYLA 507
cbs-human                    QRQMVFGVVTAIDLLNFVAAQERDQK-- 551
cbs-S.venezuelae             VEGKPTGVVSRQDLLAFLANGGAK---- 463
cysM2-M.tuberculosis         EEGKPVGVITRYDLLGFLSEGAGRR--- 464
cysK-B.subtilis              ----------------------------
```

Fig. 19.

```
cysM-E. coli                LKLEGNNPAGSVKDRAALSMIVEAEKRGEIKP--GDVLIEATSGNTGIALAMIAALKGYR 86
cysM-S.typhimurium          VKLEGNNPAGSVKDRAALSMIVEAEKRGEIKP--GDVLIEATSGNTGIALAMIAALKGYR 86
cysK-M.tuberculosis         AKLEFFNPANSVKDRIGVAMLQAAEQAGLIKP--DTIILEPTSGNTGIALAMVCAARGYR 89
CS-M.leprae                 AKLESFNPANSVKDRIGVAMIDTAEEAGLIKP--DTVILEPTSGNTGIALAMVCAARGYH 89
Csb-Synethocystis           VKLEGMNPAASVKDRIGINMINRAEQEGLIEPG-KTLLIEPTSGNTGIALAMVAAAKGYQ 111
cysK-Ecoli                  AKVESRNPSFSVKCRIGANMIWDAEKRGVLKP--GVELVEPTSGNTGIALAYVAAARGYK 87
cysK-S.typhimurium          AKVESRNPSFSVKCRIGANMIWDAEKRGVLKP--GVELVEPTNGNTGIALAYVAAARGYK 87
cysK-H.influenzae           VKIEGRNPSYSVKCRIGANMVWQAEKDGTLTK--GKEIVDATSGNTGIALAYVAAARGYK 87
CSa-S.tuberosum             AKLESMEPCSSVKDRIGYSMITDAEEKGLIKPG-ESVLIEPTSGNTGVGLAFMAAAKGYK 95
CS-potato                   AKLESMEPCSSVKDRIGYSMITDAEEKGLIKPG-ESVLIEPTSGNTGVGLAFMAAAKGYK 95
cysK-watermelon             AKLEMMEPCSSVKDRIGYSMISDAENKGLITPG-ESVLIEPTSGNTGIGLAFIAAAKGYR 95
cysK.spinich                AKLEGMEPCSSVKDRIGFSMITDAEKSGLITPG-ESVLIEPTSGNTGIGLAFIAAAKGYK 95
CS-B.juncea                 AKLEMMEPCSSVKDRIGFSMISDAEKKGLIKPG-ESVLIEPTSGNTGVGLAFTAAAKGYK 94
CSa-A.thaliana              AKLEMMEPCSSVKDRIGFSMISDAEKKGLIKPG-ESVLIEPTSGNTGVGLAFTAAAKGYK 92
cysL-spinach                AKLESMEPCCSVKDRIGYSMIDDAEQKGVITPG-KTTLVEPTSGNTGIGLAFIAAARGYK 154
cysB-spinach                AKLESMEPCCSVKDRIGYSMIDDAEQKGVITPG-KTTLVEPTSGNTGIGLAFIAAARGYK 154
CS-spinich                  AKLESMEPCCSVKDRIGYSMIDDAEQKGVITPG-KTTLVEPTSGNTGIGLAFIAAARGYK 154
csB-S.tuberosum             AKLEIMEPCCSVKDRIGFSMIVDAEEKGLISPG-KTVLVEPTSGNTGIGLAFIAASRGYK 156
CSb-A.thaliana              AKLEIMEPCCSVKDRIGYSMITDAEEKGLITPG-KSVLVESTSGNTGIGLAFIAASKGYK 162
cysK-wheat                  VKIEYMNPAGSVKDRIGAAMLAAAEKDGTVIPG-VTTLIEPTSGNTGIALAFVAAAKGYR 97
cysK-B.subtilis             LKLEYMNPGSSVKDRIGLAMIEAAEKEGKLKA--GNTIIEPTSGNTGIGLAMVAAAKGLK 90
CS-O.sativa                 GKMEAYQPLCSVKDRSALRMIEDAEEKGLITPG-VTTLIEPTSGNLGIGLVLVAVQKGYR 129
cysM-C.jejuni               AKCEFLNPSHSIKDRAAFEMIKDALDSKKINQ--DTTIVEATSGNTGISLAMICADLGLK 85
cysK-Flavobacterium         IKLEKSNPGGSIKDRIALAMIEDAEAKGLLNK--DSTIIEPTSGNTGIGLALVAAVKGYK 87
CBS-S.venezuelae            AKVEYFNPGGSVKDRIAVRMIEAAEQSGELKPG--GTIVEPTSGNTGVGLAIVAQQKGYK 89
cysM2-M.tuberculosis        AKVEYLNPGGSSKDRIAVKMIEAAEASGQLKPG--GTIVEPTSGNTGVGLALVAQRRGYK 89
CBS-human                   AKCEFFNAGGSVKDRISLRMIEDAERDGTLKPG--DTIIEPTSGNTGIGLALAAAVRGYR 164
CBS-rat                     AKCEFFNAGGSVKDRISLRMIEDAERAGTLKPG--DTIIEPTSGNTGIGLALAAAVKGYR 161
CBS-F.rubripes              VKCEFFSAGGSIKDRIALRMVEDAERAGLLKPG--DTIIEPTSGNTGIGLALVASVKGYR 168
CBS-D.discoideum            AKCEFFNAGGSVKDRIGHRMIVDAEESGRIKKG--DTLIEPTSGNTGIGLALTAAIKGYK 118
CBS-yeast                   AKLELYNPGGSIKDRIAKSMVEEAEASGRIHPS-RSTLIEPTSGNTGIGLALIGAIKGYR 99
CS-C.perfringens            AKIEGTSPGGSCKDRVGIYMVEKAEKEGKLKPG--STIIEATAGNTGIGIALAAINKGYK 89
cysK-H.pylori               AKLEHLNPGGSVKDRLGQYLIGEGFKTGKITSK--TTIIEPTAGNTGIALALVAIKHHLK 91
CSa-Synechocystis           GKAEFMNPGGSVKDRAALGIIETAEKEGKLKP--GGTVVEGTAGNTGIGLAHICNAKGYK 89
CS-R.sphaeroides            GKCEFLNPGQSVKDRAALYIIRDAVAKGLLQP--GGTIVEGTAGNTGIGLSLVGASMGFR 89
cysK-E.nidulans             AKAEFQNPGGSVKDRAALYVVKDAEERGLLKP--GGTVVEGTAGNTGIGLAHVCRSKGYK 119
CS-S.pobe                   AKAEFLNPGNSPKDRVALQMIRTAEENGDLVPYQSNAVYEGTAGSTGISIAMLCCSLGYD 130
cysM-A.aeolicus             AKLESFNPGGSVKDRPALSMFLDALKRGLIKEG--KVVIDATSGNTGIALAMVGAALGVP 110
cysM-M.tuberculosis         AKLEDRNPTGSIKDRPAVRMIEQAEADGLLRPG--ATILEPTSGNTGISLAMAARLKGYR 96
                             * *  ..  * * * .  :.  .     :       : : * *. *:.:
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Fig. 20. Sequence comparison around pyridoxal phosphate binding sites of CSs and CBSs of various organisms. The ORF1 product is identified as CBS-*S. venezuelae*. The bolded positions are amino acids at putative pyridoxal phosphate binding sides. Asterisks indicate identical amino acids for all sequences aligned; colons indicate highly conserved positions; periods indicate amino acids with similar properties. CSa and Csb are two kinds of cysteine synthases from one species; cysK, cysM, cysL are the products of corresponding genes.

V. Functional Analysis of ORF1 and ORF2

A. Construction of an apramycin resistance gene cassette

The apramycin resistance gene (*apr*) is a valuable molecular and genetic marker for vectors used in gene disruption and gene replacement in streptomycetes. One useful feature is that it can be expressed in a variety of host species, including *E. coli* and streptomycetes. For disrupting streptomycete genes, the apramycin resistance gene is inserted into or replaces part of the target DNA. A limitation in the procedure is the need for compatible restriction enzyme sites in the target and disrupting DNA. Where matching sites are not available, it is possible to construct a disruption cassette carrying the apramycin resistance gene in a fragment with ends that can be blunted. This blunt-ended fragment can then be ligated into sites within the target gene that accept blunt-ended DNA. However, ligation and transformation of streptomycete protoplasts then proceed at very low overall efficiency. In addition, the complex procedures involved are time consuming and often limit the success of gene-disruption experiments. To facilitate the disruption of ORF1 and ORF2, a novel apramycin resistance cassette (pJV225) was constructed by inserting the multi-cloning sites (MCS) of pSK+ on each side of the apramycin resistance gene in pSK+ (Fig. 21). In this cassette, the *apr* gene is flanked symmetrically by more than ten pairs of commonly used restriction enzymes. Therefore, *apr*-containing DNA fragments with more than 10 kinds of sticky ends can be created. By including among these an *Eco*RV site, a blunt-ended *apr* fragment can easily be created, allowing the cassette to be used in any situation where no commonly used restriction enzyme site is present in the target DNA region.

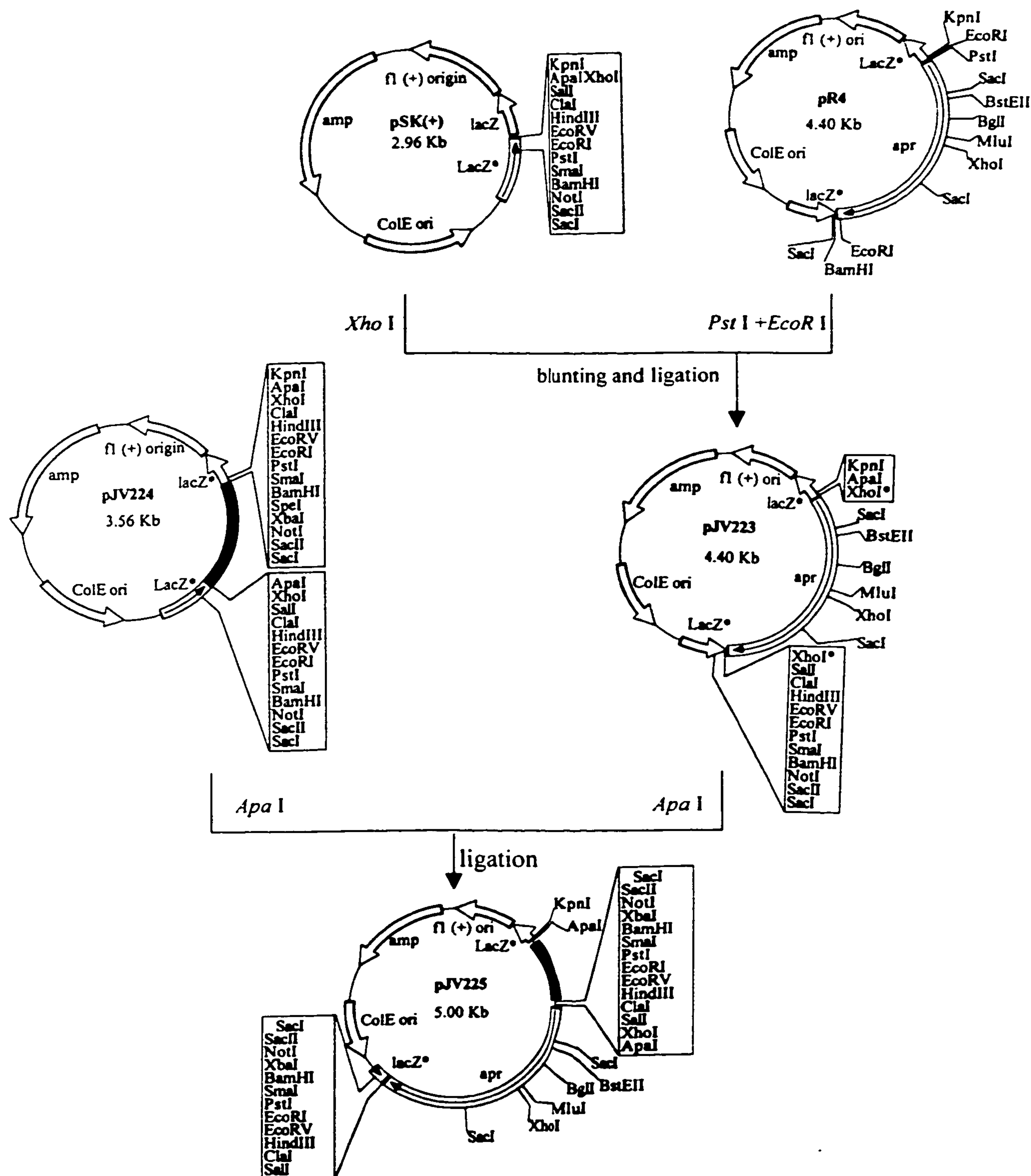


Fig. 21. Construction of plasmid pJV225 (novel apramycin resistance gene cassette). The black-filled area in pJV224 and pJV225 is a 0.6-kb *pabB* containing DNA fragment of *S. venezuelae*. *amp*: ampicillin resistance gene; *apr*: apramycin resistance gene.

This novel cassette was constructed and has been used successfully to disrupt ORF1, *pabA* and *pabB*. It has also proven helpful for disruptng *jadK* (J. He, personal communication) in *S. venezuelae*. Since apramycin is widely used in streptomycetes, the cassette should be of general value for DNA manipulation in the molecular biology of streptomycetes.

B. Complementation of *cys* mutants of *S. venezuelae* and *S. lividans* with ORF1

The 4.0-kb *Pst*I fragment containing entire ORF1 and a part of ORF2 in pJV208 was subcloned into the shuttle vector pHJL400 to give pJV215 and pJV216, with different orientations. pJV215 was used in complementation experiments to transform the *cys-28* mutant of *S. venezuelae* ISP5230 (VS263), and the *cys-107* mutant (strain CH107) of *S. lividans*. Nineteen transformants of VS263 and twenty-four transformants of CH107 were tested for growth requirements on minimal medium, but none of them exhibited prototrophy. The success of the transformations was confirmed by re-isolating pJV215 from the transformants. The inability of the plasmid to complement the host mutations should mean *a priori*, that the cloned gene is not really equivalent to the mutated gene *cys-28* or *cys107* genes. It may mean that the close amino acid sequence similarities of the products of the cloned genes to "CS" are irrelevant because the lesions in VS263 and CH107 are not in *cysK* itself, but in a regulatory gene.

C. Disruption of ORF1

a. Construction of ORF1 disruption cassettes

In another approach to establish the function of ORF1, the cloned gene in pJV216 was disrupted to give pJV217 by replacing the 0.8-kb *Bgl*II segment with a 1.5-kb *Nco* I fragment containing *apr* (Fig. 22). Wild-type *S. venezuelae* ISP5230 transformed with pJV217 gave seven transformants (CHB12-1 to CHB12-7) resistant to thiostrepton and apramycin. CHB12-6 and CHB12-7 were confirmed to be true transformants by extracting plasmid DNA from them and using it to transform an *E. coli* host. To select double crossovers, spores of CHB12-6 and CHB12-7 were harvested after several transfers to MYM medium containing only apramycin. However, no thiostrepton-sensitive presumptive double-crossover mutant colonies were isolated from about 3000 such spores. In view of the instability of pHJL400 in *S. venezuelae*, the absence of plasmid DNA, and the apramycin and thiostrepton resistant phenotypes of these transformants, it was concluded that only single crossovers had taken place. The failure to undergo double crossovers might have been due to secondary structure in the cloned DNA fragment, preventing close alignment of homologous sequences necessary for recombination.

Another pair of ORF1 disruption plasmids (pJV218 and pJV219) was constructed by inserting the *Sal*I fragment of pJV225 containing *apr* into the *Sal*I site into the middle of ORF1 in different orientations (Fig. 23). For this construction, pJV215 and pJV216 were unsuitable starting vectors because of the two *Sal*I sites in pHJL400. Therefore, pJV208 (see Fig. 23) was partially digested with *Sal*I and ligated to a pJV225 *Sal*I fragment carrying *apr*. The recombinant plasmids were screened after transformations designed to insert *apr* in

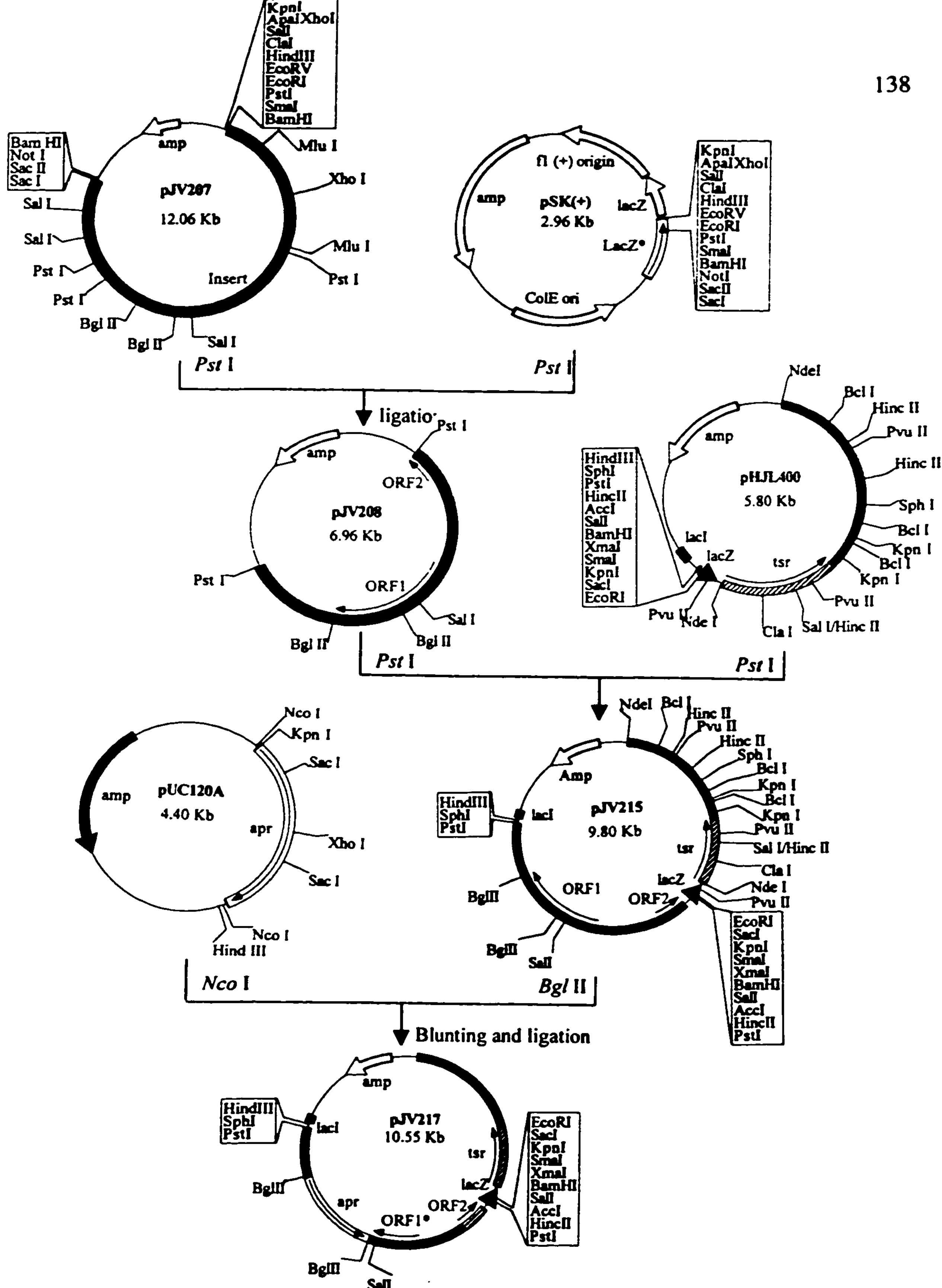


Fig. 22. Construction of pJV217 (ORF1 disruption plasmid) by replacing a *Bgl*II fragment of ORF1 with an *apr*-containing fragment. *amp*: ampicillin resistance gene; *apr*: apramycin resistance gene; *tsr*: thiostrepton resistance gene; ORF1*: disrupted ORF1.

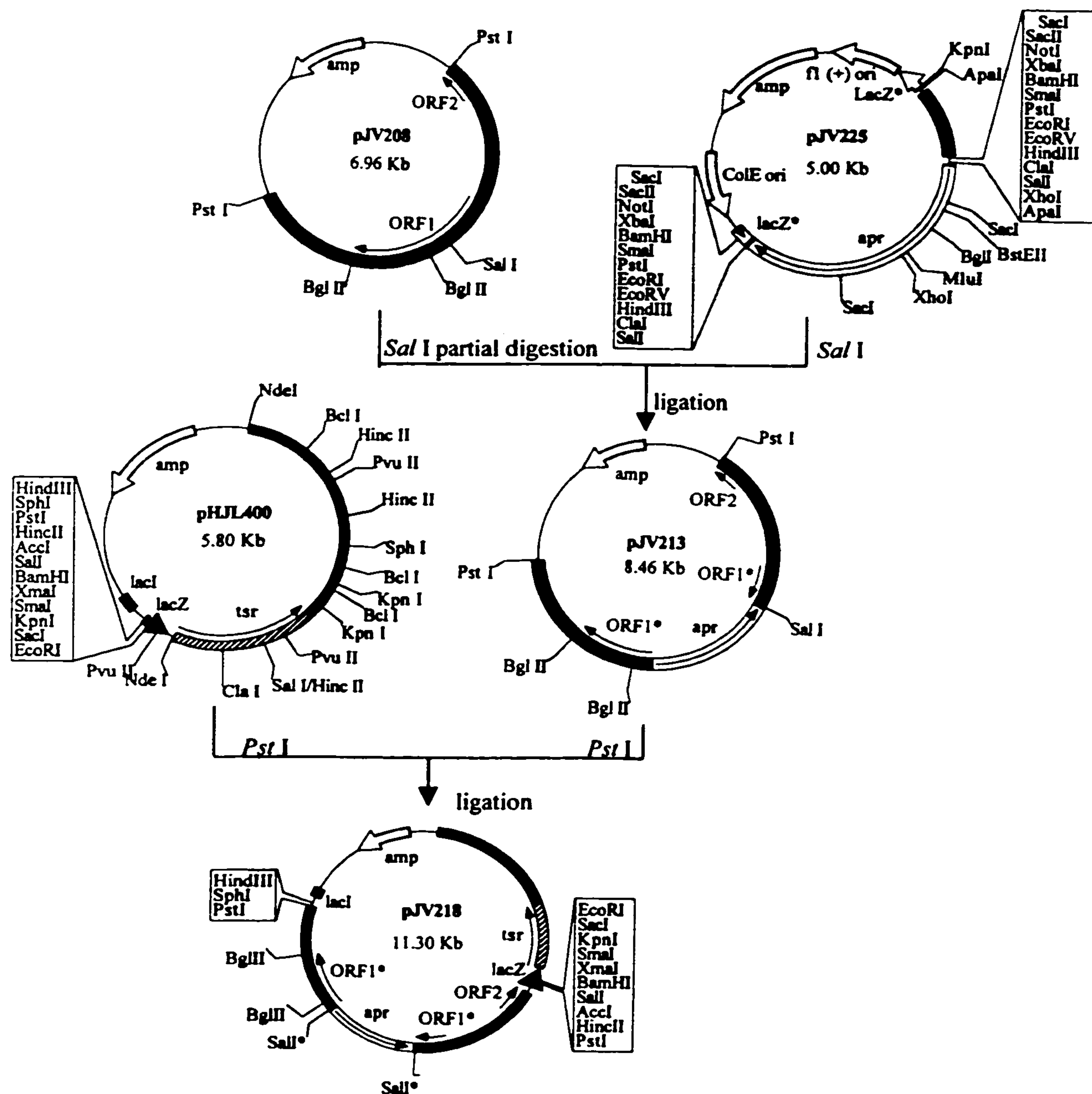


Fig. 23. Construction of pJV218 (ORF1 disruption plasmid) by inserting an *apr*-containing fragment into the *Sal*I site in ORF1: *amp*, ampicillin resistance gene; *apr*, apramycin resistance gene; *tsr*, thiostrepton resistance gene; ORF1*, disrupted ORF1.

ORF1. Plasmid pJV213 was identified as an expected result of recombination. The 5.5-kb *Pst*I fragment of pJV213 containing ORF1* disrupted by insertion of *apr* was excised after gel electrophoresis of the *Pst*I digest and inserted into the *Pst*I site of pHJL400, resulting in pJV218 and pJV219 (opposite orientations). pJV218 and pJV219 were introduced into *E. coli* ET12567 and re-isolated for transforming protoplasts of *S. venezuelae* ISP5230 and VS263.

b. Disruption of ORF1 in the *S. venezuelae* genome

Transforming *S. venezuelae* ISP5230 with pJV218 and pJV219 gave strains CHCp1 and CHCp2, respectively. Transforming the *cys-28* mutant strain VS263 with pJV218 and pJV219 gave strains CCHCp1 and CCHCp2, respectively. All these transformants were resistant to both thiostrepton and apramycin, but routine plasmid extraction of cultures yielded insuffient DNA to be visualized after electrophoresis in agarose gels, presumably because of the instability and low copy number of pHJL400 derivatives in *S. venezuelae*. The presence of the plasmids in transformants was confirmed by using plasmid DNA extracts to transform *E. coli* DH5α, and isolating the expected plasmids from the *E. coli* transformants.

To promote selection of double cross-overs between the disrupted plasmid gene and the corresponding chromosomal gene, strains CHCp1, CHCp2, CCHCp1 and CCHCp2 were grown through two rounds of sporulation on MYM agar without antibiotic selection. Spores were collected and single colonies were isolated on MYM agar containing only the apramycin supplement. When the colonies were screened for resistance to antibiotics, 91%

were apramycin resistant and thiostrepton sensitive. The apramycin-resistant and thiostrepton-sensitive strains CHC1, CHC2, CCHC1 and CCHC2, isolated from CHCp1, CHCp2, CCHCp1 and CCHCp2, respectively, were expected to have exchanged ORF1 for its disrupted counterpart through double crossovers, the occurrence of which was confirmed by Southern hybridization (Fig. 24).

Chromosomal DNA was isolated from CHC1-1, CCHC1-1, and wild-type ISP5230, and then digested with *Pst*I and electrophoresed on an agarose gel (Fig. 24 A). After transfer to a nylon membrane, the DNA bands were probed with the labeled pJV207 insert. In all these disruptants, the 4.0-kb *Pst*I band disappeared and a new *Pst*I band appeared at 5.5-kb (Fig. 24 B). In lane 1 and lane 2, the 4.0-kb *Pst*I fragment (containing ORF1) of the *S. venezuelae* chromosome was replaced by a 5.5-kb *Pst*I fragment because of the insertion of the 1.5-kb *apr* fragment. This band is formed by insertion of the apramycin resistance gene at a *Sal*I site in the middle of ORF1. This indicated that the native ORF1 in the chromosome had been replaced by the disrupted one in plasmid pJV218.

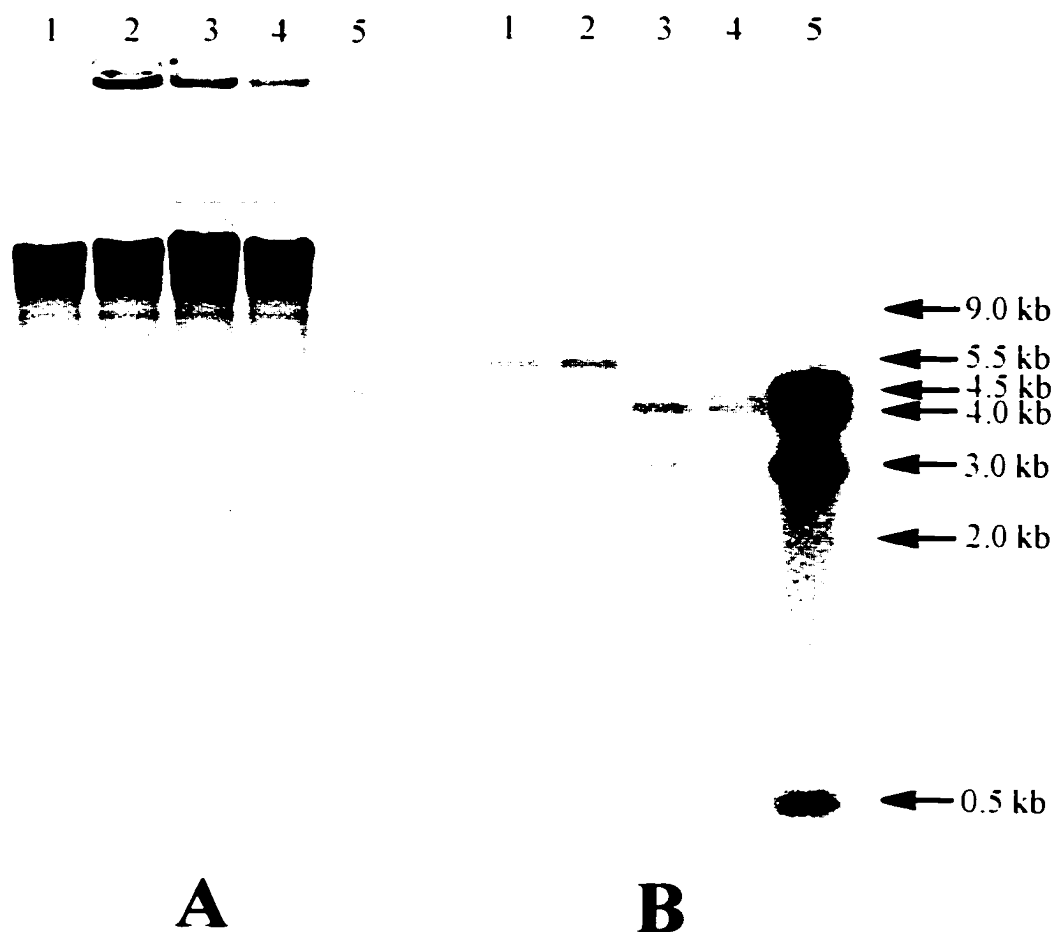


Fig. 24. Agarose gel electrophoresis (A) and Southern Hybridization (B) of *Pst*I-digested chromosomal DNA of the wild type strain and disruptants; the gel was probed with the pJV207 insert. Lane 1, CHC1-1 (disruptant of ORF1 in ISP5230) chromosomal DNA; lane 2, CCHC1-1 (disruptant of ORF1 in VS263) chromosomal DNA; lane 3, wild-type strain ISP5230 chromosomal DNA; lane 4, CHFB1 (disruptant of ORF2 in ISP5230) cromosome DNA; lane 5, pJV207 *Pst*I digest as control.

c. Characterization of ORF1 disruptants

All of the disruptants from wild-type ISP5230 (with prefix CHC) remained prototrophic under the conditions used. The VS263 disruptants (with prefix CCHC) of ORF1 showed different growth characteristics from the parent *cys-28* mutant VS263 strain (Table 6). When ORF1 was disrupted, VS263 lost the ability to grow on MM medium supplemented with methionine and homocysteine but still could grow on MM medium supplemented with cystathionine, indicating that ORF1 is responsible for the conversion of homocysteine to cystathionine catalyzed by cystathionine β-synthase (CBS). The characteristics of ORF1 disruptants also indicated that CBS is not needed for L-cysteine biosynthesis in wild-type strains, but can supply an alternative pathway in cysteine biosynthesis.

Thiosulfate stimulated growth of both VS263 and its ORF1 disruptants only at concentrations over 10 μg/ml. It might have a regulatory function in L-cysteine metabolism, but its role in *S. venezuelae* has not been determined.

Table 6 Growth requirements of ORF1 disruptants of VS263

Supplements	Strains		
	VS263	CCHC1-1	CCHC2-1
O-Acetylserine	–	–	–
L-Cysteine	+	+	+
Cystathionine	+	+	+
DL-Homocysteine	+	–	–
L-Methionine	+	–	–
O-Acetylhomoserine	–	–	–
O-Succinylhomoserine	–	–	–
Na_2S	–	–	–
$Na_2S_2O_3$	±	±	±

+ represents strong growth; - means no growth; ± indicates poor growth.

D. Disruption of ORF2

The deduced amino acid sequence for ORF2 gave a high score for similarity to acyl transferases when compared in a BlastX search with proteins in the GenBank database (Appendix IV, Table AppdxIV-2). It also showed close overall sequence similarity in ClustalW alignments when compared with acyl-CoA acyltransferases from various organisms (data not shown). Because it might play a role in Cm biosynthesis by transferring the dichloroacetyl group, a possible function for ORF2 was investigated by gene disruption. If it is involved in chlorination reactions, its disruption should cause accumulation of corynecins instead of Cm.

To disrupt ORF2, recombinant plasmids pJV220 and pJV221 containing ORF2 were first created by putting the blunted 4.0-kb *Xho*I-*Bgl*II fragment of pJV207 into the *Pst*I site of pHJL400 in opposite orientations. pJV221 was partially digested with *Nco*I and ligated with the *Nco*I fragment of pUC120A containing the apramycin resistance gene, yielding pJV222 with the *apr*-containing fragment inserted in ORF2 (Fig. 25). Transformation of *S. venezuelae* ISP5230 with pJV222 yielded CHF11, resistant to both thiostrepton and apramycin. As with pHJL400 and its derivatives, pJV222 could not be detected in plasmid DNA extracts of CHF11 by gel electrophoresis or even by Southern hybridization, presumably because of its segregational instability in *S. venezuelae*. Its existence in CHF11 was confirmed by reisolating pJV222 from *E. coli* after using the plasmid DNA extract from CHF11 to transform *E. coli* DH5α. About 500 spores harvested from MYM plates supplemented with apramycin were screened for loss of the plasmid. Eight colonies resistant to apramycin but sensitive to thiostrepton were isolated (with prefix CHFB). Disruption of

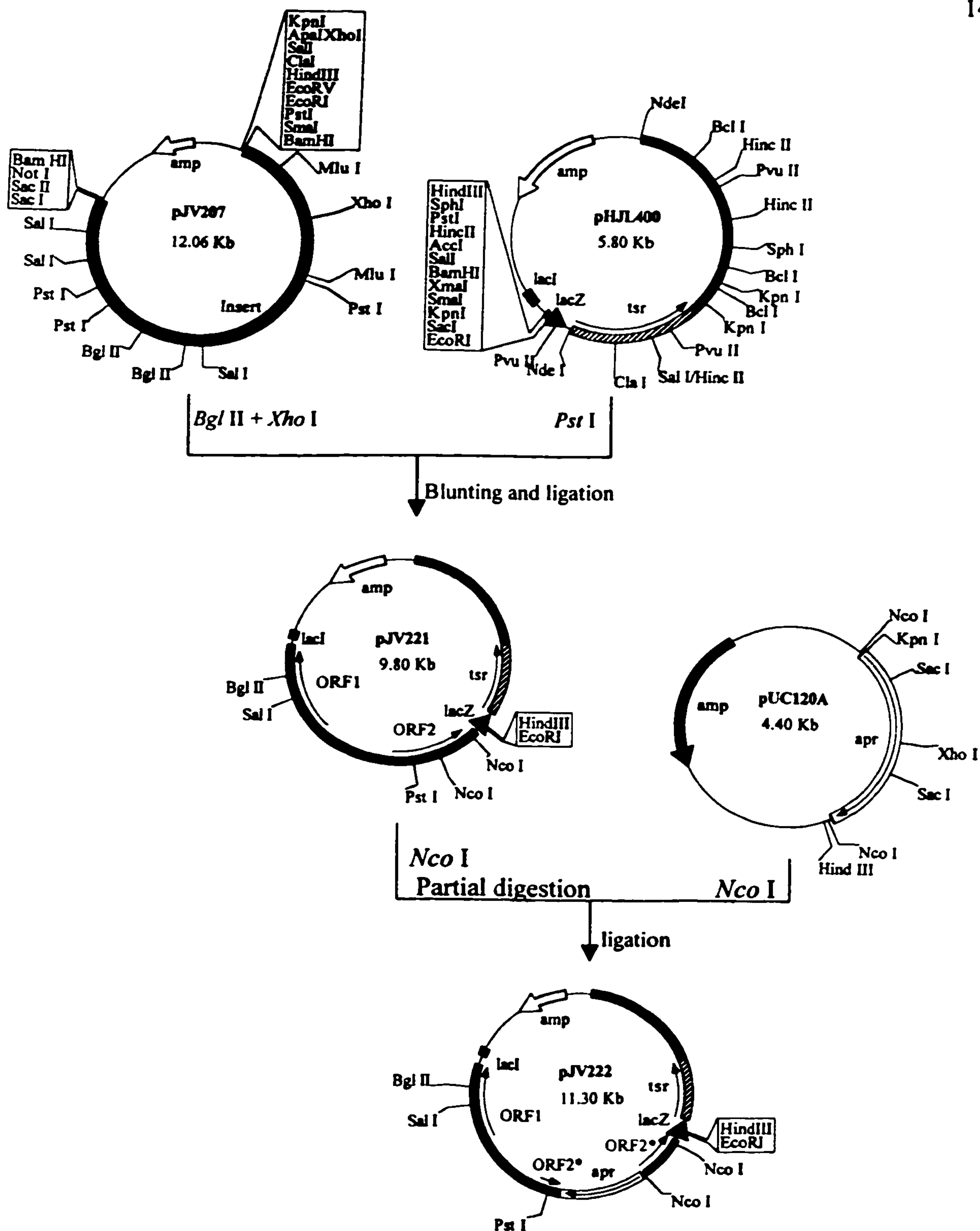


Fig. 25. Construction of an ORF2 disruption plasmid (pJV222). ORF2*: disrupted ORF2.

ORF2 in these CHFB strains was confirmed by Southern hybridization analysis. The 3.0-kb *PstI* fragment of *S. venezuelae* ISP5230 chromosome was replaced by the 4.5-kb *PstI* fragment containing *apr* in ORF2 disruptant CHFB1 (see Fig. 23 lane 4).

When the ORF2 disruptants were tested for Cm production by bioassay and HPLC, no difference was found in the products from cultures of the mutants and parent strain ISP5230 (data not shown). The disruptants were prototrophic when grown on minimum medium, suggesting that this ORF is neither a Cm pathway gene nor essential for primary metabolism under the conditions tested.

VI. Enzyme assays

A. O-Acetylserine sulfhydrylase (cysteine synthase)

O-Acetyl-L-serine sulfhydrylase (cysteine synthase) catalyzes the formation of L-cysteine using O-acetyl-L-serine and H_2S as substrates. The L-cysteine formed was measured by the modified specific spectrophotometric method (Gaitonde, 1967). Using this modified procedure the background was reduced to a minimum without affecting full development of the color reaction. No interference from homocysteine or cystathionine was detected.

Four strains, ISP5230, CHC1-1, VS263 and CCHC1-1 were tested for cysteine synthase activity when grown in MMY medium. Methionine and cystathionine (50 µg/ml) were added to MMY for strains VS263 and CCHC1-1, respectively, to maintain their fast growth. As expected, disruption of ORF1 did not affect cysteine synthase activity, as reflected in the similar amounts of cysteine synthesized by ISP5230 and in its disruptant CHC1-1. Similar enzyme activities were also detected in VS263 and CCHC1-1. Possible

repression of gene expression by cysteine was avoided by use of a poor sulfur source. The cysteine synthase activity of ISP5230 and CHC1-1 in MMY medium was 10-fold higher than that of VS263 and CCHC1-1 (Fig. 26 A).

When grown in MYM medium, the mycelium of *S. venezuelae* strains ISP5230 and CHC1-1 contained similar amounts of cysteine synthase activity, and the values were comparable to those in the mycelium of cultures grown in MMY medium (Fig. 26B). Surprisingly, the activities of this enzyme in strains VS263 and CCHC1-1 grown in MYM medium were 20 times higher than in the mycelium of cultures grown in MMY medium, even higher than that of wild-type strain (Fig. 26 B). This result indicated that the structural gene of cysteine synthase was not altered in VS263 and CCHC1-1, but expression of the enzyme was changed compared with that in the wild-type. Presumably some component (s) in MYM medium played a regulatory role and stimulated, restored or even enhanced the expression of the enzyme in VS263 and CCHC1-1. The effects of components in the medium on the cysteine synthase activity of VS263 were tested (Table 7 and Fig. 27).

In Table 7, media 6 and 7 represent MMY and MYM in composition, respectively. Yeast extract or malt extract individually did not alter the expression of cysteine synthase activity in VS263, but when combined, they greatly increased the level of the enzyme (see Table 7 and Fig. 27). Glucose had an obvious negative effect on expression of the enzyme. The mechanism by which cysteine synthase activity was influenced in VS263, as well as in the ORF1 disruptant CCHC1 remain to be uncovered.

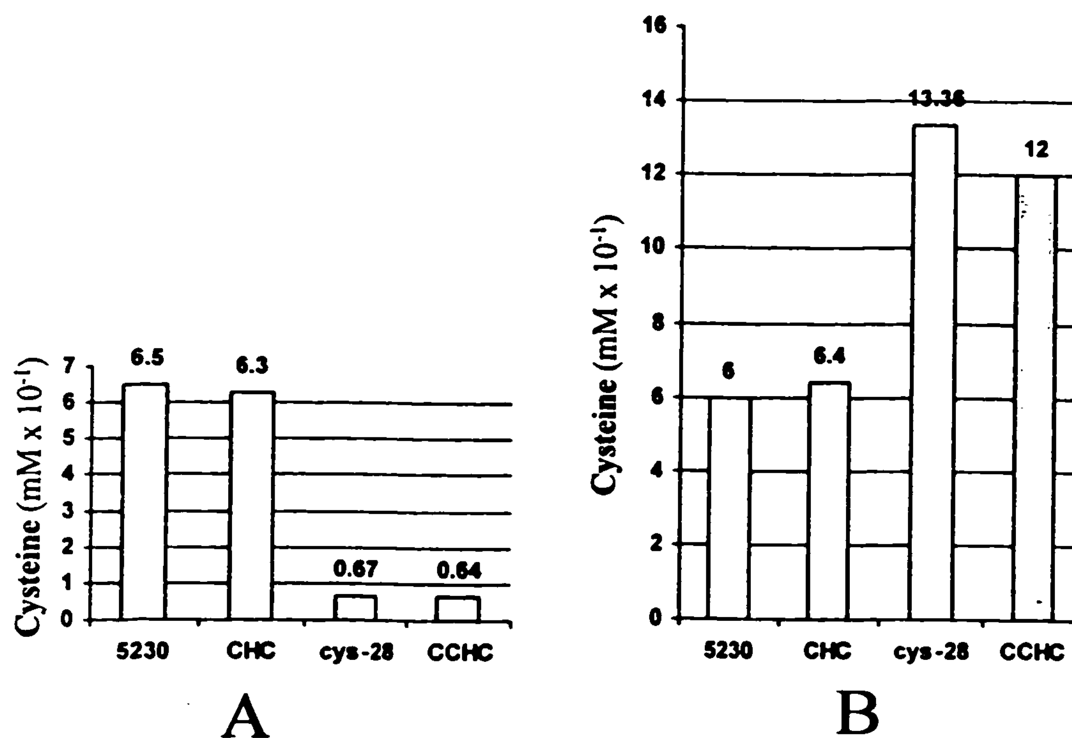


Fig. 26. Cysteine synthase activity in four representative strains. MMY (A) and MYM (B) were used in the assays. The cell extracts prepared from MMY cultures and MYM cultures were adjusted with TEPD buffer to the same total protein level.

Table 7. Media used to grow *cys-28* for cysteine synthase assays

Components	Media						
	1	2	3	4	5	6 (MMY)	7 (MYM)
Yeast extract (%)	0.1	0.5	0.1	0.1	0.1	0.1	0.5
Malt extract (%)	0	0	1	0	0	0	1
Maltose (%)	0	0	0	1	0	0	1
Glucose (%)	1	1	1	0	1	1	0
Methionine (µg/ml)	50	50	50	50	0	0	0
Acetylserine (µg/ml)	50	0	0	0	0	0	0
Cystathionine (µg/ml)	0	0	0	0	50	0	0

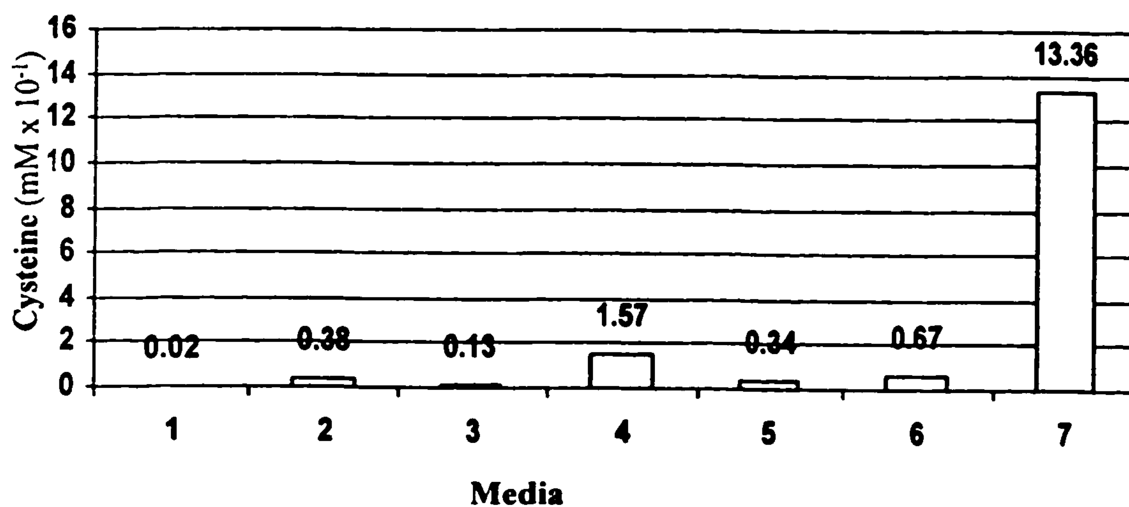


Fig. 27. Activity of cysteine synthase in VS263 (*cys-28* mutant) grown in different media. The media listed by number below each column are described in Table 9. The cell extracts prepared from all cultures was adjusted with TEPD buffer to the same total protein level.

B. Cystathionine β- and γ-lyases

Cystathionine γ-lyase catalyzes the cleavage of cystathionine into cysteine, 2-ketobutyric acid and ammonia. Cystathionine also can be cleaved into homocysteine, pyruvic acid and ammonia by cystathionine β-lyase. The latter is the main route for methionine biosynthesis in enteric bacteria. Both enzymes have been found in yeast, but in *Streptomyces* only cystathionine γ-lyase has been demonstrated (Nagasawa et al., 1984).

The 2-ketobutyric acid and pyruvic acid formed in the cleavage reactions can be converted into 3-ethyl-2-hydroxy-6,7-dimethoxyquinoxaline (EHDQ) and 3-methyl-2-hydroxy-6,7-dimethoxyquinoxaline (MHDQ) by reaction with 1,2-diamino-4,5-dimethoxybenzene (DDB), allowing cystathionine β- and γ-lyase activity to be sensitively measured simultaneously by HPLC (Ohmori et al., 1992). Under the HPLC conditions developed, pyruvic acid and 2-ketobutyric acid included as positive controls, gave sharp, well separated peaks at 4.2 min and 5.75 min, respectively (Fig. 28). Four strains tested (ISP5230, CHC1-1, VS263 and CCHC1-1) showed similar activities for the two lyases (only the result for strain ISP5230 is presented).

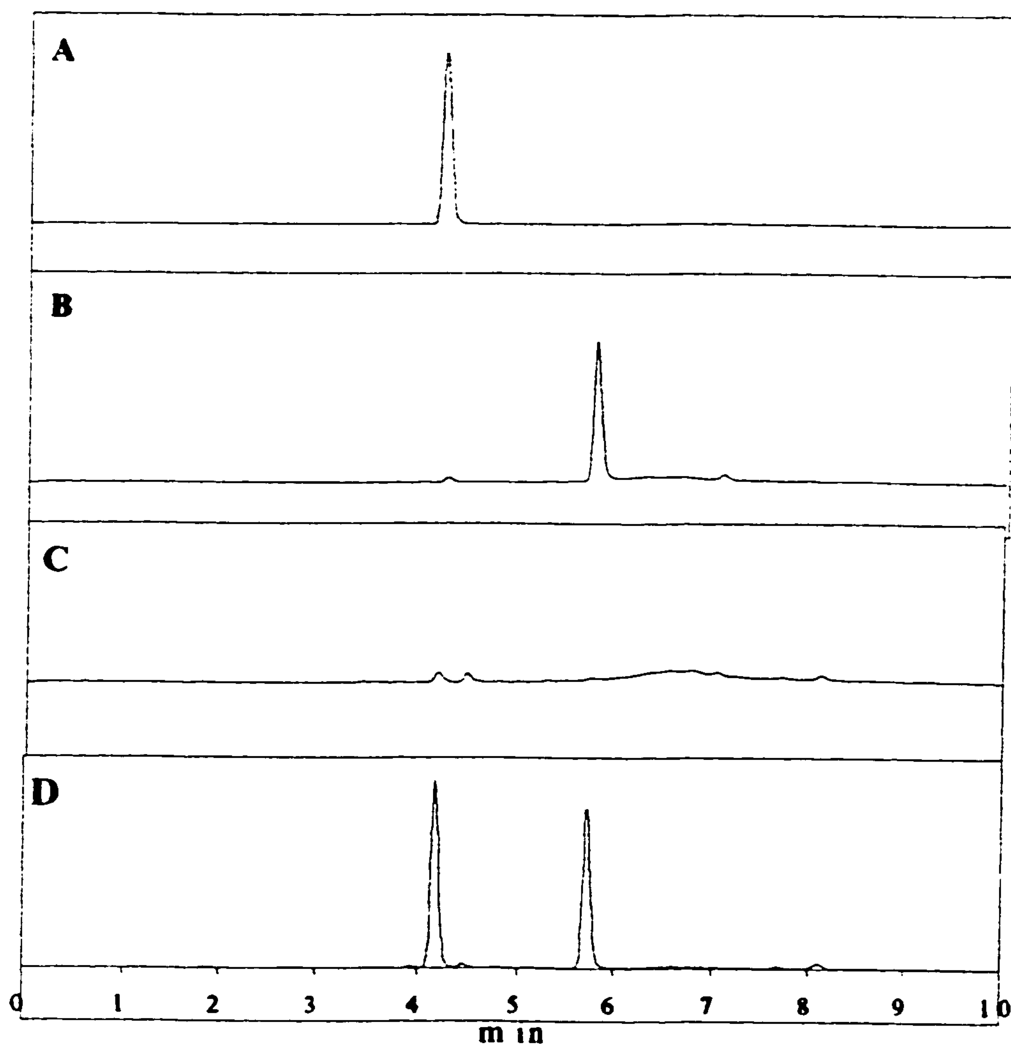


Fig. 28. HPLC analysis of cystathionine β- and γ-lyases in *S. venezuelae* ISP5230. Retention time is recorded on the X axis. The Y axis shows the levels of 2-ketobutyric acid and pyruvic acid derived from cystathionine in the cystathionase assay. A: pyruvic acid (0.5 mM); B: 2-ketobutyric acid (0.5mM); C: control for strain ISP5230 in which trichloroacetic acid was added before the substrates; D: assay of ISP5230 sample.

C. Cystathionine β-synthase

Cystathionine β-synthase is commonly found in eukaryotes (e.g. fungi and animals). It catalyzes the formation of cystathionine from homocysteine and serine, which is the main route for cysteine biosynthesis in yeast. Although it was reported to be present in *Streptomyces*, (Nagasawa et al., 1984), supporting evidence was lacking. The cloning of ORF1 and its disruption in strain ISP5230 and VS263 gave the first direct evidence that the enzyme existed in *Streptomyces venezuelae*. It is not essential for growth of the wild-type on minimum medium, presumably because the enzyme plays only an alternative role in cysteine biosynthesis through reverse transsulfuration.

Four strains, ISP5230, CHC1-1, VS263 and CCHC1-1, were tested for the activity of cystathionine β-synthase in cell extracts. The HPLC method developed to measure cystathionine formed in enzyme assay reactions can detect concentrations as low as 1μg/ml. CBS activity was not detected in any of the strains tested (Fig. 29). Even in the wild-type strain, the amount of cystathionine formed was below the limit of detection. This may be due to the low cystathionine β-synthase activity and the high activity of cystathionine β- and γ-lyases (see above). Even if only a small amount of cystathionine was formed, it would be rapidly broken down by the lyases.

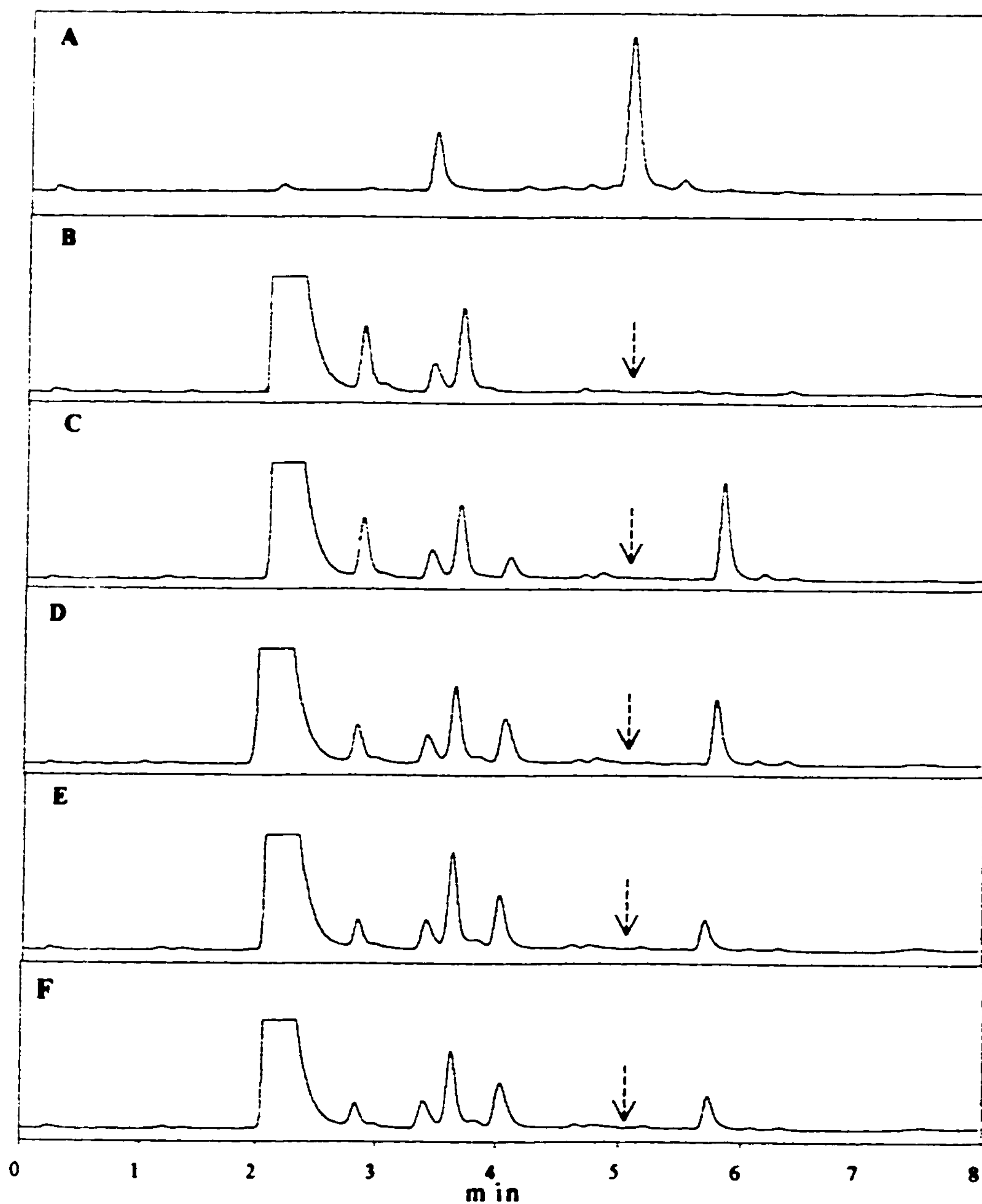


Fig. 29. HPLC analysis of cystathionine in the cystathionine β-synthase assay. Arrows point to the expected position of the cystathionine peak. A: cystathionine standard, 0.1 mM; B: negative control of strain ISP5230; C: CBS assay of ISP5230; D: CBS assay of CHC1-1; E: CBS assay of VS263; F: CBS assay of CCHC1-1.

D. Cystathionine γ-synthase

Cystathionine γ-synthase catalyzes the formation of cystathionine from cysteine and O-succinyl-L-homoserine or O-acetyl-L-homoserine. In enterobacteria, it is an essential step for biosynthesis of methionine. In *Streptomyces*, no information about the existence and possible role of the enzyme is available.

Cystathionine γ-synthase activity was assayed using cell extracts of four strains, ISP5230, CHC1-1, VS263 and CCHC1-1. Each of the strains tested showed a similar level of cystathionine γ-synthase activity so only the result for the wild-type strain is presented in Fig. 30. O-Acetyl-L-homoserine (OAH) and O-succinyl-L-homoserine (OSH) plus cysteine were used as substrates. Cystathionine formed in the assay reaction was measured by HPLC with a fluorescence detector. To confirm the accuracy of HPLC analysis, each assay sample was compared with the same sample mixed with an equal volume of 15 μg/ml cystathionine. With OAH as a substrate, adding cystathionine to the sample gave an extra peak (Fig. 30 C and D). When OSH was used as a substrate, only one peak appeared at 5 min (Fig. 30. E and F) in both the assay sample and the mixture. The results indicated that only O-succinyl-L-homoserine was used for cystathionine synthesis, and that O-acetyl-L-homoserine was not the natural substrate of cystathionine γ-synthase in *S. venezuelae*.

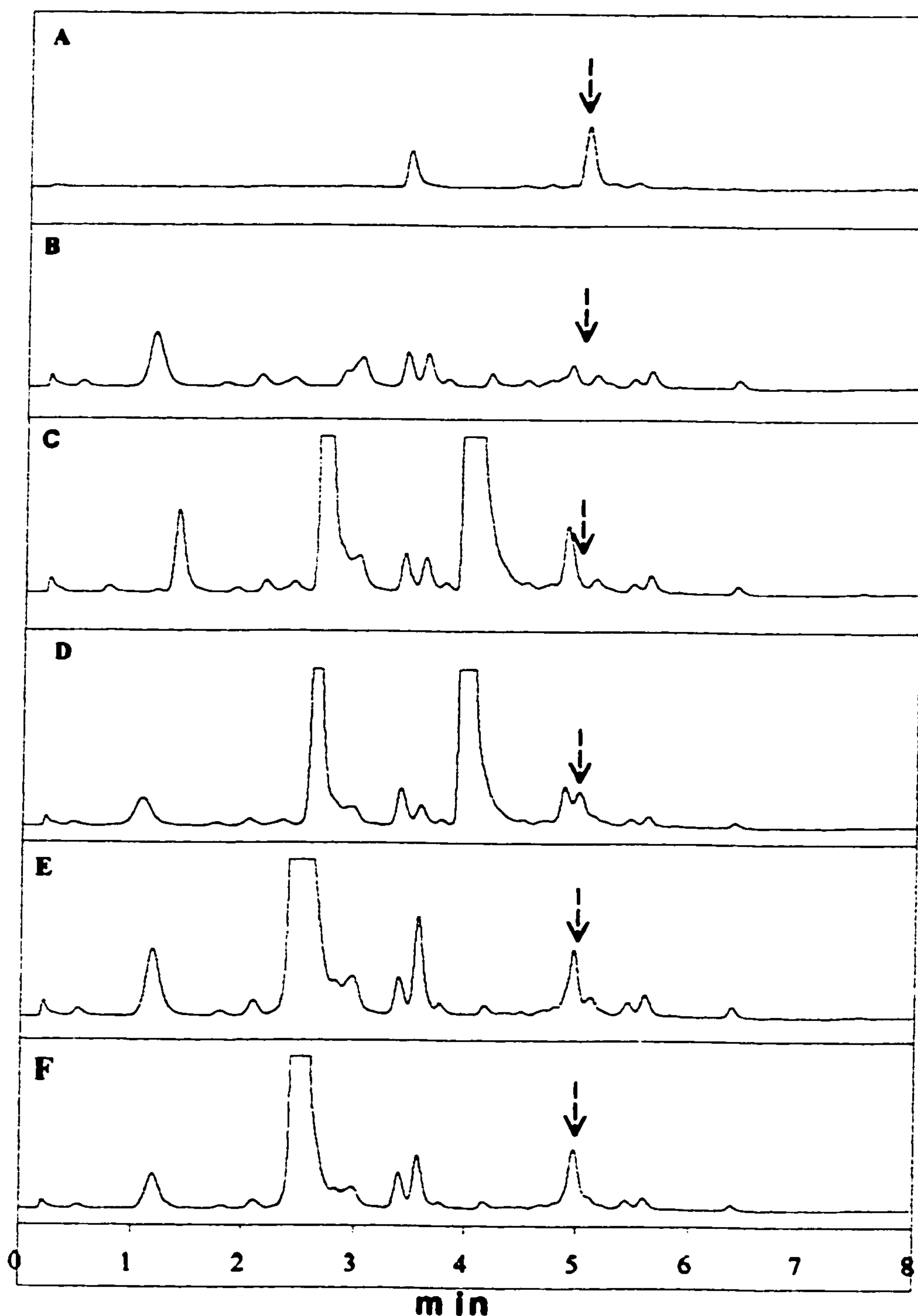


Fig. 30. HPLC analysis of cystathionine in the cystathionine γ-synthase assay. X axis represents retention time, Y axis represents peak area. Arrows point to the peak position of cystathionine. A, cystathionine standard (15 μg/ml); B, negative control of ISP5230; C, sample with OAH as substrate; D, the mixture of the sample in C and cystathionine (final concentration of 7.5 μg/ml); E, sample with OSH as substrate; F, the mixture of the sample in E and cystathionine(final concentration of 7.5 μg/ml).

E. Homocysteine Synthase

Homocysteine can be formed directly by sufhydrylation of homoserine derivatives with hydrogen sulfide. The reaction is catalyzed by specific sulfhydrylases. O-Acetylhomoserine sulfhydrylase plays a major role in metabolism of methionine and cysteine in *Brevibacterium* and yeast. O-Succinylhomoserine sulfhydrylase catalyzes the only route of methionine biosynthesis via homocysteine in *Pseudomonas* (Foglino et al., 1995; Gunther et al., 1979). The activity and possible role of the enzyme have not been reported in *Streptomyces*.

In this study, both O-Acetyl-L-homoserine and O-succinyl-L-homoserine were tested as potential substrates of the enzyme. The homocysteine formed in the assay reaction was analyzed by HPLC. High enzyme activity was present in all strains tested (ISP5230, CHC1-1, VS263 and CCHC1-1) when O-acetyl-L-homoserine was added to the assay mixture (only the result of strain ISP5230 is presented in Fig. 31). The concentration of homocysteine formed in the assay reaction reached as high as 1 mM. It is the first acetylhomoserine sulfhydrylase detected in *Streptomyces*.

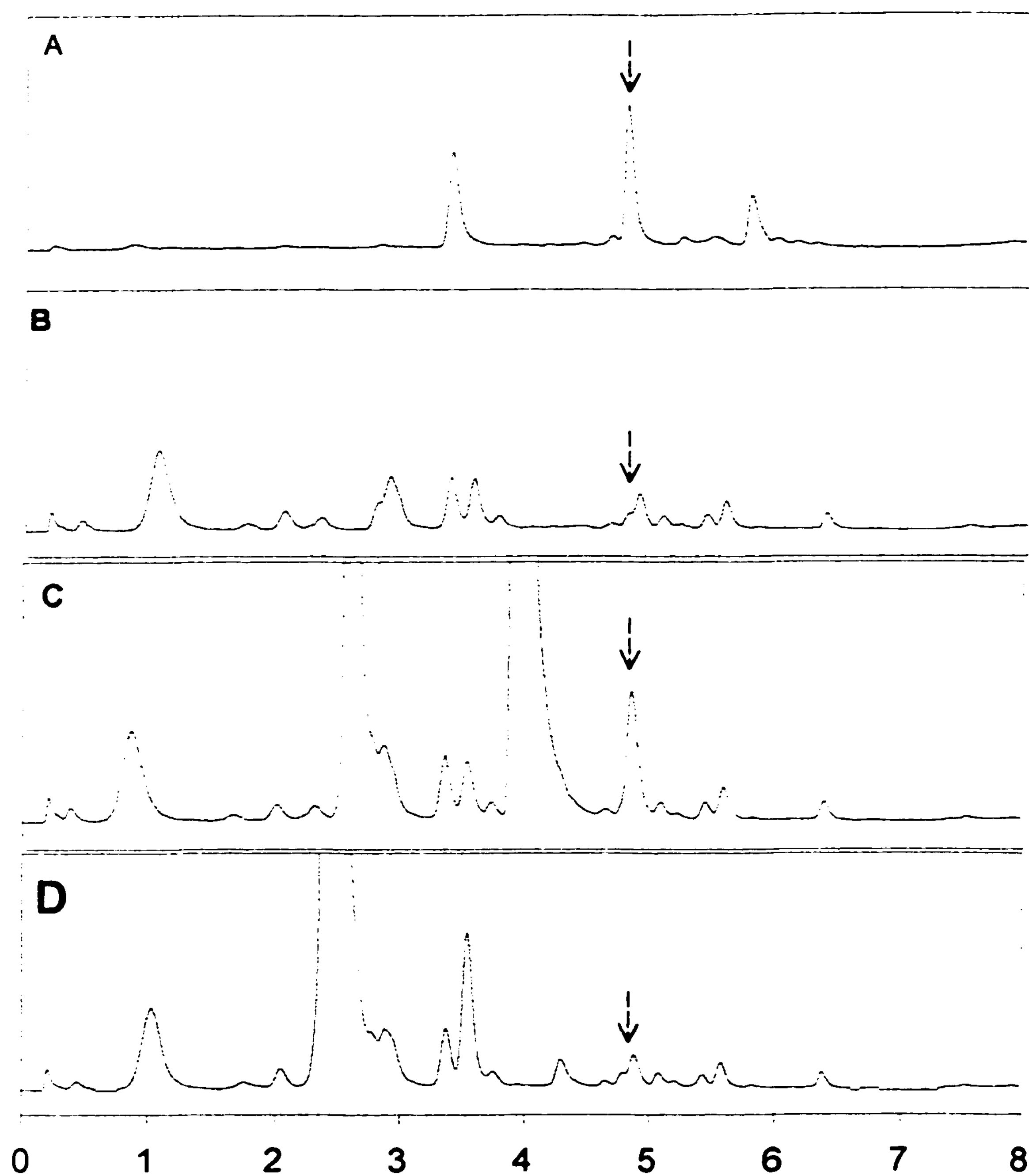


Fig. 31. HPLC assay of homocysteine synthase activity in strain ISP5230. Arrows point to the position of the homocysteine peak. A: homocysteine standard (1 mM); B: control for strain ISP5230 (no substrate added); C: sample with OAH as substrate; D: sample with OSH as substrate.

DISCUSSION AND CONCLUSIONS

Developments in the molecular genetics of streptomycetes can provide new insights into both the primary and secondary metabolic pathways of this group of microorganisms. The ability to manipulate the genes involved, and their organization, has fostered direct investigations of gene function, gene expression and gene regulation, as well as a stronger interest in evolutionary relationships within the actinomycetes. Methods available to explore the molecular genetics of streptomycetes have opened up new areas in the study of antibiotic biosynthesis, and the importance of streptomycetes in the fermentation industry has ensured that they have remained a focus of attention in a very active research field.

Our understanding of primary metabolism in streptomycetes has usually been based on studies in other organisms, such as *E. coli*, that have given us a general appreciation of biochemical processes. Although the pathways of metabolism in streptomycetes often follow similar principles to those familiar from previous studies in other bacteria, there are inevitable differences that reflect the unique position of streptomycetes in the microbial world. The research described in this thesis required a closer understanding of one aspect of the molecular biology of primary metabolism in streptomycetes that has not hitherto received particular attention - namely the biosynthesis and metabolism of the sulfur-containing amino acids.

The streptomycete used here was *Streptomyces venezuelae*, which is of interest because of its ability to produce two antibiotics, chloramphenicol and jadomycin B. It also has the advantage of relatively fast, dispersed growth at temperatures up to 42 °C, higher

than those tolerated by most other streptomycetes, and is therefore useful for large-scale fermentations. Interest in Cm biosynthesis is now centered on the cloning of genes in the Cm biosynthesis pathway, and on the development of new sources of antibiotic production genes. Genetic evidence that the Cm biosynthesis genes are clustered, and flanked by marker genes (*pdx* and *cys-28*) for two primary metabolic pathways has suggested several cloning strategies involving an initial approach through cloning genes for biosynthesis of pyridoxal and cysteine. At the outset of this research project, the gene associated with the *cys-28* marker was targeted, and much of the work described here has been devoted to characterizing the *cys-28* mutation. This has led to the cloning of several genes related to cysteine biosynthesis, as well as to a better understanding of the role of transsulfuration in the interconversion of sulfur-containing amino acids in streptomycetes. Elucidation of the transsulfuration pathway in *S. venezuelae* has provided information about the relationship between genes for cysteine and Cm biosynthesis, and about the specific metabolism of sulfur-containing amino acids in streptomycetes. The gene encoding cystathionine β-synthase (*cbs*) of *S. venezuelae* is the first such gene cloned from a prokaryotic organism. Moreover, systematic investigation of the enzymes involved in cysteine and methionine metabolism in *S. venezuelae* has demonstrated the existence of a specific transsulfuration pathway in streptomycetes.

I. The genetic map of *S. venezuelae*

Progress in sequencing the *S. coelicolor* A3(2) genome is proving to be of great benefit to all those now working on the molecular genetics of streptomycetes, not only by

revealing much more information about the structure of the streptomycete chromosome but also by pinpointing relative gene locations and offering clues about gene organization (Redenbach et al., 1996). Basing the chromosome map of *S. venezuelae* on the *S. coelicolor* model allows the gene of *cys-28* to be located at 1-2 o'clock, (corresponding to 12-1 o'clock on *the S. coelicolor* chromosomal map). The location of the 4.0-kb *Sac*I fragment is at the same position (cosmid L10 flanked by *arg A, B, C* and *pdx*), indicating the accuracy of chromosomal walking and previous mapping. By a Blast search, ORF1 and ORF2 were located on cosmid E25, which is at 10 o'clock on the *S. coelicolor* chromosome map. This indicates that the genes cloned in this study are not physically close to the *cml* cluster.

II. *cys-28* Mutation and Cysteine Synthesis

The high sequence similarity between the cloned gene for cystathionine β-synthase from *S. venezuelae* and the corresponding gene from *S. coelicolor* implies that this gene is well conserved, and may also be widely distributed in streptomycetes. The evidence from the research described here places the *cys-28* mutation in strain VS263 at the last step in cysteine biosynthesis, namely O-acetylserine sulfhydrylation. The gene mutated could have been either the structural gene for cysteine synthase or a regulatory element controlling expression of the gene, but enzyme assays indicating that the structural gene is intact in VS263 imply that expression is altered. A regulatory mutation is also suggested by the absence of cysteine synthase activity in minimal medium, which may be due to loss of an inducible component after the *cys-28* mutation. In rich (MYM) medium the activity of the enzyme in the *cys-28* mutant was increased compared with that of the wild-type strain. A

similar situation was observed for methionine synthesis in *E. coli.* Methionine adenosyltransferase, which catalyzes the conversion of methionine to adenosylmethionine is encoded by two genes, *metK* and *metX*, but the *metX* product is formed only in cells grown on rich medium (Greene, 1997). Whether there are two genes for cysteine synthase in VS263, and only the one expressed on minimal medium was altered, or whether there is only one structural gene, expression of which has been changed by mutation, is not yet clear. Previous studies of mutants affected in sulfur metabolism (Kitano et al., 1985; Donadio et al., 1990) suggested that thiosulfate may be the direct precursor of cysteine, but in *S. venezuelae* high cysteine synthase activity was detected with sulfide as a substrate, and the partial restoration of VS263's growth by thiosulfate indicates that thiosulfate may play a regulatory role in cysteine synthesis. Whether or not S-sulfocysteine synthase exists in *S. venezuelae*, and what role it may play in cysteine biosynthesis still need to be determined. Because of the easy isolation of *cys* mutants, it is unlikely that two isozymes (encoded by counterparts of *E. coli cysK* and *cysM)* are present. From the results obtained in this study, the overall strategy of shotgun cloning to complement a defective phenotype in the cloning host appears to lead more often to the selection of regulatory genes than to structural genes for the desired characteristic. If this is supported by more extensive data, it is an important factor to take into account when gene cloning strategies are being planned.

III. Complementation Strategies to Clone Both *cml* and *cys* Genes

The success of complementation as a strategy to clone *cml* and other genetic markers such as *cys-28* was limited by low protoplast transformation efficiencies and the ready incorporation of vector plasmid into the chromosomal DNA of *S. venezuelae*. Although *S.*

incorporation of vector plasmid into the chromosomal DNA of *S. venezuelae*. Although *S. lividans* mutants have been successfully used as hosts for complementation in cloning the *pdx* gene of *S. venezuelae*, the high rate of reversion of the *cys* mutation decreases the chance of success in cloning *cys-28* by using this strategy. Construction of a vector for congugal transfer of plasmids between *E. coli* and streptomycetes using the shuttle vector pHJL400 will make it easier to transfer recombinant plasmids directly from *E. coli* to streptomycetes, and recent mastery of this technique will enhance progress in this area (J. He, personal communication). During the subcloning of transposon Tn*4560* in pHJL400, insertion of the transposon appeared to stabilize the plasmid in *S. venezuelae*. A similar phenomenon was observed in other plasmids (Chung, 1987), and it is possible that a transposon segment stabilized the plasmid in *S. venezuelae* (perhaps by contributing the resolvase gene). If this segment of the transposon were inserted into the shuttle conjugal vector, a new multifunctional cloning vector could be created, suitable for use in complementation experiments.

IV. PCR Reaction and PCR Products

The primers for the PCR reaction were designed from the alignment of conserved amino acid sequences of CSs derived from the relevant genes of several bacteria. The regions aligned were all highly conserved in the ORF1 product and in CBSs of various organisms. The same primers could be suitable for cloning the CS of *S. venezuelae* as well. About 100 clones of PCR products were analyzed by restriction digestion, Southern hybridization and nucleotide sequencing. Although several of them showed digestion

patterns different from that of the *cbs* fragment (pJV205), they proved to be the result of mutation during PCR amplification. Other conserved areas could be used to design PCR primers amplifying the CS gene of *S. venezuelae* specifically.

V. Nucleotide Sequence and Amino Acid Sequence of ORF1 and ORF2

In the two ORFs identified on the 7.0-kb of sequenced DNA from *S. venezuelae*, putative RBSs preceded the start codons ATG for ORF2 and GTG for ORF1. A plausible terminator was present downstream of the ORF2 stop codon. None was seen for ORF1, though several hairpin loops were located. Thus the transcription unit may extend beyond the sequenced area. The two ORFs were located on different strands of the chromosomal DNA, oriented in opposite directions, indicating that transcription of both genes started from the central area between the ORFs. No transcriptional signals consistent with the relatively conserved –10 (tAggNT) and –35 (ttGacN) regions of streptomycete genes (Seno & Baltz, 1989) were found, making this another example where, unlike *E. coli*, -10 and –35 promoter regions of transcription are not highly conserved among streptomycetes, even among different transcriptional units of one species.

The 463 aa sequence of the ORF1 product (48.82 kDa) has high similarity to CSs of various organisms and to CBS of eukaryotes. The N terminal sequence aligned well with both kinds of enzymes whereas the C terminal region matched only the CBS sequences. The molecular size was closer to typical CBSs than to CSs. All of the sequences showed the highly conserved motif SVKDRIA containing lysine 44, corresponding to lysine 42 of *E. coli cysK* (Nalabotu et al., 1992) and lying at position 42 in the conserved SIKDRIA motif of the

Flavobacterium enzyme (Muller et al., 1992). Some other residues are also identical among all the sequences, such as lysine 33 (K), glutamic acid 35 (E), glycine 73 and 76 (G). All of these conserved residues must play important roles for the activities of the enzymes. The specific function of each remains to be determined. The pyridoxal phosphate binding site motif indicated that, like other transsulfuration enzymes, the ORF1 product (CBS) is also pyridoxal phosphate-dependent. An analysis using PILEUP to show sequence relationships among the proteins indicated that ORF1 does not belong to either the CBS of eukaryotes or the CSs of prokaryotic and eukaryotic organisms. It appears to group together with cysM2 of *M. tuberculosis* (a putative CBS), and is located at a position in the phylogenetic tree of cysteine synthase genes and cystathionine β-synthase genes (Fig. 32) that implies affinities with both CSs and CBSs. The group may represent the relatively unexplored CBSs from prokaryotes. Taken with the results of gene disruption, this evidence suggests that ORF1 encodes a CBS of *S. venezuelae* rather than a CS. It would be the first CBS gene cloned and analyzed from a prokaryote.

VI. The Function of ORF1

Under the growth conditions used, disruption of ORF1 did not change the phenotype of the wild-type *S. venezuelae* strain on minimal medium, suggesting that this gene may sometimes be dispensable. In the *cys-28* mutant, which was already impaired in the step from O-acetylserine to cysteine, disruption of ORF1 blocked the conversion of homocysteine to cystathionine, leading to the conclusion that ORF1 encoded cystathionine β-synthase catalyzing that reaction. Enzyme assay results indicated that the activity of the enzyme is

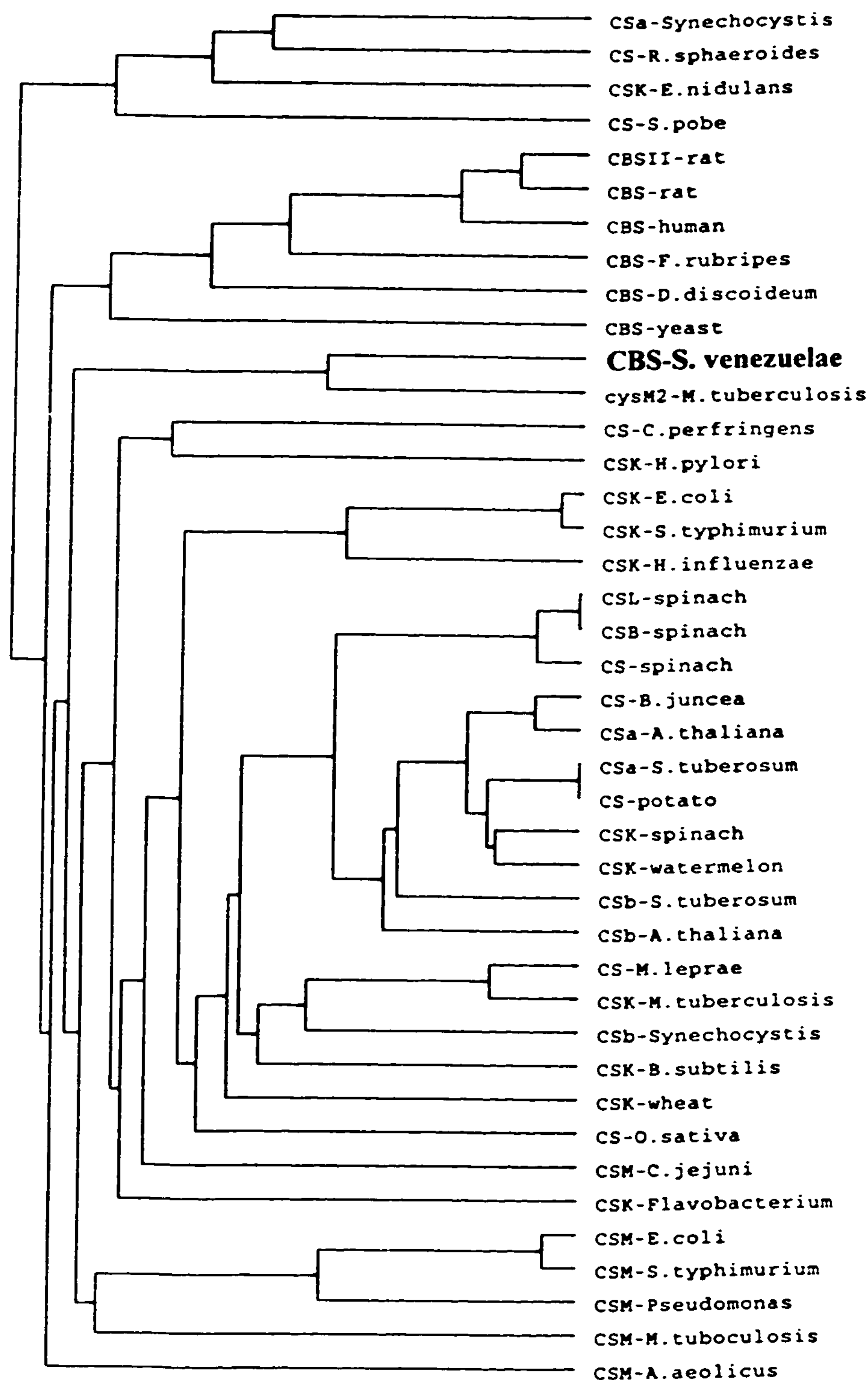


Fig. 32. Phylogenetic tree of CSs and CBSs of various organisms. CS: cysteine synthase; Csa, Csb: two kinds of cysteine synthases isolated from one organism; CSK: *cysK* product; CSM: *cysM* product; CSL, CSB: *cysL* and *cysB* products of spinach.

normally below the limits of detection for the procedure used. Even adding substrate (homocysteine) to the medium did not increase the level of the enzyme in strain VS263. The function of ORF1 was best indicated by the characteristics of VS263 and its ORF1 disruptant when these strains were grown on a medium with methionine, homocysteine or cystathionine. Although the level of the enzyme was not detectable under the conditions used, it is enough for survival of the cell on minimal medium supplemented with methionine or homocysteine. This result also indicated that cysteine is synthesized mainly by sulfhydrylation of O-acetylserine, catalyzed by cysteine synthase. Though the transsulfuration pathway exists in streptomycetes, it plays a supplementary role unless an alternative source of cysteine is required.

VII. Homocysteine and Cystathionine Synthesis

Cystathionine γ-synthase, which uses O-succinylhomoserine as a substrate, was detected in *S. phaeochromogenes* by Nagasawa et al. (1984). Similar results were obtained with *S. venezuelae*. When O-acetylhomoserine and O-succinylhomoserine were used as substrates in the enzyme assay, only O-succinylhomoserine was converted to cystathionine by the high level of cystathionine γ-synthase present. This is consistent with previous results in that, unlike other Gram-positive bacteria, which use O-acetylhomoserine, streptomycetes resemble Gram-negative bacteria in using O-succinylhomoserine. Few bacteria have been found to directly sulfhydrylate acylhomoserine to form homocysteine. The examples are: *Brevibacteriun falvum* and *P. aeruginosa* (Ozaki & Shiio 1982, Gunther et al., 1979). Streptomycetes give us another example of an O-acetylhomoserine sulfhydrylase. They are

the only organisms that use parallel pathways (transsulfuration from O-succinylhomoserine via cystathionine, and direct sulfhydrylation of O-acetylhomoserine) to synthesize homocysteine Because the enzymes for both routes were present at similar high levels, it is still too early to say they play the same role, or that one plays a more important role than the other in the metabolism of sulfur-containing amino acids.

VIII. Cystathionine Lyases

Wide distribution of cystathionine γ-lyase in streptomycetes was detected by Nagasawa et al. (1984), and the enzyme was later purified. In contrast, cystathionine β-lyase, which degrades cystathionine to homocysteine, eventually giving methionine, was not found in *S. phaeochromogenes* (Nagasawa et al., 1984; Nagasawa et al., 1987). In *S. venezuelae*, high levels of both lyases were detected by the sensitive and specific HPLC method. The high level of cystathionine γ-synthase activity is consistent with the presumption that cystathionine, formed by a high level of cystathionine γ-synthase, should be converted to cysteine or methionine. Because in *S. venezuelae* cysteine appears to be made mainly by the direct sulfhydrylase route, it would be expected that most of the cystathionine formed would be converted to homocysteine by cystathionine β-lyase and then methylated to give methionine. If so, the similar and high levels of cystathionine β- and γ-lyases are not easy to explain without further study; they may mean that the conversion of cystathionine to cysteine is under regulatory control.

IX. Conversions of Sulfur-Containing Amino Acids in *S. venezuelae*

My overall conclusions on the routes through which sulfur-containing amino acids are made available by biosynthesis and metabolism in the streptomycetes are summarized in Fig. 33. *Streptomyces venezuelae* seems to possess all the mechanisms for interconversion of sulfur-containing amino acids that have been reported to exist in both prokaryotes, including the cystathionine synthase and eukaryotic homocysteine synthase pathways. The presence of the latter pathway is consistent with the position of the *S. venezuelae* CBS in the evolutionary tree (Fig. 32), at a location between the CBSs of eukaryotes and the CSs of various organisms.

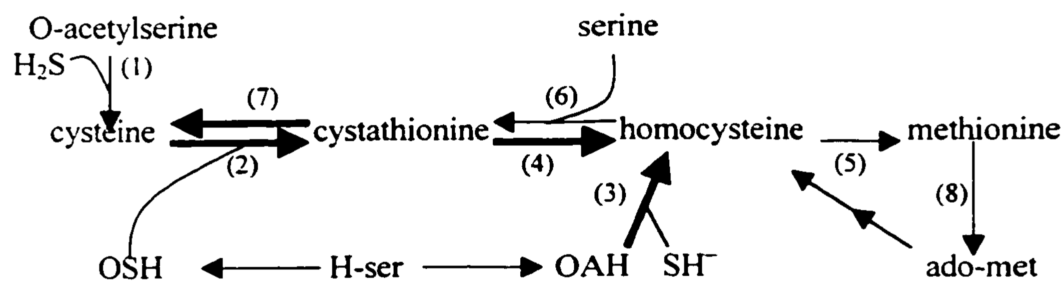


Fig. 33. Biosynthesis of sulfur-containing amino acids in *S. venezuelae*. Thick arrows represent high levels of enzyme activity. Enzymes: (1), O-acetylserine sulfhydrylase; (2), cystathionine γ-synthase; (3), O-acetylhomoserine sulfhydrylase; (4), cystathionine β-lyase; (5), methionine synthase; (6), cystathionine β-synthase; (7), cystathionine γ-lyase; (8), S-adenosylmethionine synthase.

APPENDICES

APPENDIX I. Minimal Inhibitory Concentrations (MIC) of Common Antibiotics used in This Study

Antibiotics are widely used in molecular biology for maintenance and selection of plasmid transformants, as genetic or molecular markers in cloning, and for the disruption of genes of interest. Proper concentrations of the antibiotics used are important for the efficiency and success of molecular biological experiments. The MICs of thiostrepton, apramycin and viomycin were screened for different strains used in this study (Tables AppdxI-1, AppdxI-2 and AppdxI-3).

Table AppdxI-1. Mic of thiostrepton for *Streptomyces*

Strains	Concentration of thiostrepton (μg/ml)								
	0	1	2	4	6	8	10	15	20
S. venezuelae ISP5230	++	±	–	–	–	–	–	–	–
S. venezuelae 13(s)	++	±	±	–	–	–	–	–	–
S. lividans TK23	++	++	++	+	±	–	–	–	–
S. lividans UC8882	++	++	++	++	++	++	++	++	++
CHG*	++	++	++	++	++	++	++	++	++
VS258	++	±	±	–	–	–	–	–	–

*, pJV231 transformant of ISP5230

Table AppdxI-2. MIC of viomycin for *E. coli* and *Streptomyces*

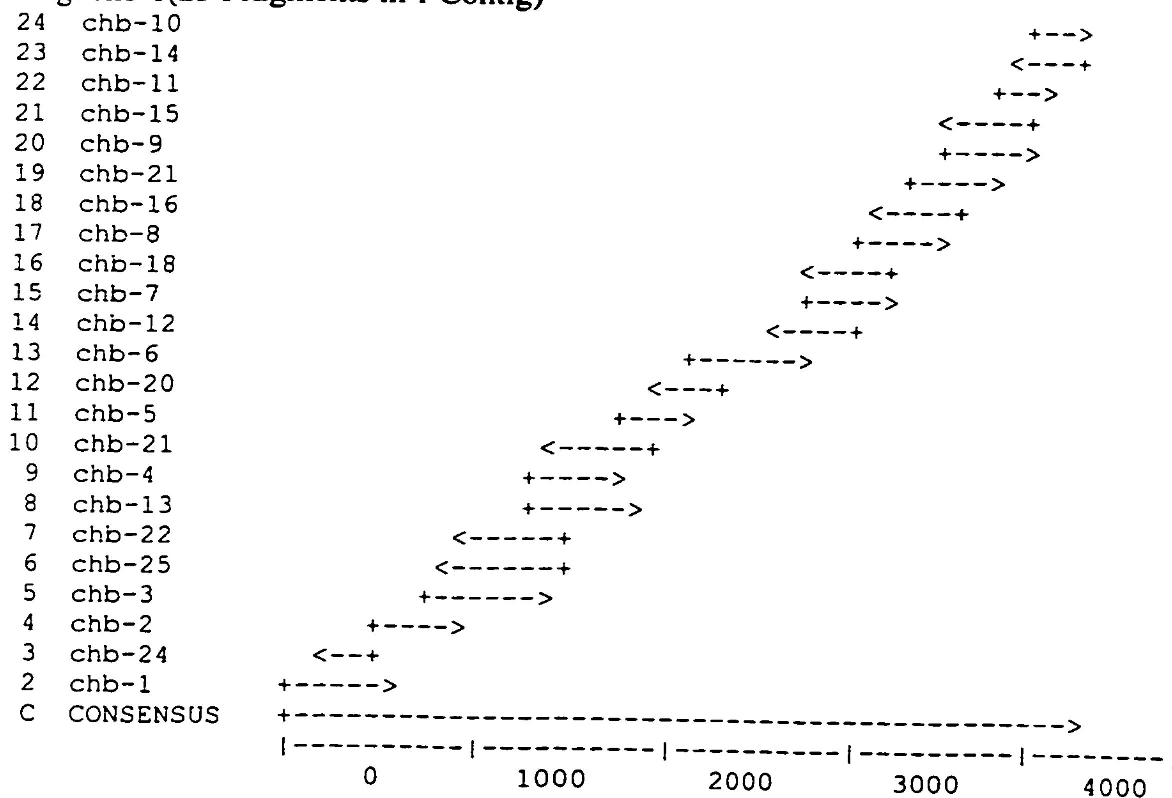
Strains	Concentration of viomycin (μg/ml)							
	0	2	4	6	8	10	15	20
S. venezuelae ISP5230	++	++	+	±	±	–	–	–
S. venezuelae 13(s)	++	++	+	±	±	–	–	–
S. lividans TK23	++	++	+	±	±	±	–	–
S. lividans UC8882	++	++	++	++	++	++	++	+
S. venezuelae CHG5	++	++	++	++	++	++	++	+
S. venezuelae LG222	++	++	++	++	++	++	++	++
S. venezuelae LG221	++	++	++	++	++	++	++	+
E. coli DH5	++	++	++	++	++	++	±	–

Table AppdxI-3. MIC of apramycin for *E. coli* and *Streptomyces*

Strains	Concentration of Apramycin (μg/ml)							
	0	2	4	6	8	10	50	100
S. venezuelae ISP5230	++	++	++	+	–	–	–	–
S. venezuelae 13(s)	++	++	++	+	±	–	–	–
S. lividans TK23	++	++	±	–	–	–	–	–
S. lividans 1326	++	++	–	–	–	–	–	–
TK23-pJV218[a]	++	++	++	++	++	++	++	±
CHC1-1[b]	++	++	++	++	++	++	++	±
E. coli DH5 -ppJV218[c]	++	++	++	++	++	++	++	++
E. coli DH5	++	++	±	–	–	–	–	–
E. coli LE392	++	++	++	++	++	++	–	–

[a] pJV218 transformant of TK23; [b] disruptant of ORF1 by inserting *apr* into *Sal* I site inside the gene; [c] pJV218 transformant of *E. coli* DH5α.

APPENDIX II. FRAGMENT ASSEMBLY OF A SEQUENCED CHROMOSOMAL REGION



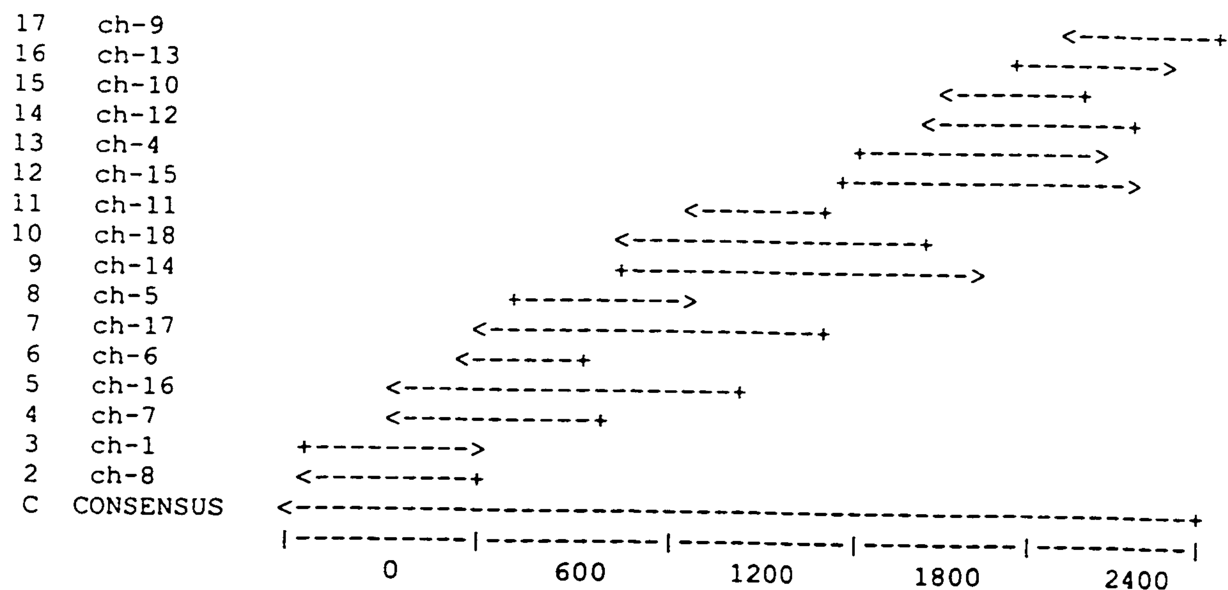


Fig. 34. Fragment assembly contigs of cys7.0.

APPENDIX III. CODON USAGE AND AMINO ACID USAGE IN *STREPTOMYCES* GENES

Table AppdxIII-1. Comparison of base composition of ORF1, ORF2 and average *S. venezuelae* genes.

Base	Codon position								
	ORF1			ORF2			*S. venezuelae*[a]		
	1	2	3	1	2	3	1	2	3
A	20.3	27.5	0.7	21.4	24.6	0.5	18.8	23.2	2.8
T	12.4	27.9	1.5	11.5	28.5	0.7	11.6	25.5	2.0
G	45.6	18.8	36.7	41.5	19.7	34.2	43.9	21.7	33.9
C	21.6	25.8	61.1	25.6	27.3	64.6	25.7	29.7	61.3
G + C	67.2	44.5	97.8	67.1	46.9	98.8	69.6	51.4	95.2
Overall G + C	69.7			70.6			71.9		

[a] calculated from data in the NCBI Codon Usage Database

Table AppdxIII-2. Comparison of codon usage in ORF1 and ORF2 with average usage in 18 *S. venezuelae* genes (SV) and 2008 genes in the genus *Streptomyces* (ST) (The data are from the NCBI codon usage database)

Amacid	Codon	Number		/1000				Fraction			
		ORF1	ORF2	ORF1	ORF2	SV[a]	ST[b]	ORF1	ORF2	SV	ST
Gly	GGG	3	0	6.55	0.00	13.81	17.14	0.06	0.00	0.14	0.18
Gly	GGA	2	0	4.37	0.00	6.81	7.22	0.04	0.00	0.07	0.08
Gly	GGT	3	3	6.55	7.37	6.98	10.48	0.06	0.06	0.07	0.11
Gly	GGC	45	44	98.25	108.11	73.28	59.45	0.85	0.94	0.73	0.63
Glu	GAG	31	22	67.69	54.05	53.39	48.04	1.00	0.96	0.90	0.83
Glu	GAA	0	1	0.00	2.46	5.93	9.73	0.00	0.04	0.10	0.17
Asp	GAT	0	0	0.00	0.00	1.05	4.33	0.00	0.00	0.02	0.07
Asp	GAC	28	26	61.14	63.88	57.06	57.68	1.00	1.00	0.98	0.93
Val	GTG	17	10	37.12	24.57	26.35	34.24	0.30	0.33	0.32	0.40
Val	GTA	0	0	0.00	0.00	1.57	2.64	0.00	0.00	0.02	0.03
Val	GTT	0	0	0.00	0.00	0.17	2.01	0.00	0.00	0.00	0.02
Val	GTC	39	20	85.15	49.17	54.49	45.78	0.70	0.67	0.66	0.54
Ala	GCG	13	7	28.38	17.20	42.22	46.72	0.32	0.16	0.31	0.35
Ala	GCA	0	0	0.00	0.00	4.01	5.78	0.00	0.00	0.03	0.04
Ala	GCT	0	0	0.00	0.00	1.57	3.91	0.00	0.00	0.01	0.03
Ala	GCC	28	36	61.14	88.45	89.16	75.86	0.68	0.84	0.65	0.57
Arg	AGG	1	0	2.18	0.00	2.62	3.51	0.06	0.00	0.07	0.04
Arg	AGA	0	0	0.00	0.00	1.40	0.74	0.00	0.00	0.02	0.01
Ser	AGT	0	0	0.00	0.00	0.52	1.74	0.00	0.00	0.00	0.03
Ser	AGC	7	2	15.28	4.91	15.53	13.01	0.21	0.08	0.28	0.25
Lys	AAG	19	12	41.48	29.94	22.51	20.63	1.00	1.00	0.99	0.95
Lys	AAA	0	0	0.00	0.00	0.17	1.16	0.00	0.00	0.01	0.05
Asn	AAT	0	0	0.00	0.00	0.35	1.00	0.00	0.00	0.01	0.05
Asn	AAC	13	13	28.38	31.94	24.95	18.31	1.00	1.00	0.99	0.95
Met	ATG	12	14	26.20	34.40	17.62	15.78	1.00	1.00	1.00	1.00
Ile	ATA	0	0	0.00	0.00	0.17	0.78	0.00	0.00	0.01	0.03
Ile	ATT	1	0	2.18	0.00	0.17	0.93	0.06	0.00	0.01	0.03
Ile	ATC	16	23	34.93	56.51	36.47	29.07	0.94	1.00	0.99	0.94
Thr	ACG	4	7	8.73	17.20	17.97	18.71	0.17	0.30	0.28	0.26
Thr	ACA	1	0	2.18	0.00	0.87	1.94	0.04	0.00	0.01	0.03
Thr	ACT	0	0	0.00	0.00	0.17	1.28	0.00	0.00	0.00	0.02
Thr	ACC	19	16	41.48	39.31	46.24	41.78	0.79	0.70	0.71	0.66
Trp	TGG	4	3	8.73	7.37	13.26	14.34	1.00	1.00	1.00	1.00
End	TGA	0	1	0.00	2.46	2.62	2.13	0.00	1.00	0.83	0.80

Table AppdxIII-2 continued on page 179

Table AppdxIII-2 continued

AmAcid	Codon	Number		/1000				Fraction			
		ORF1	ORF2	ORF1	ORF2	SV	ST	ORF1	ORF2	SV	ST
Cys	TGT	0	0	0.00	0.00	0.35	0.93	0.00	0.00	0.04	0.12
Cys	TGC	4	6	8.73	14.74	8.20	7.14	1.00	1.00	0.96	0.88
End	TAG	1	0	2.18	0.00	0.52	0.39	1.00	0.00	0.17	0.15
End	TAA	0	0	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.05
Tyr	TAT	0	0	0.00	0.00	0.17	1.25	0.00	0.00	0.01	0.06
Tyr	TAC	13	3	28.38	7.37	20.76	19.73	1.00	1.00	0.99	0.94
Leu	TTG	0	0	0.00	0.00	0.87	3.15	0.00	0.00	0.01	0.03
Leu	TTA	0	0	0.00	0.00	0.17	0.15	0.00	0.00	0.00	0.00
Phe	TTT	0	0	0.00	0.00	0.17	0.58	0.00	0.00	0.01	0.02
Phe	TTC	9	11	19.65	27.03	29.14	27.10	1.00	1.00	0.99	0.98
Ser	TCG	9	9	19.65	22.11	12.91	14.76	0.27	0.36	0.23	0.28
Ser	TCA	0	0	0.00	0.00	0.52	1.23	0.00	0.00	0.01	0.02
Ser	TCT	0	0	0.00	0.00	1.05	0.74	0.00	0.00	0.02	0.01
Ser	TCC	17	14	37.12	34.40	25.48	20.39	0.52	0.56	0.46	0.39
Arg	CGG	6	6	13.10	14.74	29.66	29.97	0.33	0.29	0.40	0.37
Arg	CGA	0	0	0.00	0.00	1.92	2.65	0.00	0.00	0.03	0.03
Arg	CGT	3	0	6.55	0.00	3.49	6.25	0.17	0.00	0.05	0.08
Arg	CGC	8	15	17.47	36.86	35.77	37.35	0.44	0.71	0.48	0.46
Gln	CAG	12	15	26.20	36.86	23.21	25.92	1.00	1.00	0.98	0.94
Gln	CAA	0	0	0.00	0.00	0.52	1.78	0.00	0.00	0.02	0.06
His	CAT	0	0	0.00	0.00	1.05	2.24	0.00	0.00	0.05	0.09
His	CAC	9	8	19.65	19.66	19.54	21.94	1.00	1.00	0.95	0.91
Leu	CTG	18	20	39.30	49.14	36.12	56.66	0.53	0.53	0.41	0.57
Leu	CTA	0	0	0.00	0.00	0.00	0.48	0.00	0.00	0.00	0.00
Leu	CTT	0	0	0.00	0.00	1.22	2.02	0.00	0.00	0.01	0.02
Leu	CTC	16	18	34.93	44.23	49.38	36.81	0.47	0.47	0.56	0.37
Pro	CCG	18	14	39.30	34.40	25.30	32.05	0.67	0.64	0.47	0.53
Pro	CCA	0	0	0.00	0.00	1.05	1.45	0.00	0.00	0.02	0.02
Pro	CCT	0	0	0.00	0.00	1.74	1.83	0.00	0.00	0.03	0.03
Pro	CCC	9	8	19.65	19.66	26.17	25.09	0.33	0.36	0.48	0.42

Table AppdxIII-3. Amino acid usage in ORF1 and ORF2.

ORF1				ORF2			
AA	Count	% By #	% By Wt	AA	Count	% By #	% By Wt
Ala A	41	8.84%	7.48%	Ala A	43	10.57%	8.98%
Xxx X	0	0.00%	0.00%	Xxx X	0	0.00%	0.00%
Arg R	18	3.88%	6.42%	Arg R	21	5.16%	8.57%
Asn N	13	2.80%	3.52%	Asn N	13	3.19%	4.02%
Asx B	0	0.00%	0.00%	Asx B	0	0.00%	0.00%
Asp D	29	6.25%	7.91%	Asp D	26	6.39%	8.11%
Cys C	4	0.86%	0.99%	Cys C	6	1.47%	1.70%
Glu E	31	6.68%	9.34%	Glu E	23	5.65%	7.93%
Gln Q	13	2.80%	3.89%	Gln Q	15	3.69%	5.14%
Glx Z	0	0.00%	0.00%	Glx Z	0	0.00%	0.00%
Gly G	53	11.42%	8.15%	Gly G	47	11.55%	8.27%
His H	10	2.16%	3.18%	His H	8	1.97%	2.91%
Ile I	17	3.66%	4.57%	Ile I	23	5.65%	7.07%
Leu L	34	7.33%	9.13%	Leu L	38	9.34%	11.68%
Lys K	19	4.09%	5.69%	Lys K	12	2.95%	4.11%
*** *	0	0.00%	0.00%	*** *	0	0.00%	0.00%
Met M	12	2.59%	3.67%	Met M	14	3.44%	4.89%
Phe F	10	2.16%	3.38%	Phe F	11	2.70%	4.26%
Pro P	27	5.82%	6.37%	Pro P	22	5.41%	5.93%
Ser S	34	7.33%	7.32%	Ser S	25	6.14%	6.16%
Amb @	1	0.22%	0.00%	Amb @	0	0.00%	0.00%
Och #	0	0.00%	0.00%	Och #	0	0.00%	0.00%
Umb &	0	0.00%	0.00%	Umb &	1	0.25%	0.00%
Thr T	24	5.17%	5.86%	Thr T	23	5.65%	6.42%
Trp W	4	0.86%	1.67%	Trp W	3	0.74%	1.44%
Tyr Y	13	2.80%	4.82%	Tyr Y	3	0.74%	1.27%
Val V	57	12.28%	13.68%	Val V	30	7.37%	8.23%

ORF1:
Acidic = 70, Basic = 54
Non-Polar = 256, Polar = 208
MW = 48825.3
From 1 to 464, total = 464

ORF2:
Acidic = 57, Basic = 42
Non-Polar = 232, Polar = 175
MW = 42681.8
From 1 to 407, total=407

APPENDIX IV. PROTEINS RANKED IN BLAST SEARCHES OF THE GENBANK DATABASE FOR SIMILARITY TO THE DEDUCED AMINO ACID SEQUENCES OF ORF1 AND ORF2.

Table AppdxIV-1. Proteins ranked by a BlastP search of the GenBank database for similarity to the deduced amino acid sequence ORF1.

Sequences producing significant alignments:	Score (bits)	E Value
emb|CAA17193.1| (AL021897) cysM2 [Mycobacterium tuberculosis]	568	e-161
pir||JX0145 hemoprotein H-450 precursor - rat >gi|220759|db...	274	8e-73
ref|NP_000062.1|PCBS| cystathionine-beta-synthase >gi|54395...	274	8e-73
emb|CAA61252| (X88562) cystathionine beta-synthase [Homo sa...	274	8e-73
pir||B42790 cystathionine beta-synthase (EC 4.2.1.22) type ...	274	1e-72
gi|206600 (M88346) cystathionine beta-synthase [Rattus norv...	273	1e-72
sp|P32232|CBS_RAT CYSTATHIONINE BETA-SYNTHASE (SERINE SULFH...	273	2e-72
pir||C42790 cystathionine beta-synthase (EC 4.2.1.22) type ...	272	4e-72
gi|3776243 (AF042836) cystathionine beta-synthase minor iso...	272	5e-72
gi|4204469 (AF090120) cystathionine beta-synthetase; CBS [F...	270	2e-71
pir||A55760 cystathionine beta-synthase (EC 4.2.1.22) - human	268	6e-71
sp|P46794|CBS_DICDI CYSTATHIONINE BETA-SYNTHASE (SERINE SUL...	262	4e-69
dbj|BAA80212.1| (AP000061) 393aa long hypothetical cystathi...	245	6e-64
dbj|BAA81640.1| (AB028629) cysteine synthase [Clostridium p...	231	1e-59
sp|P32582|CBS_YEAST CYSTATHIONINE BETA-SYNTHASE (SERINE SUL...	224	1e-57
dbj|BAA03952| (D16502) cystathionine beta-synthase [Sacchar...	224	1e-57
pir||D42790 cystathionine beta-synthase (EC 4.2.1.22) type ...	223	2e-57
dbj|BAA03947| (D16496) cystathionine beta-synthase [Sacchar...	219	5e-56
sp|P37887|CYSK_BACSU CYSTEINE SYNTHASE (O-ACETYLSERINE SULF...	216	2e-55
gi|393279 (L14578) cystathionine beta-synthase [Saccharomyc...	215	5e-55
dbj|BAA16664| (D90899) cysteine synthase [Synechocystis sp.]	215	7e-55
pir||S29733 cysteine synthase (EC 4.2.99.8) B - spinach >gi...	214	1e-54
sp|P32260|CYSL_SPIOL CYSTEINE SYNTHASE CHLOROPLAST PRECURSO...	214	1e-54
emb|CAB06151| (Z83860) cysK [Mycobacterium tuberculosis]	212	5e-54
emb|CAA71800| (Y10847) O-acetylserine(thiol) lyase [Brassic...	211	1e-53
emb|CAA71798| (Y10845) O-acetylserine(thiol) lyase [Brassic...	210	2e-53
gi|3290022 (AF044173) cysteine synthase; CS-B; O-acetylseri...	210	2e-53
sp|Q00834|CYSK_SPIOL CYSTEINE SYNTHASE (O-ACETYLSERINE SULF...	209	3e-53
gi|3342569 (AF078693) putative O-acetylserine(thiol)lyase p...	208	7e-53
emb|CAB11412| (Z98741) cysteine synthase [Mycobacterium lep...	207	2e-52
pir||S65533 cysteine synthase (EC 4.2.99.8) 3A - Arabidopsi...	206	2e-52
emb|CAB10267.1| (Z97337) cytosolic O-acetylserine(thiol)lya...	206	2e-52
gi|2281095 (AC002333) cysteine synthase, cpACS1 [Arabidopsi...	206	3e-52
gb|AAD23909.1|AF073697_1 (AF073697) cysteine synthase [Oryz...	204	1e-51
pir||S49587 cysteine synthase (EC 4.2.99.8) cpACS1 - Arabid...	203	2e-51
emb|CAB01676| (Z78415) Similarity to Wheat cysteine synthas...	203	2e-51
gb|AAD23908.1|AF073696_1 (AF073696) cysteine synthase [Oryz...	203	3e-51
sp|Q43317|CYSK_CITVU CYSTEINE SYNTHASE (O-ACETYLSERINE SULF...	202	5e-51
gi|3290020 (AF044172) cysteine synthase; CS-A; O-acetylseri...	201	9e-51
sp|P50867|CYSK_EMENI CYSTEINE SYNTHASE (O-ACETYLSERINE SULF...	200	1e-50
gi|464226 (L05184) O-acetylserine-(thiol)-lyase [Spinacia o...	198	1e-49
gb|AAB52276.1| (U97004) contains similarity to Pfam domain ...	196	2e-49
dbj|BAA78561.1| (AB024283) cysteine synthase [Arabidopsis t...	196	2e-49
emb|CAA88980| (Z49131) similar to cystathionine beta-syntha...	196	4e-49
sp|P31300|CYSL_CAPAN CYSTEINE SYNTHASE CHLOROPLAST PRECURSO...	195	7e-49
gi|2293314 (AF008220) putative cysteine synthase [Bacillus ...	194	9e-49
sp|P38076|CYSK_WHEAT CYSTEINE SYNTHASE (O-ACETYLSERINE SULF...	194	1e-48
gb|AAB65342.1| (AF016425) Contains similarity to Pfam domai...	194	1e-48
gb|AAD23907.1|AF073695_1 (AF073695) cysteine synthase [Oryz...	192	4e-48
sp|Q43725|CYSM_ARATH CYSTEINE SYNTHASE MITOCHONDRIAL PRECUR...	192	6e-48
dbj|BAA17450| (D90906) cysteine synthase [Synechocystis sp.]	192	6e-48
sp|P47999|CYSL_ARATH CYSTEINE SYNTHASE CHLOROPLAST PRECURSO...	191	1e-47
pir||S48695 cysteine synthase (EC 4.2.99.8) isoform 7-4 pre...	191	1e-47
emb|CAA19052| (AL023589) cysteine synthase [Schizosaccharom...	190	2e-47

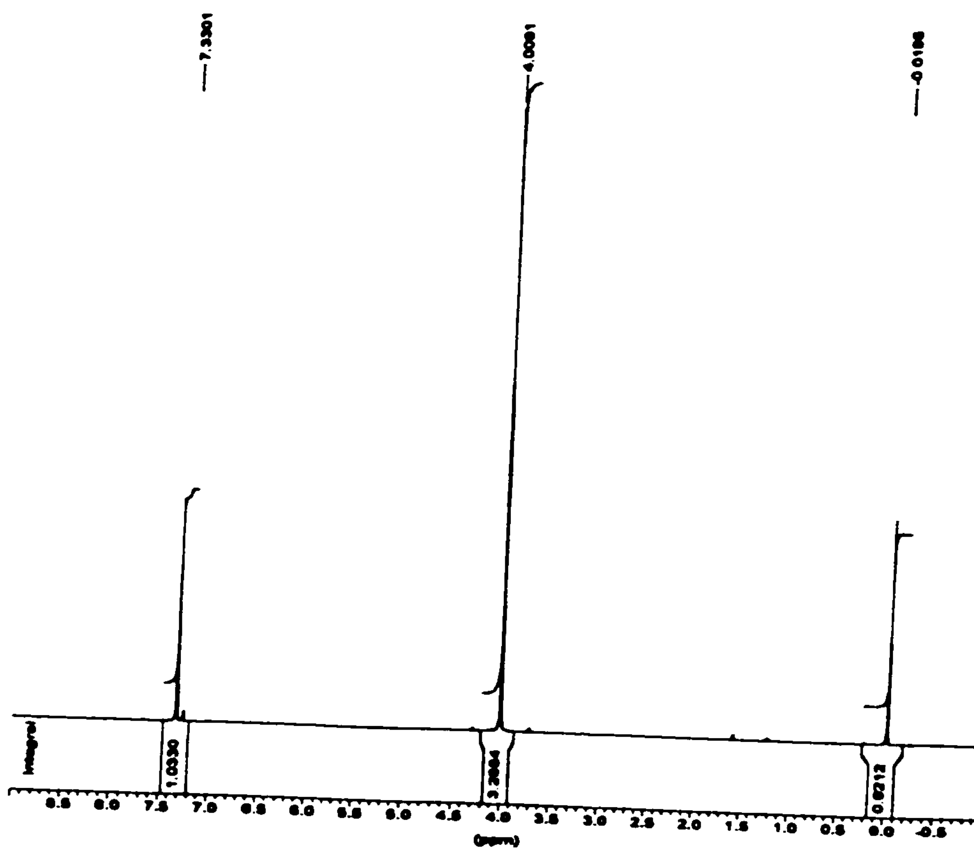
Table AppdxIV-2. Proteins ranked by a BlastP search of the GenBank database for similarity to the deduced amino acid sequence of ORF2.

Sequences producing significant alignments:	Score (bits)	E Value
emb|CAA17190.1| (AL021897) fadA3 [Mycobacterium tuberculosis]	599	e-170
sp|P45359|THL_CLOAB ACETYL-COA ACETYLTRANSFERASE (ACETOACET...	296	2e-79
emb|CAB15272| (Z99120) similar to acetyl-CoA C-acyltransfer...	290	1e-77
sp|P14611|THIL_ALCEU ACETYL-COA ACETYLTRANSFERASE (ACETOACE...	288	5e-77
emb|CAA66099| (X97452) acetyl-CoA acetyltransferase (thiola...	283	2e-75
dbj|BAA15002| (D90777) 3-ketoacyl-CoA thiolase (EC 2.3.1.16...	283	2e-75
sp|P44873|ATOB_HAEIN ACETYL-COA ACETYLTRANSFERASE (ACETOACE...	282	2e-75
pir||A64092 acetyl coenzyme A acetyltransferase (thiolase) ...	282	2e-75
sp|P76461|ATOB_ECOLI ACETYL-COA ACETYLTRANSFERASE (ACETOACE...	282	3e-75
sp|P45369|THIL_CHRVI ACETYL-COA ACETYLTRANSFERASE (ACETOACE...	282	4e-75
sp|P45363|THIL_THIVI ACETYL-COA ACETYLTRANSFERASE (ACETOACE...	280	9e-75
pir||B48376 beta-ketothiolase=poly(3-hydroxyalkanoate) synt...	280	1e-74
gi|3309206 (AF072735) thiolase B [Clostridium acetobutylicum]	277	1e-73
gb|AAC46434.1| (AF009224) beta-ketoadipyl CoA thiolase [Aci...	276	2e-73
gi|141777 (L05770) beta-ketoadipyl CoA thiolase [Acinetobac...	276	2e-73
gi|3253200 (AF029714) PhaD [Pseudomonas putida]	273	2e-72
emb|CAB40681.1| (AL049587) putative thiolase [Streptomyces ...	273	2e-72
emb|CAB39721.1| (AL049485) probable acetyl coA acetyltransf...	270	1e-71
pir||XUEC acetyl-CoA C-acyltransferase (EC 2.3.1.16) - Esch...	269	3e-71
sp|P21151|THIK_ECOLI 3-KETOACYL-COA THIOLASE (FATTY OXIDATI...	268	4e-71
emb|CAB40810.1| (X52837) fadA gene product (AA 1 - 387) [Es...	267	1e-70
sp|P28790|THIK_PSEFR 3-KETOACYL-COA THIOLASE (FATTY OXIDATI...	266	2e-70
gi|148245 (M87049) small (beta) subunit of the fatty acid-o...	266	3e-70
dbj|BAA36197| (AB014757) beta-ketothiolase [Pseudomonas sp....	266	3e-70
emb|CAB04793| (Z82038) acetyl coenzyme A acetyltransferase ...	266	3e-70
gb|AAD22035.1| (AF109386) beta-ketoadipyl-CoA thiolase; Pca...	265	3e-70
sp|P54810|THIL_PARDE ACETYL-COA ACETYLTRANSFERASE (ACETOACE...	265	4e-70
gi|2649384 (AE001021) 3-ketoacyl-CoA thiolase (fadA-2) [Arc...	262	4e-69
gi|576786 (L37761) beta-ketothiolase [Acinetobacter sp.]	261	5e-69
emb|CAB45575.1| (AL079355) beta-ketoadipyl-CoA thiolase [St...	261	9e-69
gb|AAD34967.1|AF143489_1 (AF143489) acetyl-CoA acetyltransf...	259	3e-68
gi|3982782 (AF078795) ketothiolase protein PhaA [Alcaligene...	259	3e-68
gi|2649566 (AE001032) 3-ketoacyl-CoA thiolase (fadA-1) [Arc...	256	2e-67
gi|506695 (U10895) PcaF [Pseudomonas putida]	254	7e-67
gi|4097187 (U47026) 3-ketothiolase [Alcaligenes latus]	252	3e-66
dbj|BAA33156| (AB009273) beta-ketothiolase [Comamonas acido...	251	6e-66
sp|P50174|THIL_RHIME ACETYL-COA ACETYLTRANSFERASE (ACETOACE...	249	3e-65
pir||XXGZAC acetyl-CoA C-acetyltransferase (EC 2.3.1.9) - Z...	245	4e-64
sp|P07097|THIL_ZOORA ACETYL-COA ACETYLTRANSFERASE (ACETOACE...	245	6e-64
gb|AAC38322.1| (AF026544) beta-ketothiolase [Ralstonia eutr...	244	7e-64
sp|P21775|THIJ_RAT 3-KETOACYL-COA THIOLASE PEROXISOMAL A PR...	244	7e-64
pir||JT0551 acetyl-CoA C-acyltransferase (EC 2.3.1.16), per...	243	1e-63
gi|3169568 (AF062589) 3-keto-acyl-CoA-thiolase 1 [Arabidops...	243	1e-63
gi|3169569 (AF062589) 3-keto-acyl-CoA thiolase 2 [Arabidops...	243	1e-63
sp|P07871|THIK_RAT 3-KETOACYL-COA THIOLASE PEROXISOMAL B PR...	243	2e-63
gb|AAD34966.1|AF143488_1 (AF143488) acetyl-CoA acetyltransf...	242	4e-63
dbj|BAA14107| (D90063) 3-ketoacyl-CoA thiolase B [Rattus no...	242	4e-63
emb|CAB04455| (Z81546) similar to Thiolases; cDNA EST yk345...	242	5e-63
gi|205097 (J02749) peroxisomal 3-ketoacyl-CoA thiolase prec...	240	1e-62
gi|2306817 (AF002013) beta-ketothiolase [Alcaligenes sp. SH...	240	1e-62
gi|1262448 (U37723) FipA [Fusobacterium nucleatum]	239	2e-62
gi|2924779 (AC002334) putative 3-ketoacyl-CoA thiolase [Ara...	238	5e-62

APPENDIX V. NMR SPECTRA OF COMPOUNDS SYNTHESIZED IN THIS STUDY

Fig. 35. The [1]H NMR spectrum (A) and the [13]C NMR spectrum (B) of 1,2-dinitroveratrole. The signals in A at 4.03 and 7.35 ppm are due to O-CH3 and C-H; signals at 0.00 and 7.27 ppm are due to tetramethylsilane and $CHCl_3$, respectively. The signals in B at 57.1, 107.0, 136.7 and 151.9 ppm are due to -OCH_3, C-H, C-NO_2 and C-OCH_3, respectively. The three signals at approximately 77 ppm are due to the solvent CH_3COCH_3.

A



B

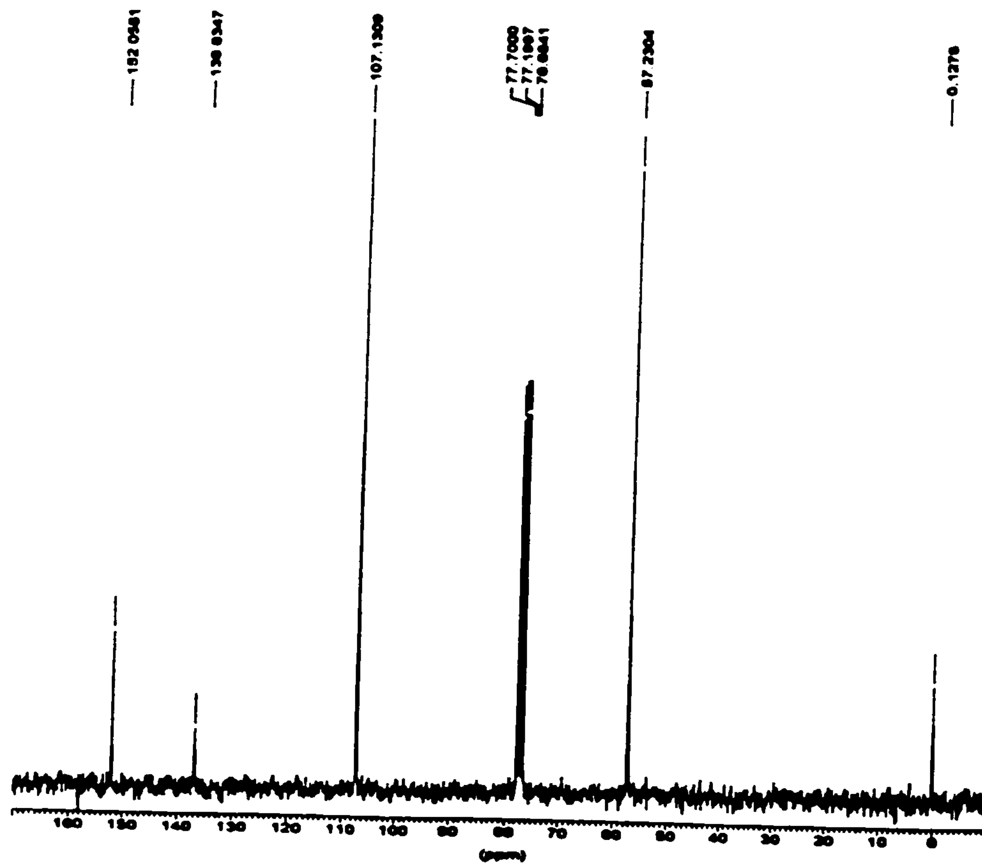
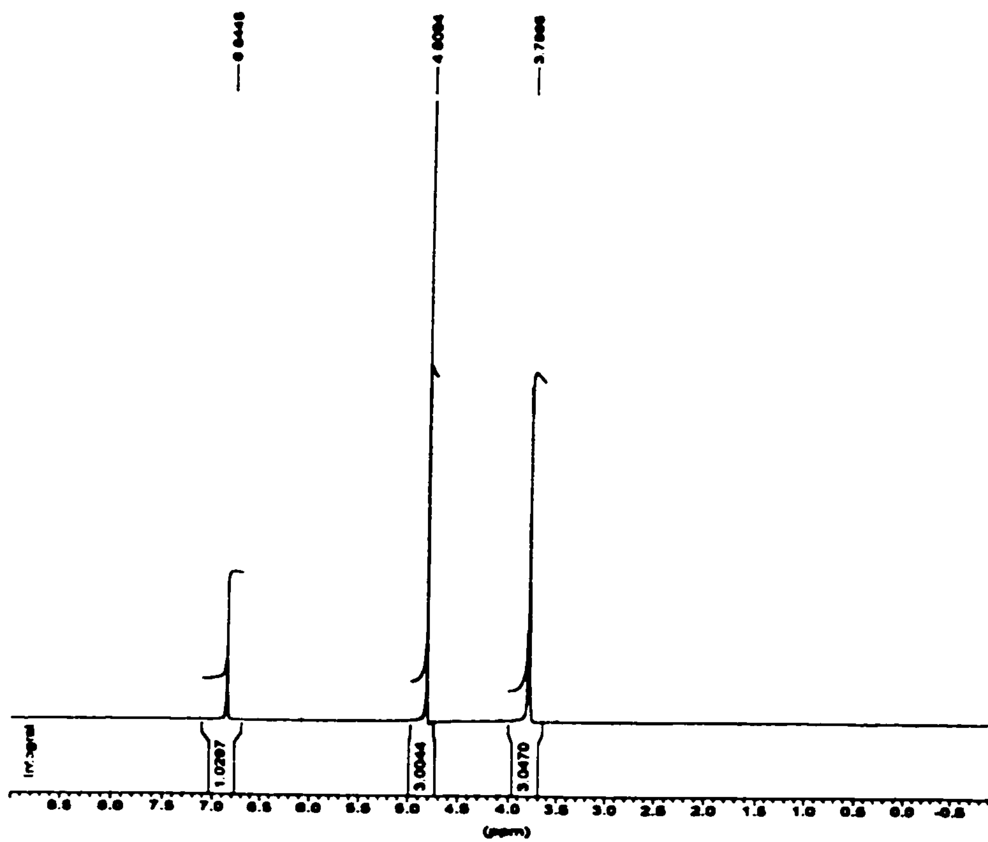


Fig. 35

Fig. 36. The ^{1}H NMR spectrum (A) and the ^{113}C NMR spectrum (B) of 1,2-diamino-4,5-dimethoxylbenzene (DDB). The signals in A at 3.78 and 6.84 ppm are due to OCH_3 and C-H respectively. The signals in B for OCH_3, C-H, C-$^{+}NH_3$ and C-OCH_3 are at 58.9, 109.2, 122.8 and 149.5 ppm, respectively.

A



B

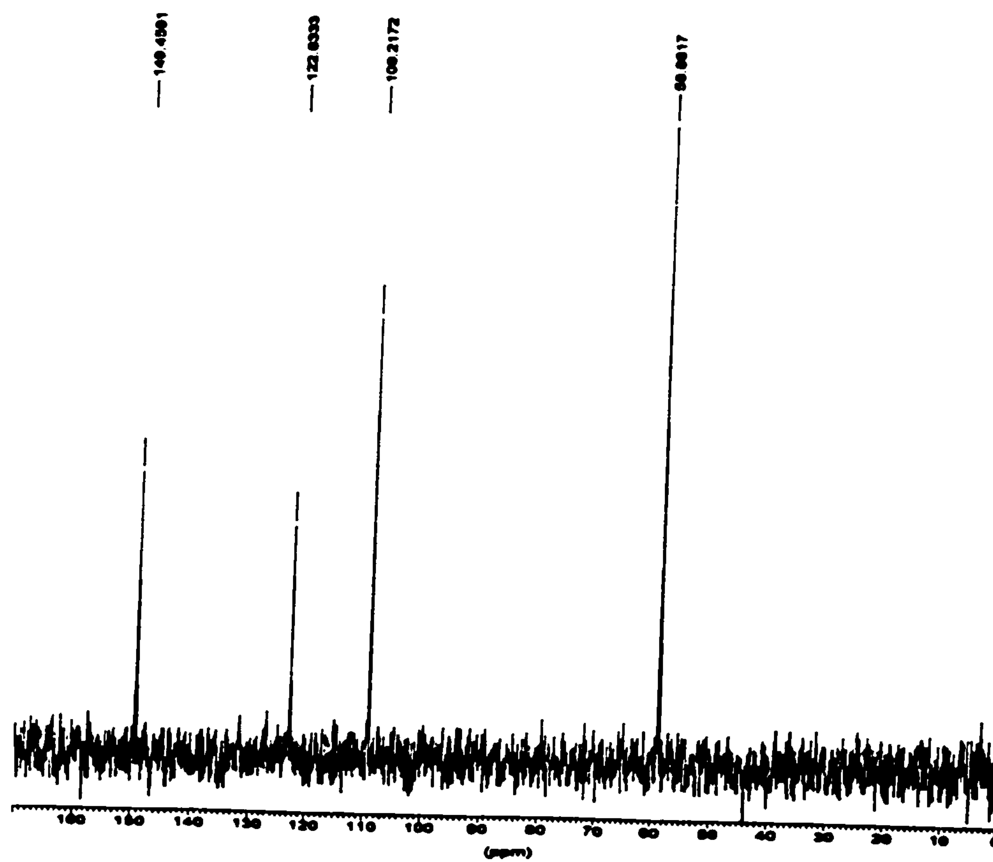
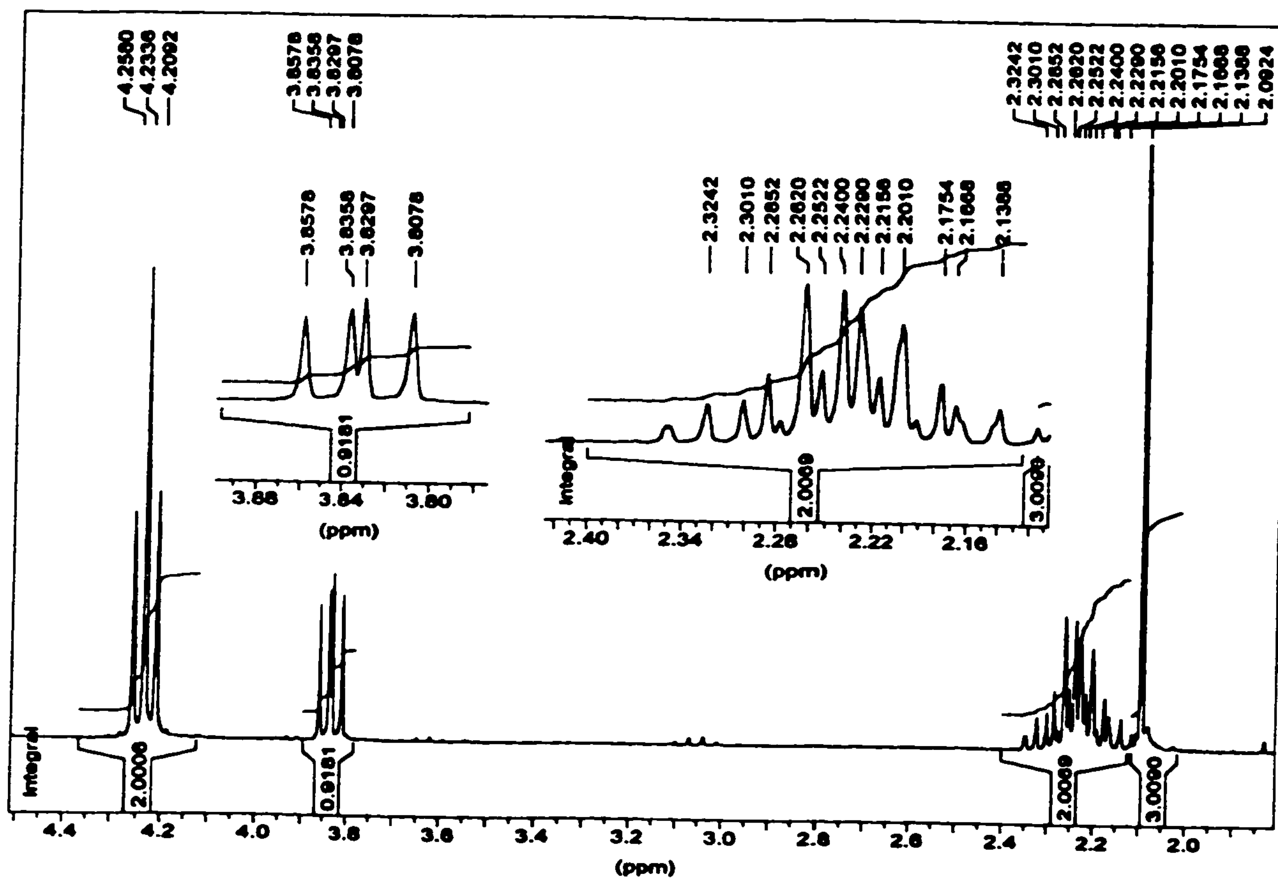


Fig. 36.

Fig. 37. The [1]H NMR spectrum (A) and the [13]C NMR spectrum (B) of O-acetylhomoserine. The signals in A are at 2.08 ppm (3H, s, CH3), 2.10-2.34 ppm (2H, m, CH2), 3.82 ppm (1H, dd, J'=7.0 and 5.5 H3, CH) and 4.23 ppm (2H, t, J=6.1 H3, CH_2O). In B the signals are at 23.1 ppm (-CH3), 32.0 ppm (CH2), 55.4 ppm (CH), 64.3 ppm (CH2O), 176.7 ppm and 176.9 ppm (acid and ester C=O).

A



B

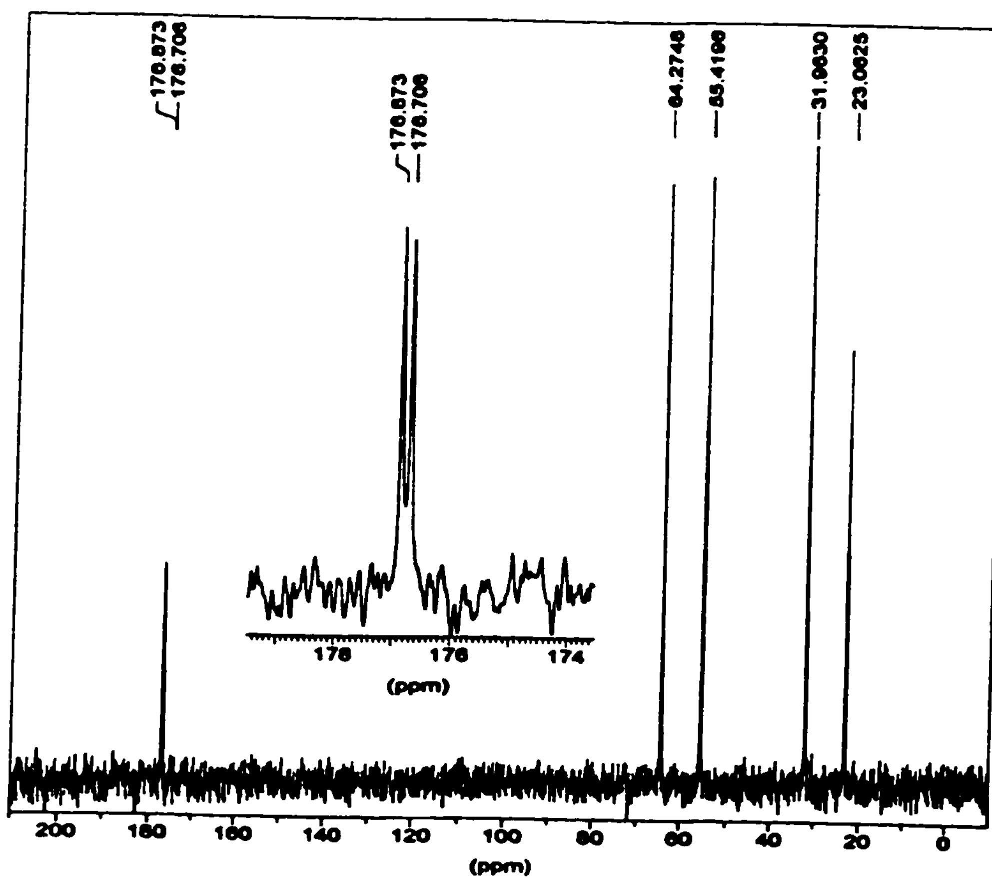


Fig. 37

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