***Streptomyces akiyoshiensis* differs from other Gram-positive bacteria in the organization of a core biosynthetic pathway gene for aspartate family amino acids**

Yunzheng Le, Jianyong He† and Leo C. Vining

Author for correspondence: Leo C. Vining. Tel. +1 902 494 2040. Fax: +1 902 494 3736.

Department of Biology,
Dalhousie University,
Halifax, Nova Scotia,
Canada B3H 4J1

A partial *Sau3AI* digest of genomic DNA from *Streptomyces akiyoshiensis* was cloned in a *Streptomyces*–*Escherichia coli* shuttle vector, and the recombinant plasmids were used to transform *E. coli* CGSC 6212, which carries a mutation in the gene for aspartate semialdehyde dehydrogenase (Asd). One of 39 000 transformants tested grew on LB medium lacking diaminopimelate. A 17 kb plasmid (pJV21) isolated from this strain conferred prototrophy when used to transform *E. coli* CGSC 6212. The gene responsible was located on a 2.2 kb DNA fragment by subcloning. Nucleotide sequencing and codon preference analysis of the subcloned insert and of the 3.3 kb insert in the Asd⁺-complementing plasmid pJV36 located three complete and two incomplete open reading frames (ORFs). One of these (ORF3), encoding a polypeptide of 338 amino acids (*M_r* 35 484), was identified as the gene for Asd by comparing its sequence with database sequences of *asd* from other bacteria. The inability of pJV30, in which a segment of ORF3 had been deleted, to transform *E. coli* CGSC 6212 to prototrophy supported this assignment. Southern hybridization indicated that the sequenced region of the cloned DNA fragment represented a continuous segment of the *S. akiyoshiensis* chromosome. The deduced amino acid sequences of the ORFs adjacent to *asd* showed no similarity to sequences for aspartate kinase (Ask); also, transformation with plasmids containing *asd* and adjacent regions from the *S. akiyoshiensis* chromosome did not complement the *ask* mutant *E. coli* CGSC 5074. It is concluded that *asd* and *ask* in *S. akiyoshiensis* are not present in an operon, and thus are organized differently from these genes in the Gram-positive bacteria previously examined.

Keywords: aspartate pathway, aspartate semialdehyde dehydrogenase, *Streptomyces akiyoshiensis*, *asd*

INTRODUCTION

Cultures of *Streptomyces akiyoshiensis* accumulate 5-hydroxy-4-oxonorvaline (HON), a nonprotein amino acid active against tuberculous bacilli (Kanazawa *et al.*, 1960)

† **Present address:** Pharmaceuticals Department, Pharmacy University of Shenyang, 103 Wenhua Road, Shenyang 110015, People's Republic of China.

Abbreviations: Asd, aspartate semialdehyde dehydrogenase; Ask, aspartate kinase; DAP, diaminopimelic acid; HON, 5-hydroxy-4-oxonorvaline.

The GenBank accession number for the nucleotide sequence reported in this paper is U29446.

and some fungi (Yamaguchi *et al.*, 1990). From the pattern of isotope incorporation into the metabolite from labelled aspartate and acetate, White *et al.* (1988) postulated that the first step in HON biosynthesis is the condensation of an acetate derivative (e.g. acetyl- or malonyl-coenzyme A) with the activated β -carboxyl of aspartate. Potential forms of aspartate would be aspartyl phosphate or aspartate semialdehyde. Subsequent loss of the acetate-derived carboxyl group would generate HON from the six-carbon β -keto or β -hydroxy acid (or thioester) initially formed. Isolation of mutants altered in the biosynthesis of aspartate-family amino acids as well as in HON production supported the conclusion from precursor labelling that aspartate is a key biosynthetic intermediate. Efforts to characterize the mutations indicated that the

Fig. 1. Biosynthetic pathway for the aspartate family of amino acids.

pathway to HON diverged at an early step from the branching aspartate family pathway, but did not identify the branch-point (Le, 1994).

Amino acids of the aspartate family are synthesized by a similar series of biochemical reactions in all bacteria (Fig. 1); in particular, the core pathway from aspartic acid to homoserine is well conserved. However, differences in the regulation of the pathway give rise to multiple forms of some enzymes, and enzyme multiplicity can vary with the organism. In *Escherichia coli* there are three isozymes of aspartate kinase (Ask), regulated specifically by lysine, methionine or threonine (Theze *et al.*, 1974). In bacilli there are also three Asks, but they are regulated by diaminopimelic acid (DAP), lysine, or lysine and threonine (Zhang *et al.*, 1990). Only one Ask is present in *Pseudomonas*, *brevibacteria* and *corynebacteria* (Shiio & Miyajima, 1969; Cohen *et al.*, 1969; Cremer *et al.*, 1988). Aspartate semialdehyde dehydrogenase (Asd), which catalyses the conversion of aspartyl phosphate to aspartate semialdehyde, and thus is required for the biosynthesis of DAP, lysine, methionine, threonine and isoleucine, has so far been found in only one form in all micro-organisms examined.

In streptomycetes, where aspartate is a precursor not only of the related protein amino acids but sometimes also of secondary metabolites, there is evidence that regulatory mechanisms acting on core reactions in the aspartate pathway control the flow of metabolic intermediates to such products as the β -lactam antibiotics, which are derived from lysine via L- α -amino adipic acid (Whitney *et al.*, 1972; Madduri *et al.*, 1989; Vining *et al.*, 1990). Both Ask and Asd have been partially purified from *Streptomyces clavuligerus* (Mendelovitz & Aharonowitz, 1982). Concerted feedback inhibition of Ask was demonstrated with a mixture of lysine and threonine, while lysine alone caused activation. No feedback effects on Asd by pathway end-products have been reported, but the regulation of this and other core enzymes of the aspartate pathway in streptomycetes has not been examined in depth. To better understand the role of the core reactions in the biosynthesis of aspartate family amino acids and related

secondary metabolites in *S. akiyoshiensis*, we have cloned and sequenced the gene for Asd and its adjacent regions.

METHODS

Micro-organisms, plasmids and media. The characteristics and sources of bacteria, phage and plasmids used are given in Table 1. *Streptomyces akiyoshiensis* was maintained on maltose/yeast extract/malt extract (MYM) agar (Stuttard, 1982) adjusted to pH 7.0. For isolation of genomic DNA, *S. akiyoshiensis* was grown in a medium containing (per litre): Trypticase Soy Broth (BBL; 30 g), yeast extract (10 g), sucrose (100 g) and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (10.1 g). Sterile CaCl_2 solution (7.36%, w/v; 20 ml l⁻¹) was added immediately before use. *Escherichia coli* strains were grown in L-broth; for *asd* auxotrophs, and in screening for *asd* mutants, the medium was supplemented with DAP.

Manipulation of DNA. Except where selection for Asd⁻ strains was involved, *E. coli* DH5 α was used for plasmid construction and maintenance; competent cells were prepared and transformed as described by Sambrook *et al.* (1989). Plasmids from *E. coli* and *S. akiyoshiensis* were isolated by the method of Lee & Rasheed (1990); single-stranded DNA was obtained as described by Sambrook *et al.* (1989). DNA fragments from plasmids digested with restriction enzymes were separated by electrophoresis in 0.8% agarose gels; individual fragments were purified with the GeneClean kit (BIO 101). Genomic DNA from *S. akiyoshiensis* was isolated as described by Hopwood *et al.* (1985); for use in a genomic library it was partially digested with *Sau*3AI and size-fractionated by centrifugation in a sucrose density gradient (Hopwood *et al.*, 1985).

DNA cloning. Sized 6–15 kb fragments from *Sau*3AI partial digestion of *S. akiyoshiensis* genomic DNA were ligated behind the *lac* promoter in pHJL400 (Larson & Hershberger, 1986) linearized at its *Bam*HI site and dephosphorylated with calf-intestinal alkaline phosphatase (Promega). The recombinant plasmid library was used to transform competent cells of the *E. coli asd* mutant strain CGSC 6212. Transformed cells were plated on L-broth agar containing ampicillin (100 $\mu\text{g ml}^{-1}$), and examined for prototrophic colonies. Similar transformation and screening methods were used to sub-clone *S. akiyoshiensis* DNA inserts.

Hybridization. Genomic DNA, pJV21 and pJV36 were digested with *Pvu*II. The digests were fractionated in 1% (w/v) agarose gels, and DNA was transferred to a nylon membrane (Zeta-Probe, Bio-Rad). The 0.75 kb *Sal*I fragment of pJV24 was labelled with [α -³²P]dCTP by the random priming procedure (Feinberg & Vogelstein, 1983) for use as the probe. Hybridization was carried out at 65 °C overnight, after which the membrane was washed at 65 °C with a solution containing 15 mM sodium chloride, 1.5 mM sodium citrate and 0.1% sodium dodecyl sulphate.

DNA sequencing and sequence analysis. The DNA inserts in pJV24 and pJV36 were each subcloned in the phagemids pBluescript II SK(+) and SK(-), allowing both strands to be sequenced; nested deletions were generated by the DNase I method (Sambrook *et al.*, 1989). Single-stranded DNA produced from the phagemids was sequenced by the dideoxynucleotide-mediated chain-termination method (Sanger *et al.*, 1977). Sequenase version 2.0 (US Biochemical) was used for DNA synthesis. The sequence data were analysed with 'DNA strider' (Marck, 1988), and programs (version 7.1) developed by the Genetics Computer Group, University of Wisconsin Biotechnology Center (Devereux *et al.*, 1984). Amino acid sequences deduced from open reading frames (ORFs) were

Table 1. Bacteria, plasmids and phage used

Genotype/phenotype		Source/reference
Strains		
<i>Streptomyces akiyoshiensis</i> ATCC 1348	Wild-type	American Type Culture Collection
<i>Escherichia coli</i> CGSC 5074	F ⁻ <i>thrA1101 supE44 λ⁻ rpsL9 malT1 (λ^R) xyl-7 mtl-2 ilvA296 metL1000 arg-1000 thi-1 lysC1001</i>	<i>E. coli</i> Genetic Stock Center
CGSC 6212	F ⁻ <i>φ80dlacZΔ(lacZYA-argF)4169 supE44 λ⁻ gyrA recA1 relA1 endA1 Δasd4 Δ[γhf-2::Tn10] hsdR17</i>	R. Curtiss III, Biology Department, Washington University
DH5α	F ⁻ <i>φ80dlacΔM15Δ(lacZYA-argF)U169 supE44 λ⁻ thi-1 gyrA recA1 relA1 endA1 hsdR17</i>	Sambrook <i>et al.</i> (1989)
Plasmids and phage		
pHJL400	Amp ^r <i>lacZ'</i> <i>tsr</i> ; bifunctional	Larson & Hershberger (1986)
pJV21	Amp ^r <i>lacZ'</i> <i>asd⁺</i> <i>tsr</i> ; bifunctional	This study
pJV24, pJV36	Amp ^r <i>lacZ'</i> <i>asd</i> ; bifunctional	This study
pJV30	Amp ^r ; bifunctional	This study
pTZ18R, pTZ19R	Amp ^r <i>lacZ'</i>	Mead & Kemper (1988)
pJV31, pJV32	Amp ^r <i>lacZ'</i> <i>asd⁺</i>	This study
pBluescript II SK(+) and SK(-)	Amp ^r <i>lacZ'</i>	Stratagene
pJV25-29, pJV34-35	Amp ^r	This study
VCSM13	Kan ^r	Stratagene

compared with sequences in the GenBank database using BLAST (Altschul *et al.*, 1990). Amino acid sequences were aligned using CLUSTAL software (Higgins & Sharp, 1988).

RESULTS

Cloning of *asd* from *S. akiyoshiensis*

A library of *S. akiyoshiensis* DNA fragments in the *Streptomyces-E. coli* shuttle vector pHJL400 (Larson & Hershberger, 1986) was used to transform the *E. coli asd* mutant strain CGSC 6212. Screening approximately 39 000 transformants for prototrophy yielded one Asd⁺ colony, from which we recovered a 17 kb plasmid (pJV21; Fig. 2) that conferred the Asd⁺ phenotype. To locate the *asd* gene within the 11.2 kb insert, pJV21 was partially digested with *Sa*I, and the fragments were ligated with the 4.9 kb vector fragment generated when pHJL400 was digested with *Sa*I. Transformation of *E. coli* CGSC 6212 with the ligation mixture, selection of Asd⁺ transformants, and screening of these for the smallest plasmid present gave pJV24. This plasmid contained a 2.2 kb insert but lacked a 0.9 kb region of pHJL400 containing *lacZ'* and *tsr* sequences that had been removed during the digestion with *Sa*I (see Fig. 2).

Approximately 0.2 kb of DNA adjacent to the *lac* promoter was excised from the pJV24 insert by digesting the plasmid with a *Bam*HI/*Kpn*I mixture and subcloning the remainder of the insert in pHJL400 similarly digested with *Bam*HI and *Kpn*I. The plasmid (pJV30) obtained by transforming *E. coli* CGSC 6212 in the presence of DAP failed to complement the *asd* mutation in this host when DAP was omitted from the medium. Since the *Kpn*I site, like the *Sa*I site, is located within the *tsr* sequence of

pHJL400, and thus should not affect *asd* expression, the results positioned *asd* near the end of the insert adjoining the vector promoter in pJV24. To obtain a fragment containing *asd* with a larger flanking region of *S. akiyoshiensis* DNA at this end, pJV24 was digested with *Hind*III and *Bam*HI; the 6.9 kb fragment in the digest was ligated to the 1.2 kb fragment of insert DNA recovered by gel electrophoresis from a *Hind*III/*Bam*HI double digest of pJV21 (see Fig. 2); transformation of *E. coli* CGSC 6212 and selection for *asd* yielded pJV36, containing a 3.3 kb insert.

Sequencing and analysis of the DNA cloned in pJV36

The *S. akiyoshiensis* DNA insert cloned in pJV24 was excised as a mixture of 0.75 and 1.4 kb fragments by digesting the plasmid with *Sa*I. The fragments were separated by gel electrophoresis, and each was cloned in the *Sa*I site of pBluescript II SK(+) to give pJV25 and pJV27, respectively; the single-stranded DNA generated from them was sequenced. To obtain the opposite strands, the inserts in pJV25 and pJV27 were excised with *Hind*III/*Kpn*I, and ligated to similarly digested pBluescript II SK(-), giving pJV26 and pJV28, respectively. The sequence at the junction of the 0.75 and 1.4 kb fragments was confirmed by subcloning the 0.75 kb *Bam*HI-*Sst*I fragment of pJV24 in *Bam*HI/*Sst*I-digested pBluescript II SK(+), and sequencing single-strand DNA obtained from the resulting phagemid pJV29. The region of pJV36 not already sequenced in pJV24 was subcloned as a 1.2 kb *Hind*III-*Bam*HI fragment in *Hind*III/*Bam*HI-digested pBluescript II SK(+) and pBluescript II SK(-) to give pJV34 and pJV35, respectively. ORFs in the nucleotide sequence were detected with the

Fig. 2. Construction of recombinant plasmids. A partial *Sau3AI* digest of *S. akyoshiensis* DNA was ligated with *Bam*HI-linearized pHJL400 to give pJV21. Partial *Sal*I digestion of pJV21 and ligation with a *Sal*I digest of pHJL400 yielded pJV24. To construct pJV36, the 1.2 kb insert fragment obtained by digesting pJV21 with *Hind*III and *Bam*HI was ligated to pJV24, from which a 0.2 kb fragment had been removed by *Hind*III/*Bam*HI digestion. All plasmids were isolated from transformants of *E. coli* CGSC 6212 selected for prototrophy. Asterisks identify restriction enzymes for which sites additional to those shown are present in a plasmid.

Fig. 3. Map of selected restriction sites in the *S. akyoshiensis* DNA inserts of pJV21, pJV24 and pJV36. For *Sal*I, only sites present in all three plasmids are included. For *Pvu*II and *Bst*XI, only sites present in both pJV21 and pJV36 are included; *Hind*III is in the vector polylinker. ORFs and their orientation are shown as boxed arrows; incomplete ORF segments are shown by broken outlines.

CODONPREFERENCE program (Devereux *et al.* 1984) using the table for frequency of codon usage in streptomycetes compiled by Wright & Bibb (1992). Two incomplete ORFs (1 and 5) and three complete ORFs were present in the pJV36 insert (Fig. 3).

Sequence of *asd* from *S. akyoshiensis*

A comparison of amino acid sequences in the GenBank database with sequences deduced from ORFs 1–5 in the cloned *S. akyoshiensis* DNA showed strong similarities

```

1 Sali
GTCGAGACCGCAGGTCGCGAACCATGGATGGAGACGCGCTCCCATCTCTCGGGCAGACCGGGGAAAGAGTGGACAGG
81
CGGGCCGTCGGTCGGCGCATGATGGAAAGCCTCGGCCAAGCAACGAGCAGCTGAAGAGACGCGAGGAGAGGAGTCCACC
160/1
GTG AGG GTC GGA ATC GTC GGA GCC ACC GGA CAG GTG GGC ACG GTC ATG CGC AGG ATC CTC
Met arg val gly ile val gly ala thr gly gln val gly thr val met arg arg ile leu
220/21
ACG GAG CGG AAC TTC CCG GTC ACG GAG CTG CGC CTG TTC GCC TCG GCC CGT TCC GCG GGC
thr glu arg asn phe pro val thr glu leu arg leu phe ala ser ala arg ser ala gly
280/41
ACG GAG CTG GAC GGC GTG ACG GTG GAG GAC GCG GCG ACC GCC GAC TAC ACC GGC CTG GAC
thr glu leu asp gly val thr val glu asp ala ala thr ala asp tyr thr gly leu asp
340/61
ATC GTG CTG TTC TCC GCG GCG GCG GCG ACC TCC AAG GCG CTG GCG GAG AAG GTC GCC TCC
ile val leu phe ser ala gly gly ala thr ser lys ala leu ala glu lys val ala ser
400/81
CAG GGC GCG GTC GTG ATC GAC AAC TCC TCC GCG TGG CGC AAG CAC CCG GAG GTC CCC CTG
gln gly ala val val ile asp asn ser ser ala trp arg lys his pro glu val pro leu
460/101
GTC GTC TCC GAG GTC AAC CCG CAC GCG ATC AAG GAC CGC CCC AAG GGC ATC ATC GCC AAC
val val ser glu val asn pro his ala ile lys asp arg pro lys gly ile ile ala asn
520/121
CCG AAC TGC ACG ACG ATG GCC GCG ATG CCG GTG CTG CGT CCG CTG CAC GAG GCG GCG
pro asn cys thr thr met ala ala met ser val leu arg pro leu his asp ala gly
580/141
CTG GAA GCC CTC GTC GTC GCC ACG TAC CAG GCG GTC TCC GGT TCC GGC CTC GCG GCG GTC
leu glu ala leu val val ala thr tyr gln ala val ser gly ser gly leu ala gly val
640/161
GCC GAG CTG CAC GGC CAG ACG CAG AAG GTC GTC GCC GAC GCC GAG AAG CTC ACC CAC GAC
ala glu leu his gly gln thr gln lys val val ala asp ala glu lys leu thr his asp
700/181
GGT GAG GCG GTG GAC TTC CCG GAG CCG GGC GTC TAC AAG CGC CCC ATC GCC TTC AAC GTG
gly glu ala val asp phe pro glu pro gly val tyr lys arg pro ile ala phe asn val
760/201
CTG CCG CTG GCG GCG AGC ATC GTC GAC GAC GCG CTG AAC GAG ACC GAC GAG GAG CAG AAG
leu pro leu ala gly ser ile val asp asp gly leu asn glu thr asp glu glu gln lys
820/221
CTC CGC AAC GAG TCC CGC AAG ATC CTG GAG ATC CCC GGG CTG AAG GTC TCC GGC ACC TGC
leu arg asn glu ser arg lys ile leu glu ile pro gly leu lys val ser gly thr cys
880/241
GTG CGC GTC CCG GTC TTC TCC GGC CAC TCC CTC CAG ATC AAC GCC CGT TTC GCC CGC CCG
val arg val pro val phe ser gly his ser leu gln ile asn ala arg phe ala arg pro
940/261
ATC TCG GCG GAC GGC GCG ACG GAG CTC CTG AAG GAC GCC CCC GGC GTC GAG CTC TCC GAC
ile ser ala asp gly ala thr glu leu leu lys asp ala pro gly val glu leu ser asp
1000/281
ATC CCG ACC CCC CTC CAG GCC GCC GGC AAG GAC CCG TCC TAC GTC GGC CGC ATC CGC AGT
ile pro thr pro leu gln ala ala gly lys asp pro ser tyr val gly arg ile arg ser
1060/301
GAC GAG ACG GTG GAC AAC GGC CTG GCC CTC TTC GTC TCC AAC GAC AAC CTC CGC AAG GGC
asp glu thr val asp asn gly leu ala leu phe val ser asn asp asn leu arg lys gly
1120/321
GCG GCG CTG AAC GCC GTA CAG ATC GCG GAG CTG GTG GCG GCG GAG CTG AAG GGC TAG TCC
ala ala leu asn ala val gln ile ala glu leu val ala ala glu leu lys gly *
1180
ACGCCCCCTCAGCCCTCGCGCACCTCGTAGAGAAAGCAGCGTACTCGACGGCCCGGACGTGGACCGCCACGCGC
----->
1259
CGGGTCGTCGAATGCTTCTGTGAAGGCTCGCCGAAACCGGTGGGTCTCGACACCGCGCCCGAGGATGTGCCCC
-----

```

Fig. 4. Nucleotide sequence of the 1337 bp lefthand segment of pJV24 containing ORF3 (see Fig. 3); the deduced amino acid sequence for ORF3 is shown. Restriction sites are underlined; the putative ribosome-binding site (RBS) for ORF3 is underlined and in boldface. A downstream inverted repeat is marked with broken arrows.

between the ORF3 sequence and Asd from a variety of micro-organisms. Fig. 4 shows the nucleotide and deduced amino acid sequences of the region in pJV24 containing ORF3. In a CLUSTAL alignment with the ORF3 polypeptide (Fig. 5), the amino acid sequences of Asd from Gram-positive bacteria showed the following frequency of identical amino acids: *Mycobacterium smegmatis* (Cirillo *et al.*, 1994), 66% over 200 amino acids; *Corynebacterium glutamicum* (Kalinowski *et al.*, 1990), 60% over 271 amino acids; *Bacillus subtilis* (Chen *et al.*, 1993), 48% over 60 amino acids; *Streptococcus mutans* (Cardineau & Curtiss, 1987), 43% over 62 amino acids. The sequence identity was lower (38% over 42 amino acids) with Asd from *E. coli* (Biellmann *et al.*, 1980), and also (24% over 62 amino acids) with Asd from *Saccharomyces cerevisiae* (Thomas & Surdin-Kerjan, 1989).

The mean G + C content in ORF3 was 70.1 mol%, with 69.1, 44.7 and 96.5 mol% in the first, second and third

positions, respectively, of the codons; the values are similar to those reported for streptomycete ORFs (Seno & Baltz, 1989). ORF3 has a potential start codon (GTG) at nt 160–162, which is preceded at nt 148–151 by a plausible ribosome-binding site [GAGG; ΔG –9.4 kcal mol⁻¹ (–39.0 kJ mol⁻¹); Tinoco *et al.*, 1973]. A comparison of the region upstream of the translational start codon with the sequences of promoter recognition sites in streptomycetes (Strohl, 1992) did not identify any sequences corresponding to *E. coli* σ^{70} -like –10 and –35 hexamers. The translational stop codon (TAG) is at nt 1174–1176. Beginning 45 nt downstream of this, sequences at nt 1227–1244 and 1252–1269 were identified as an imperfect inverted repeat by the STEMLOOP program (Devereux *et al.*, 1984); the binding strength for the corresponding mRNA, calculated by MFOLD, is –29.2 kcal mol⁻¹ (–122.2 kJ mol⁻¹), suggesting that the inverted repeat forms a transcriptional terminator (Adhya & Gottesman, 1978).

The sequence analysis indicated that ORF3 codes for a polypeptide of M_r 35484 containing 338 amino acids. The conclusion from comparisons with database sequences that it constitutes the gene for Asd in *S. akiyoshiensis* is supported by the failure of pJV30 to complement the *asd* mutation in *E. coli* CGSC 6212. Sequence analysis indicates that the *Bam*HI–*Kpn*I segment of pJV24 subcloned in this plasmid lacks the first 18 codons of ORF3 and an upstream region that includes the putative ribosome-binding site.

Expression of *asd* from the *lac* promoter

The absence of a recognizable promoter sequence upstream of *asd* in the DNA fragment cloned from *S. akiyoshiensis* suggested that the gene was expressed in *E. coli* from the *lac* promoter of the vector. This was investigated by inserting DNA fragments containing *asd* in opposite orientations into an *E. coli* vector. The *S. akiyoshiensis* DNA inserts in pJV21 and pJV24 were excised by double digestion with *Hind*III/*Kpn*I, and recloned downstream of the *lac* promoter in *Hind*III/*Kpn*I-digested pTZ18R and pTZ19R; these vectors differ in the opposite orientation of their multiple cloning regions. Only pJV31 and pJV32, in which the inserts from pJV21 and pJV24, respectively, were cloned in pTZ19R, complemented the *asd* mutation in *E. coli* CGSC 6212. Since the orientation of *asd* to the *lac* promoter in these plasmids was the same as in pJV21 and pJV24, the results indicate that in the initial shotgun cloning, *asd* from *S. akiyoshiensis* was expressed from the *lac* promoter of pHJL400.

Regions adjacent to *asd*

The amino acid sequences deduced from ORF4 and the incomplete ORF1 did not show significant similarity to sequences in the GenBank database. The amino acid sequence deduced from ORF2 showed a marked similarity to sigma factors (Lonetto *et al.*, 1994); this is being investigated with M. Buttner and M. Paget at the John

```

S. a. M-R---VGIVGATGQVGTVMRR--ILTERNFPVTELRFL-ASARSAGTE-----LD---GVTVEDAATA-----DVTGL---DIVLFSAGGATSKALAEEKV
M. s. MVN---IGVVGATGQGRQVMRN--LLEQRNFPATSVRFF--ASPRSEGK-----LTFRGQEIIEVENAETA-----DPSGL---DIALFSAGATMSRVQAPRF
C. g. MTT---IAVVGATGQVGQVMRT--LLEERNFPADTVRFF--ASPRSAGR-----IEFRGTIEVEDITQA-----TEESLKIDIVLFSAGGTASKQYAPLF
B. s. MGRGLHVAUVGATGAVGQQLMLK--TLEDNRFEMDTLL--SSKRSAGTK-----VTFKGQELTVQEASP-----ESFEGVNIALFSAGGSVSQALAPEA
S. m. MG--YTVAIVGATGAVGTIRMIQ--QLEQSTLPVDKVRLL--SSRSAGKV-----LQYKDQDVTVELTTK-----DSFEAVDIALFSAGGSVSQAFAPYA
S. c. MAGKKIAGVLGATGSGVGRFIL--LLANH--PHFELKVLGASSRSAGKKYVDAVNWKQTDLLPESATDIIVSECKSEFFKECDIVFSGLDADYAGATIEKEF
E. c. MKN---VGFIGWRGMVGSVLMQRMVEERDFDAIRPVFFSTSQLGQA-----APSFGGTGTGLQDAFDL-----EALKALDIIVTCQGGDYTNEIYPKL
*      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *

S. a. ASQGAVV--IDNSSAWRKHEVPLVVSEVN-----PHAIKDRPKG-----IIANPNCTTMAAMPVLRPLHDEAG--LEALVVATYQAVSGSGLAGVA
M. s. AEAGVIV--VDNSSAFRKDPDPLVVSEVN--FDRDVRGKKLAG-----IIANPNCTTMAAMPVLRPLHDEAG--LQRLIVSSYQAVSGSGLAGVE
C. g. AAGATV--VDNSSAWRKDEVLIVSEVN-----PSDKDSLVRG-----IIANPNCTTMAAMPVLRPLHDAAG--LVKLHVSSYQAVSGSGLAGVE
B. s. VKRGAIV--IDNTSAFRMDENTPLVVPEVN-----EADLHEHNG-----IIANPNCTTMAAMPVLRPLHDAAG--LVKLHVSSYQAVSGSGLAGVE
S. m. VKAGAVV--VDNTSHFRQNPDPVPLVVSEVN-----AYAMDAHNG-----IIACPNCTTMAAMPVLRPLHDAAG--LVKLHVSSYQAVSGSGLAGVE
S. c. MEAGIAI--VSNAKNYRREQDPLVIVPVNPEHLDIVAQKLDATAQKQKPRPGFIICISNCSTAGLVAPLPLIEKFGPIDALTTTTLQAIISAGFSPGV
E. c. RESGWQGYWIDAASSLRMKDDAIILDPVNQDVIT-----DGLNNGIRTFV--GGNCTVSLMLSLGGLFAN--DLVDWVSVATYQAASGGGARHMR
*      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *

S. a. ELHGQTQKVVADEAKLTHDGEAVDFP-----EPGVYKRIAFNVLPAGSIVDDGLNETDEEQKLRNESRKILEIPGL--KVSQTCVRVPVF
M. s. ELAQARPVIDGVEQLVHDGSAEQYP-----APNKYVAPIAFNIVPLAGNYVDDGSGETDEDQKLRNESRKILGIPEL--LVSGTCVRVPVF
C. g. TLAKQVAAGVDHNVFVHDGQAADAG-----DVGPIVSPAIYVLPFAGNLVDDGTFETDEEQKLRNESRKILGLPDL--KVSQTCVRVPVF
B. s. ELYSQTQAILNKE---EIEPEIMPV-----KGDKKHYQIAFNAIPQIDKPDNGY--TFEEMKMINETKKIMHMPDL--QVAATCVRLPIQ
S. m. ETVREIKVVDGVDPAKVAHIDFPPS-----GGDKKHYPIAFNALAQIDVFTDNDY--TYEEMKMTNETKKIMEEPEL--PVSAHCVRVPI
S. c. P---GIDILDNIIPYIGGEED-----KMEWETKKILAPLAED-----KTHVKLLTPEEI--KVSQTCVRVPVF
E. c. ELLTQMGLHYGHVADELATPSSAILDIERKVTTLTRSGELPVDNFGVPLAGSLIPWIDKQLDNG--QSRWEWKQAEATNKILNTSSVIPVDGLCVRGAL
*      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *

S. a. SGHSLQINARF--ARPI--SADGATELLKD-----APGVELSD-----IPTPLQAAGKDPSTY--VGRIRSDETVDN--GL--ALFVSDNLRKGA
M. s. SGHSLSINAEF--SQPI--SVERTKELLSA-----AAGVKLVN-----VPTPLAAGIDDCD--VGRIRQDPGVDPGRGL--ALFVSGDNLKGA
C. g. TGHTLTIIAEF--DKAI--TVDQAQELGA-----ASGVKLVN-----VPTPLAAGIDESL--VGRIRQDSTVDDNRL--VLVSGDNLKGA
B. s. TGHSESIVYIEI--DRDDATVEDIKNLLKE-----APGVTLQDDPSQQLYMPADAVGKNDVF--VGRIRK--LDRANGF--HLVWVSDNLLKGA
S. m. FSHSEAVYIET--KDVAPIEEVKAAIAA-----FPGAVLEDDIKHQIYPQAAAVGSRF--F--VGRIRK--LDIENGI--HMWVSDNLLKGA
S. c. DGHTECISLRFKNRPAVSVEQVKTCLKEYVCDAYKLGCBSAPKQTIHVLQPDPRQPRLDNRDSDGYGVSIGVRIEDPLD--F--KMWVLSHNTIIGA
E. c. RCHSQAFTIKLKKD--VSIPTVEELLAA-----HNPWAKVVPN--DREITMRELTPAAVTGTLTTPVGRLRK--LNMGEPELSAFTVGDQLLWGA
*      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *

S. a. ALNAVQIAELV-----AAELK---G
M. s. ALNTQIAELL-----AADL---
C. g. ALNTQIAELL-----VK-----
B. s. AWNSVQIAESL-----KLNLV
S. m. AWNSIITANRLHERGLVRSTSELKFELK
S. c. AGSGVLIAELLARNLI-----
E. c. A-----EPL--RRMLR-----QLA
*      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *      . . . *

```

Fig. 5. CLUSTAL alignment (Higgins & Sharp, 1988) of deduced amino acid sequences for *asd* cloned from bacteria. Identical and similar amino acids are indicated below the alignment by asterisks and dots, respectively. *S. a.*, *Streptomyces akiyoshiensis*; *M. s.*, *Mycobacterium smegmatis*; *C. g.*, *Corynebacterium glutamicum*; *B. s.*, *Bacillus subtilis*; *S. m.*, *Streptococcus mutans*; *S. c.*, *Saccharomyces cerevisiae*; *E. c.*, *Escherichia coli*.

Innes Institute, Norwich, UK. The deduced amino acid sequence for the incomplete ORF5 showed strong similarity (85% identity over 119 amino acids) to the N-terminal region of aminopeptidase N from *Streptomyces lividans* (Butler *et al.*, 1994).

Southern analysis of cloned and chromosomal DNA

S. akiyoshiensis genomic DNA and the plasmids pJV21 and pJV36 were digested with *Pvu*II and probed by Southern hybridization with an α -³²P-labelled 0.75 kb *Sal*I–*Sal*I fragment from pJV24. The probe detected a single 2.8 kb fragment in all three digests, indicating that the cloned *Pvu*II fragment represented the true chromosomal sequence.

Transformation of an *ask* mutant of *E. coli*

pJV21 and pJV24 were used to transform *E. coli* CGSC 5074, an auxotrophic strain carrying mutations in each of the three genes encoding Ask isozymes (Theze *et al.*, 1974). None of the transformants was prototrophic. Since complementation of any of the defective isozyme genes would have supported growth, the result indicated that *ask* was either absent from the plasmids or, if present, was not expressed.

DISCUSSION

Of the possible start codons for ORF3, the GTG at nt 160–162 gives an N-terminal amino acid sequence corresponding closely to those deduced for *asd* from other

Gram-positive organisms. In addition, this location offers a plausible ribosome-binding site appropriately spaced from the translational start codon (Strohl, 1992). Expression of the cloned *S. akiiyoshiensis asd* in *E. coli* from the *lac* promoter of the vector is consistent with the apparent lack of an *E. coli*-like promoter in the region upstream of ORF3. It was also noted (data not shown) that expression in *E. coli* increased with addition of IPTG to the culture medium, and that expression was higher from *asd* cloned in pJV24 than from the gene in pJV21, where it is more distant from the *lac* promoter.

The deduced amino acid sequence of ORF3 shows significant similarity to the Asds in low % G + C Gram-positive bacteria (*Bacillus* and *Streptococcus*), as well as to those in *E. coli* and *Saccharomyces cerevisiae*. However, it is most similar to the Asds of mycobacteria and corynebacteria, which like streptomycetes are high % G + C Gram-positive bacteria. To date, no well-characterized mutant blocked in a core reaction of the aspartate pathway in streptomycetes has been reported, and thus the loci for these genes have not been mapped. The marked similarity in deduced amino acid sequence between ORF3 of *S. akiiyoshiensis* and the Asd sequences of other high % G + C Gram-positive bacteria raised the possibility that the aspartate pathway genes might be similarly organized. In mycobacteria, corynebacteria and bacilli, *asd* is clustered in an operon with *ask* (Kalinowski *et al.*, 1990; Chen *et al.*, 1993; Cirillo *et al.*, 1994). However, no *ask*-like sequence was detected by analysis of the DNA cloned from regions immediately upstream or downstream of *asd* in *S. akiiyoshiensis*. The absence of *ask* was consistent with the failure of either pJV21 or pJV24 to complement an *E. coli ask* mutant, but this failure might also be attributed to lack of expression (Seno & Baltz, 1989). Since a probe excised from the *asd* gene hybridized with 2.8 kb *PvuII* fragments in pJV21, pJV36 and the *S. akiiyoshiensis* chromosome, the sequenced region of the cloned fragments had probably not undergone deletion or rearrangement, and represents the native sequence. A comparison of streptomycete genomes has shown extensive structural conservation of the genes for primary metabolism (Stuttard, 1988); thus the difference in organization between *ask* and *asd* in *S. akiiyoshiensis* and in other Gram-positive bacteria examined is likely to apply to streptomycetes generally.

In the lysine branch of the aspartate pathway in streptomycetes, DAP is not only a metabolic intermediate but also represents an essential cell wall component. Most culture media lack DAP; thus in mutants where its endogenous synthesis is blocked, plasmids carrying a gene complementing the mutation are maintained as a condition for survival. The use of *asd* mutants in such plasmid maintenance systems has been demonstrated in a *Salmonella* vaccine strain (Nakayama *et al.*, 1988). Since streptomycetes are potentially useful organisms for producing proteins with recombinant vectors (Takaguchi *et al.*, 1989; Baltz, 1990; Bender *et al.*, 1990), we attempted to develop a similar system ensuring vector maintenance in streptomycete hosts without antibiotic selection. Chemical mutagenesis and screening of *S. akiiyoshiensis*

spores failed to yield a specific *asd* mutation. *S. akiiyoshiensis* could be efficiently transformed with pHJL400, and efforts were then directed towards replacement of *asd* with a cloned and disrupted vector copy of the gene. However, we were unable to select transformants with an Asd⁻ phenotype. Attempts in other laboratories to isolate *asd* mutants by chemical mutagenesis of corynebacteria (Yeh *et al.*, 1988), and by gene disruption of mycobacteria (Cirillo *et al.*, 1991) and bacilli (Chen *et al.*, 1993), have also been unsuccessful, and Chen *et al.* (1993) suggested that absence of an uptake system for DAP in these bacteria makes *asd* a lethal mutation. We have observed that *S. akiiyoshiensis* shows little growth with DAP as a sole nitrogen source, but normal growth with lysine (data not shown). Thus here as well, inability to transport DAP may account for the absence of *asd* mutants.

ACKNOWLEDGEMENTS

We are grateful to Dr R. Curtis III for the culture of *E. coli* CGSC 6212, and to Dr B. J. Bachmann for *E. coli* CGSC 5074. This research was supported by the Natural Sciences and Engineering Research Council of Canada, and by the World University Service of Canada.

REFERENCES

- Adhya, S. & Gottesman, M. (1978). Control of transcription termination. *Annu Rev Biochem* **47**, 967–996.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Baltz, R. H. (1990). Gene expression using streptomycetes. *Curr Opin Biotechnol* **1**, 12–20.
- Bender, E., Koller, K.-P. & Engels, J. W. (1990). Secretory synthesis of human interleukin-2 by *Streptomyces lividans*. *Gene* **86**, 227–232.
- Biellmann, J.-F., Eid, P., Hirth, C. & Jornavall, H. (1980). Aspartate-β-semialdehyde dehydrogenase from *Escherichia coli*. *Eur J Biochem* **104**, 59–64.
- Butler, M. J., Aphale, J. S., Binnie, C., DiZonno, M. A., Krygsman, P., Soltes, G. A., Walczyk, E. & Malek, L. T. (1994). A gene encoding an aminopeptidase N from *S. lividans* 66. *Gene* **141**, 115–119.
- Cardineau, G. A. & Curtiss, R., III (1987). Nucleotide sequence of the *asd* gene of *Streptococcus mutans*. *J Biol Chem* **262**, 3344–3553.
- Chen, N.-Y., Jiang, S.-Q., Klein, D. A. & Paulus, H. (1993). Organization and nucleotide sequence of the *Bacillus subtilis* diaminopimelate operon, a cluster of genes encoding the first three enzymes of diaminopimelate synthesis and dipicolinate synthase. *J Biol Chem* **268**, 9448–9465.
- Cirillo, J. D., Barletta, R. G., Bloom, B. R. & Jacobs, W. R., Jr (1991). A novel transposon for mycobacteria: isolation and characterization of IS1096. *J Bacteriol* **173**, 7772–7780.
- Cirillo, J. D., Weisbrod, T. R., Pascopella, L., Bloom, B. R. & Jacobs, W. R., Jr (1994). Isolation and characterization of the aspartokinase and aspartate semialdehyde dehydrogenase operon from mycobacteria. *Mol Microbiol* **11**, 629–639.
- Cohen, G. N., Stanier, R. Y. & LeBras, G. (1969). Regulation of the biosynthesis of amino acids of the aspartate family in coliform bacteria and pseudomonads. *J Bacteriol* **99**, 791–801.
- Cremer, J., Treptow, C., Eggeling, L. & Sahm, H. (1988). Regulation of enzymes of lysine biosynthesis in *Corynebacterium glutamicum*. *J Gen Microbiol* **134**, 3221–3229.

- Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Feinberg, A. P. & Vogelstein, B. (1983). A technique for radio-labeling restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**, 6–13.
- Higgins, D. G. & Sharp, P. M. (1988). Clustal: a package for performing multiple sequence alignments on a microcomputer. *Gene* **73**, 237–244.
- Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M. & Schrempf, H. (1985). *Genetic Manipulation of Streptomyces: a Laboratory Manual*. Norwich: John Innes Foundation.
- Kalinowski, J., Bachmann, B., Thierbach, G. & Puhler, A. (1990). Aspartokinase genes *lysC α* and *lysC β* overlap and are adjacent to the aspartate β -semialdehyde dehydrogenase gene *asd* in *Corynebacterium glutamicum*. *Mol & Gen Genet* **224**, 317–324.
- Kanazawa, K., Tsuchiya, K. & Araki, T. (1960). A new anti-tuberculosis amino acid (5-hydroxy-4-oxo-L-norvaline). *Am Rev Resp Dis* **81**, 924.
- Larson, J. L. & Hershberger, C. L. (1986). The minimum replicon of a streptomycete plasmid produces an ultrahigh level of plasmid DNA. *Plasmid* **15**, 199–209.
- Le, Y. (1994). *Biosynthesis of 5-hydroxy-4-oxonorvaline and aspartate family amino acids in Streptomyces akiyoshiensis*. PhD thesis, Dalhousie University, Halifax, Canada.
- Lee, S.-Y. & Rasheed, S. (1990). A simple procedure for maximum yield of high quality plasmid DNA. *BioTechniques* **9**, 676–679.
- Lonetto, M., Brown, K. L., Rudd, K. & Buttner, M. J. (1994). Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase sigma factors involved in the regulation of extracytoplasmic functions. *Proc Natl Acad Sci USA* **91**, 7573–7577.
- Madduri, K., Stuttard, C. & Vining, L. C. (1989). Lysine catabolism in *Streptomyces* spp. is primarily through cadaverine: β -lactam producers also make α -aminoadipate. *J Bacteriol* **171**, 299–302.
- Marck, C. (1988). 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. *Nucleic Acids Res* **16**, 1829–1836.
- Mead, D. A. & Kemper, B. (1988). Chimeric single-strand DNA phage-plasmid cloning vectors. In *Vectors*, pp. 85–102. Edited by R. L. Rodriguez & D. T. Denhardt. Toronto: Butterworths.
- Mendelovitz, S. & Aharonowitz, Y. (1982). Regulation of cephamycin C synthesis, aspartokinase, dihydrodipicolinic acid synthetase and homoserine dehydrogenase by aspartic acid family amino acids in *Streptomyces clavuligerus*. *Antimicrob Agents Chemother* **21**, 74–84.
- Nakayama, K., Kelly, S. & Curtiss, R., III (1988). Construction of an Asd⁺ expression vector: stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. *Bio/Technology* **6**, 693–697.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbour Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Seno, E. T. & Baltz, R. H. (1989). Structural organization of antibiotic biosynthesis and resistance genes in actinomycetes. In *Regulation of Secondary Metabolites in Actinomycetes*, pp. 1–48. Edited by S. Shapiro. Boca Raton, FL: CRC Press.
- Shiio, I. & Miyajima, R. (1969). Concerted inhibition and its reversal by end-products of aspartate kinase in *Brevibacterium flavum*. *J Biochem* **65**, 849–859.
- Strohl, W. R. (1992). Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. *Nucleic Acids Res* **20**, 961–974.
- Stuttard, C. (1982). Temperate phages of *Streptomyces venezuelae*: lysogeny and host specificity shown by phages SV1 and SV2. *J Gen Microbiol* **128**, 115–121.
- Stuttard, C. (1988). Transduction and genome structure in *Streptomyces*. *Dev Ind Microbiol* **29**, 69–76.
- Takaguchi, S., Kumagai, I., Nakayama, J., Suzuki, A. & Miura, K. (1989). Efficient extracellular expression of a foreign protein in *Streptomyces* using secretory protease inhibitor (SSI) gene fusion. *Bio/Technology* **7**, 1063–1066.
- Theze, J., Margarita, D., Cohen, G. N., Borne, F. & Patte, J. C. (1974). Mapping of the structural genes of three aspartokinases and of the two homoserine dehydrogenases of *Escherichia coli* K12. *J Bacteriol* **117**, 133–143.
- Thomas, D. & Surdin-Kerjan, Y. (1989). Structure of the HOM2 gene of *Saccharomyces cerevisiae* and regulation of its expression. *Mol & Gen Genet* **217**, 149–154.
- Tinoco, I., Jr, Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M. & Gralla, J. (1973). Improved estimation of secondary structure in ribonucleic acids. *Nature New Biol* **246**, 40–41.
- Vining, L. C., Shapiro, S., Madduri, K. & Stuttard, C. (1990). Biosynthesis and control of β -lactam antibiotics: the early steps in the 'classical' tripeptide pathway. *Biotechnol Adv* **8**, 159–183.
- White, R. L., DeMarco, A. C. & Smith, K. C. (1988). Biosynthesis of the unusual amino acid 5-hydroxy-4-oxonorvaline. *J Am Chem Soc* **110**, 8228–8229.
- Whitney, J. G., Brannon, D. R., Mabe, J. A. & Wicker, K. J. (1972). Incorporation of labeled precursors into A16886B, a novel β -lactam antibiotic produced by *Streptomyces clavuligerus*. *Antimicrob Agents Chemother* **1**, 247–251.
- Wright, F. & Bibb M. J. (1992). Codon usage in the G+C rich *Streptomyces* genome. *Gene* **113**, 55–65.
- Yamaguchi, M., Yamaki, H., Shinoda, T., Yoshitaka, T., Suzuki, H., Nishimura, T. & Yamaguchi, H. (1990). The mode of antifungal action of (S)-2-amino-4-oxo-5-hydroxypentanoic acid RI-331. *J Antibiot* **43**, 411–416.
- Yeh, P., Sicard, A. M. & Sinskey, A. J. (1988). General organization of the genes specifically involved in the diaminopimelate-lysine biosynthetic pathway of *Corynebacterium glutamicum*. *Mol & Gen Genet* **212**, 105–111.
- Zhang, J.-J., Hu, F.-M., Chen, N.-Y. & Paulus, H. (1990). Comparison of the three aspartokinase isoenzymes in *Bacillus subtilis* Marburg and 168. *J Bacteriol* **172**, 701–708.

Received 14 July 1995; revised 27 November 1995; accepted 4 December 1995.