BIOSYNTHESIS OF 5-HYDROXY-4-OXONORVALINE AND ASPARTATE FAMILY AMINO ACIDS IN STREPTOMYCES AKIYOSHIENSIS

by

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Psychometrics 0632
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TABLE OF CONTENTS

TABLES

FIGURES

ABSTRACT

Cultures of Streptomyces akiyoshiensis accumulate 5-hydroxy-4-oxonorvaline (HON), which is an antifungal antibiotic and a chiral synthon biosynthesized from aspartate and acetate. To develop the potential for economic production of HON by fermentation, links between the biosynthesis of HON and aspartate family amino acids have been investigated.

Bioassays for HON based on its antifungal activity were developed and used to screening NTG-treated spores for mutants blocked in the biosynthesis of HON. Cross-feeding between pairs of blocked mutants identified four co-synthetic groups of mutants. One consisted of regulatory and/or non-pathway mutants. The remaining three groups, which were blocked in the biosynthetic pathway, were placed in biosynthetic sequence. Since blocked mutants were not auxotrophic, aspartate pathway auxotrophs were isolated by screening NTG-treated spores; putative mutants in ask, hsd and lysA were obtained. Their phenotypes indicated that the HON pathway does not require the steps after homoserine dehydrogenase and diaminopimelate decarboxylase, but probably includes aspartate kinase and aspartate semialdehyde dehydrogenase. To examine this further, asd from S. akiyoshiensis was cloned by complementing a mutation in E. coli. The gene was localized to a 2.2-kb fragment, which was completely sequenced. The sequence data suggested that the gene organization for aspartate kinase and aspartate semialdehyde dehydrogenase in S. akiyoshiensis is different from that in other high %G+C Gram-positive bacteria. To facilitate molecular genetic analysis procedures for DNA transformation in S. akiyoshiensis were developed.

ABBREVIATIONS AND SYMBOLS

Asd	aspartate semialdehyde dehydrogenase
Ask	aspartate kinase
bp	base pair
°C	degrees Celsius
ccc	covalently closed circular
cfu	colony forming unit
ATP	adenosine 5'-triphosphate
DAP	diaminopimelic acid
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanidine 5'-triphosphate
dNTP	dATP, dCTP, dGTP, dTTP
dTTP	deoxythymidine 5'-triphosphate
DMSO	dimethysulfoxide
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
HON	5-hydroxy-4-oxonorvaline
HPLC	high performance liquid chromatography
Hsd	homoserine dehydrogenase
IPTG	isopropyl-ß-thiogalactopyranoside
kb	kilobase
MOPS	morpholinopropane sulfonate

nt	nucleotide
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
OD	optical density
ORF	open reading frame
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
RBS	ribosomal binding site
R_f	relative mobility
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TE	Tris-EDTA buffer
TEMED	N', N', N', N-tetramethylenediamine
TES	tris-(hydroxymethyl) methylaminoethanesulfonic acid
Tris	tris-(hydroxymethyl) aminomethane
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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INTRODUCTION

Streptomycetes are Gram-positive soil bacteria well known for their ability to produce secondary metabolites. Cultures of Streptomyces akiyoshiensis accumulate an unusual nonprotein amino acid active against tuberculous bacilli (Kanazawa et al., 1960) and some fungi (Watanabe et al., 1987). The amino acid was first discovered in S. akiyoshiensis during screening for antituberculous antibiotics (Tatsuoka, et al., 1961); it was isolated from broth filtrates by cation exchange chromatography and purified by recrystallization from acetone-water. Elemental analysis and molecular weight measurements indicated the molecular formula $C_5H_9O_4N$ (Miyake, 1960a). Van Slyke nitrogen determinations suggested that the substance contained a primary amine, supporting the conclusion from the infrared absorption spectrum that the substance was an amino acid. The ultraviolet absorption spectrum was consistent with the presence of a nonconjugated carbonyl group. Treatment of the substance with sodium metaperiodate gave two compounds: formaldehyde and L-aspartic acid, which could have arisen from two potential structures. The correct one (Fig. 1) was determined by chemical synthesis to be L-5-hydroxy-4-oxonorvaline (HON; Miyake, 1960b). HON was subsequently reisolated as antibiotic RI-331, and investigated because of its activity against many yeasts, including a number of pathogenic Candida species (Yamaguchi et al., 1988; Yamaki et al., 1988).

HON inhibited the biosynthesis of protein to a greater extent than it inhibited the synthesis of RNA and DNA in growing Saccharomyces cerevisiae cells (Yamaguchi et al., 1990). The inhibition was attributed to a demonstrated decrease in the rate of de novo biosynthesis of the aspartate family amino acids threonine, methionine and isoleucine. HON was later shown to act as an antimetabolite of homoserine, inhibiting homoserine dehydrogenase activity (Yamaguchi et al., 1990). Because threonine, methionine and

isoleucine are essential dietary components in animals, HON is nontoxic in humans and is potentially useful as an antifungal drug. It also has potential value as a chiral synthon (R. L. White, personal communication). To develop an inexpensive production of HON by fermentation, more information about its biosynthetic pathway is needed.

A convenient and sensitive HPLC analysis for HON developed by White et al. (1989) facilitated biosynthetic studies, which were pursued by feeding isotopically labelled aspartate and acetate to S. akiyoshiensis (White et al., 1988). The results with [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]acetate suggested that C-1 to C-4 of HON are derived from a four-carbon Krebs cycle intermediate, and C-5 is derived from the methyl carbon of acetate. Experiments with DL-[4-^{13}C]aspartate were consistent with an asymmetric Krebs cycle intermediate such as oxaloacetate, malate or aspartate. Subsequent feeding experiments with DL-[2-^{13}C,^{15}N]aspartate indicated that the C-N bond remained intact during incorporation of the precursor into HON. Based on this information, White et al. (1988, 1990) postulated that HON is biosynthesized from L-aspartate as the closest four-carbon precursor (Fig. 1). The initial reaction is likely to be a condensation of acetyl coenzyme A or malonyl coenzyme A with a ß-activated aspartate, possibly aspartyl phosphate or aspartate semialdehyde. At some stage, the acetate carboxyl is lost and the methyl group is hydroxylated. A similar condensation reaction between acetyl coenzyme A and γ-activated glutamate (or glutamic semialdehyde) has been postulated in the biosynthesis of thienamycin, a carbapenem antibiotic (Williamson et al., 1985).

Although the pathways by which aspartate family amino acids are biosynthesized use similar biochemical reactions, they differ in details. Variations occur in the number of isoenzymes catalyzing the early steps of the pathways, the gene organizations, and the regulation of the pathway. In streptomycetes, these details are not well documented. In fact,

none of the genes in the core pathway (i.e. the reactions shared by lysine, methionine and threonine) have been cloned in streptomycetes. Cloning and analysis of the gene will not only provide a better understanding of the molecular genetics of the pathway, but also facilitate studies on the biosynthesis of antibiotics, including groups of medically important ß-lactams, derived from the aspartate pathway.

The present study was designed (1) to extend the results of White et al. (1990) by revealing more information about the biosynthesis of HON and its relationship to the aspartate pathway, and (2) to obtain more information about the aspartate pathway in S. akiyoshiensis. The goal in the latter study was to clone the aspartate semialdehyde dehydrogenase (asd) gene, to explore the gene organization for this enzyme and aspartate kinase. In the overall plan, S. akiyoshiensis mutants blocked in HON production were isolated and were arranged in sequences in the biosynthetic pathway. Auxotrophic mutants blocked in the aspartate pathway were isolated, and the potential role of the early part of this pathway in HON biosynthesis was explored. To clone asd from S. akiyoshiensis, a genomic library was made by ligating Sau3AI-digested and size fractionated genomic DNA into the BamHI site of a shuttle vector, pHJL400. The genomic library was used to transform an E. coli asd mutant (CGSC 6212) to prototrophy. From a transformant, a recombinant plasmid (pJV21), carrying an 11.3-kb insert was isolated. The asd complementing DNA in pJV21 was localized by subcloning to a 2.2-kb SalI-SalI fragment (carried in pJV24). The nucleotide sequence of the 2.2-kb segment of DNA was determined and the asd gene was identified. Analysis of the nucleotide sequence of asd and its adjacent regions indicated that, in S. akiyoshiensis this gene is not clustered with an aspartate kinase gene.

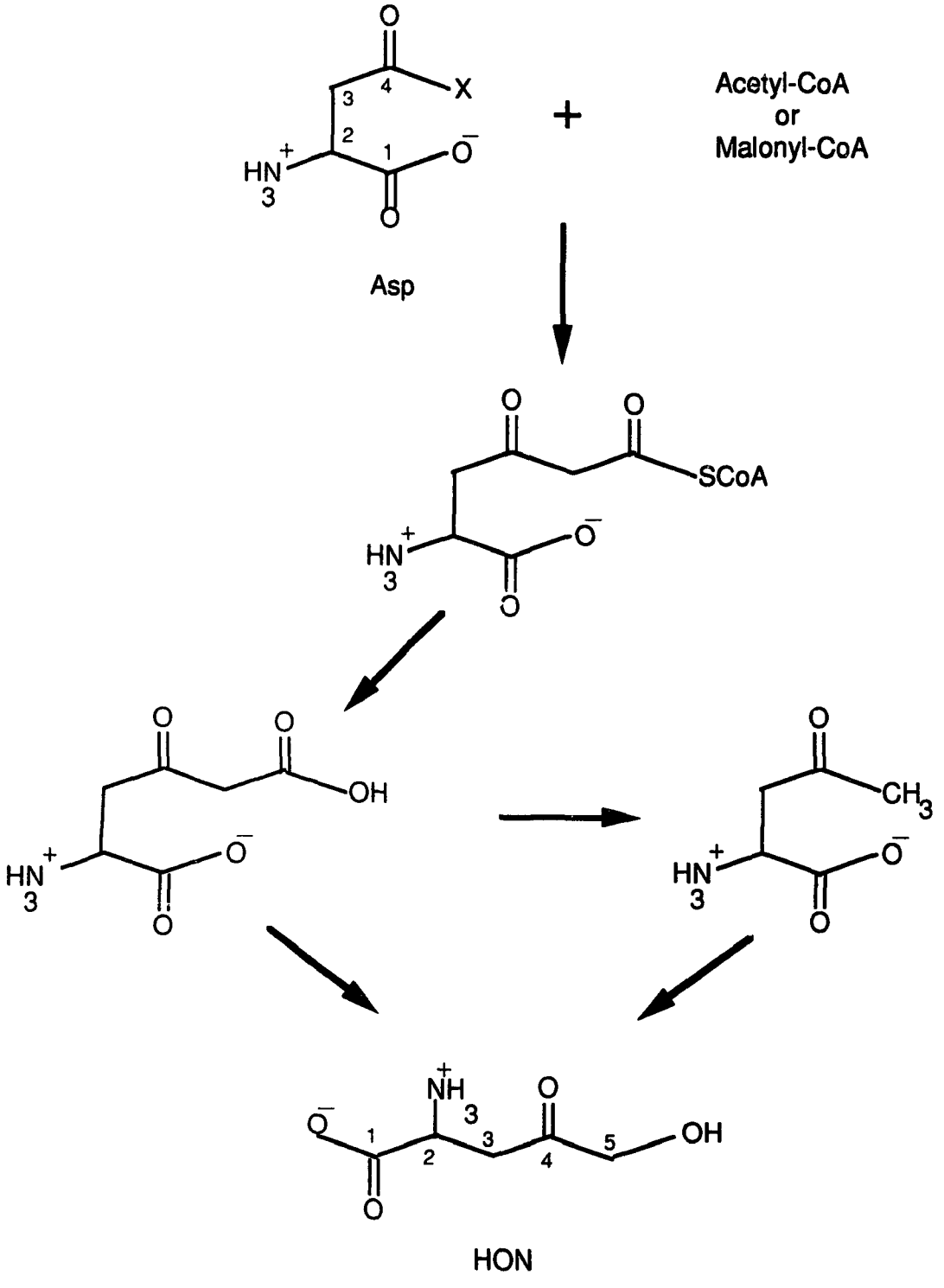


Figure 1. Postulated biosynthesis of HON.

LITERATURE REVIEW

I. Biosynthesis of aspartate family amino acids

In prokaryotes the aspartate family of protein amino acids (Fig. 2) includes aspartate, asparagine, lysine, methionine, threonine and isoleucine. Lysine and isoleucine can also be considered as members of the pyruvate family because they are synthesized by pathways that require pyruvate as well as aspartate as a precursor. Aspartate is the sole precursor for the carbon skeleton of the other amino acids and is biosynthesized from oxaloacetate, an intermediate in the Krebs cycle; glutamate serves as the amino donor. Asparagine may be synthesized by transamidation of aspartate by glutamine, or more commonly, by an ATP-requiring amidation with ammonia. The remaining amino acids in the family are biosynthesized from aspartate through specific pathways that branch from a core pathway through which the γ-carboxyl group is modified.

In all prokaryotic organisms the pathways leading from aspartate to lysine, methionine, threonine and isoleucine differ only in detail. There is a sequence of "core" reactions that is shared by two or more amino acids. This core sequence (Fig. 2) leads from aspartate to homoserine and has two branch-point intermediates that give the end products by further amino acid-specific reactions. The first branch point is at aspartate semialdehyde; the branch leads to diaminopimelate and lysine by a series of specific reactions. The second branch point in the core sequence is at homoserine. By specific pathways, homoserine is converted to either methionine or threonine. Threonine is both an end-product protein amino acid and an intermediate en route to isoleucine. The biosynthesis of aspartate family amino acids in E. coli has been extensively studied. The information about the pathway in other bacteria is less detailed.

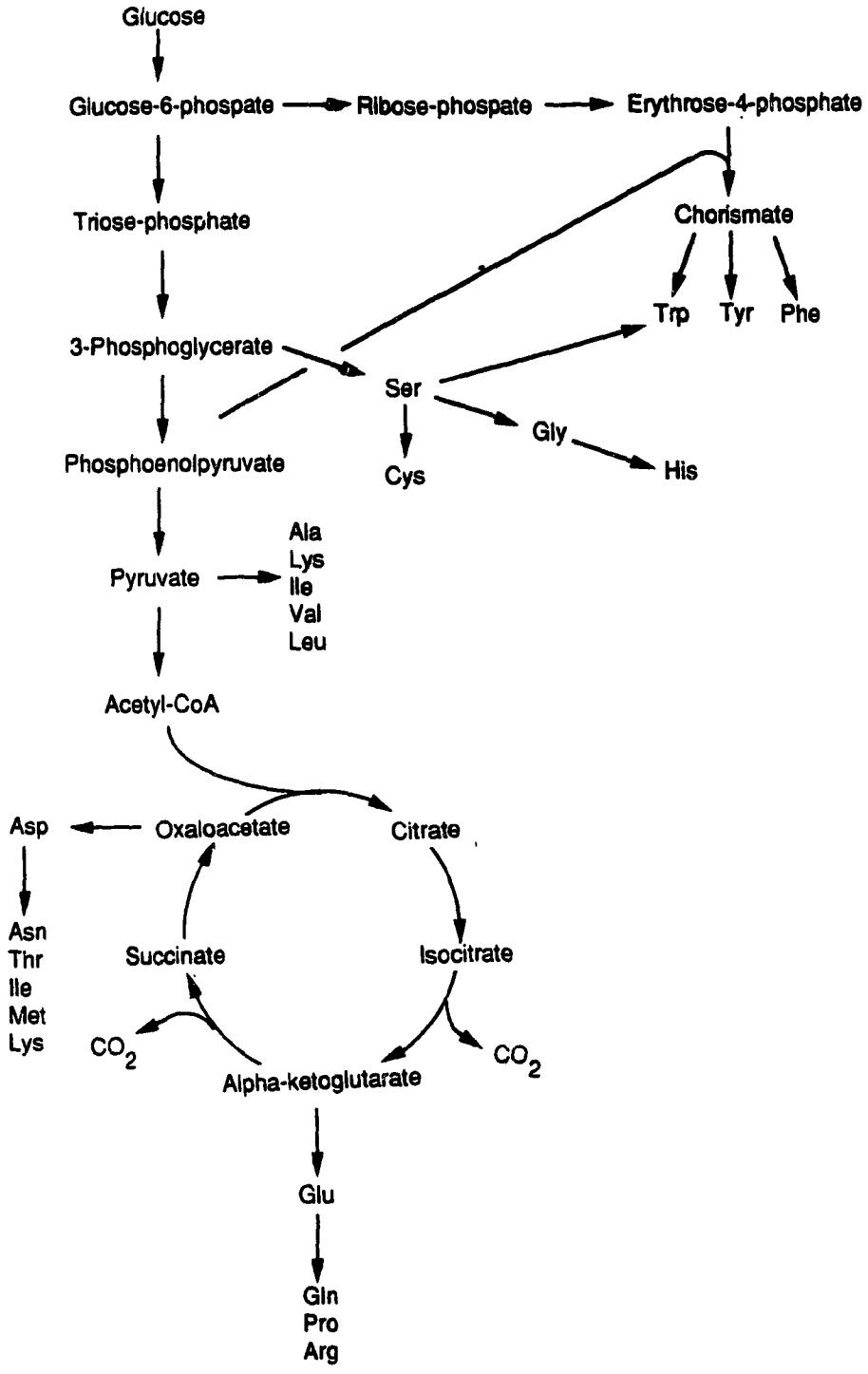


Figure 2. Biosynthetic routes for protein amino acids.

A. Core pathway

The core pathway (Fig. 3) begins with activation of ß-carboxyl of aspartate to give aspartyl phosphate. The reaction is catalyzed by aspartate kinase and ATP is required. In the next reaction, aspartate semialdehyde dehydrogenase catalyzes the NADPH-dependent reduction of aspartyl phosphate to aspartate semialdehyde. A second NADPH-dependent reduction, catalyzed by homoserine dehydrogenase yields homoserine.

B. Aspartate kinase and homoserine dehydrogenase

In all organisms so far examined, the biochemical reactions are identical. However, the regulation of the pathway and the number of isozymes for aspartate kinase and homoserine dehydrogenase are different.

1. In E. coli

Three aspartate kinases and two homoserine dehydrogenases have been identified (Stadtman et al., 1961; Patte et al., 1963; Patte et al., 1967). Each isoenzyme is regulated by the end-product amino acid(s) of a specific pathway. Aspartate kinase I and homoserine dehydrogenase I activities are present on the same protein, encoded by thrA (Patte et al., 1963). Synthesis of the protein is influenced by the intracellular levels of both threonine and isoleucine (Katinka et al., 1980). Both enzyme activities are inhibited by threonine (Cohen et al., 1965).

Aspartate kinase II and homoserine dehydrogenase II activities are also located on a single protein, encoded by metL (Patte et al., 1967). Synthesis of the protein is repressed by methionine (Patte et al., 1967), but neither enzyme activity is inhibited by methionine or threonine.

Aspartate kinase III is encoded by lysC (Stadtman et al., 1961). This enzyme is

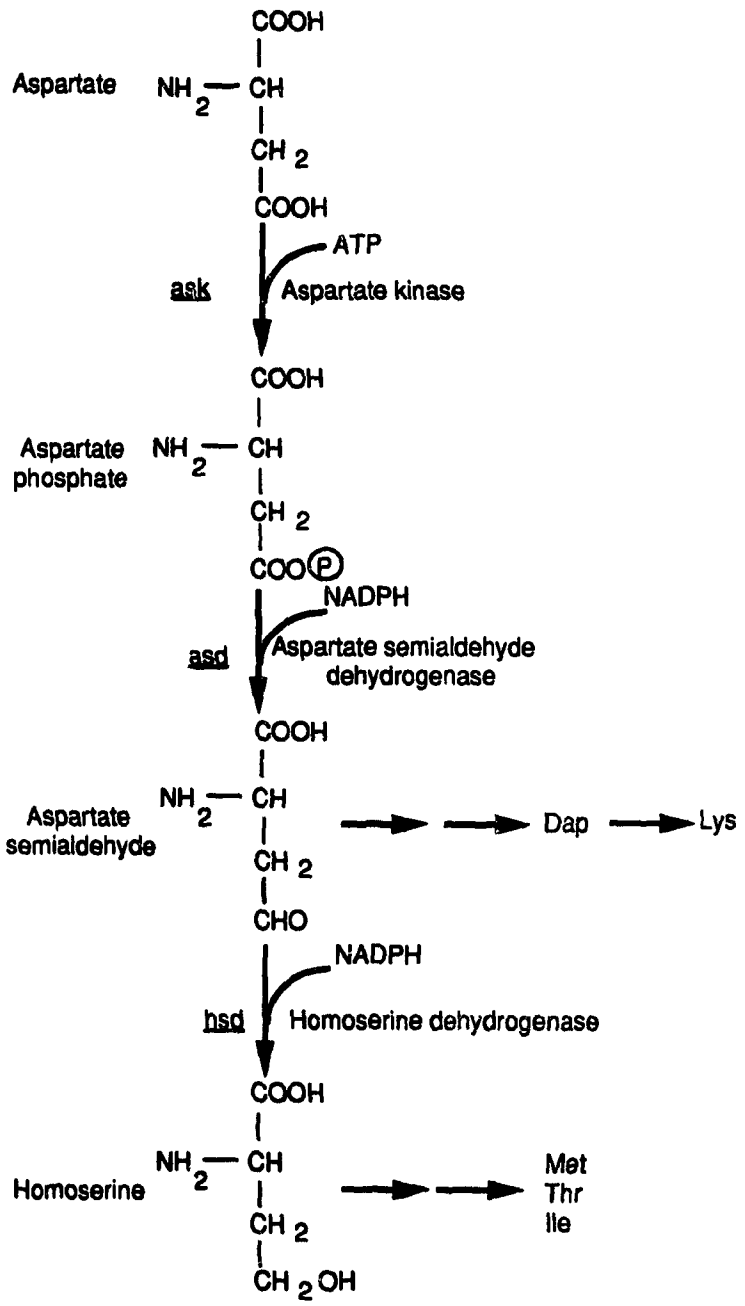


Figure 3. The core pathway to lysine, methionine and threonine.

regulated by lysine, which inhibits the enzyme activity and represses synthesis of the enzyme (Cohen et al., 1983).

2. In other bacteria

Little information regarding homoserine dehydrogenase has been obtained from strains other than E. coli. However, information is available on aspartate kinase and its isozyme. Isofunctional enzymes subject to independent end-product inhibition have been found in other coliform bacteria (Cohen et al., 1969). In bacilli, where three aspartate kinases have been identified (Zhang et al., 1990), aspartate kinase I is inhibited by diaminopimelate, and the level of the enzyme remains relatively constant throughout growth and sporulation; aspartate kinase II is inhibited by lysine, and aspartate kinase III is subject to concerted inhibition by lysine and threonine.

In mycobacteria, the aspartate kinase activity is inhibited by threonine, lysine, methionine or homoserine (Sritharan et al., 1990). Inhibition of aspartate kinase by methionine or homoserine has not yet been found in other organisms. In Pseudomonas, Corynebacteria and Brevibacteria, only one aspartate kinase has been found in cell extracts (Cohen et al., 1969; Cremer et al., 1988; Shiio and Miyajima, 1969). The enzyme in this preparation was subject to concerted inhibition by lysine and threonine. In Streptomyces clavuligerus also, aspartate kinase has been investigated only in cell extracts or desalted cell extracts (Mendelovitz and Aharonowitz, 1982). The enzyme preparations were activated by lysine or threonine, but lysine and threonine together exerted concerted inhibition.

C. Aspartate semialdehyde dehydrogenase

Aspartate semialdehyde dehydrogenase is not inhibited by lysine. However, excess lysine represses synthesis of the enzyme. In E. coli, lysine limitation causes 20-30 fold

derepression (Cohen, 1983). DNA sequence data suggested that the asd gene from E. coli and Corynebacteria does not contain an attenuator (Haziza et al., 1982; Kalinowski, et al., 1990); however, an attenuator-like sequence has been described in the asd gene from Streptococcus mutans (Cardineau and Curtiss III, 1987). No bacteria have yet been found to possess isozymes of aspartate semialdehyde dehydrogenase. Interestingly, no clear-cut asd mutant has been isolated by classical mutagenesis from Gram-positive bacteria. Attempts to replace or disrupt the wild-type asd gene in Mycobacterium smegmatis (Cirillo et al., 1991) and Bacillus subtilis (Chen et al., 1993) were unsuccessful, possibly due to a lethal event. It has been suggested that bacilli lack a transport system for diaminopimelate (Chen et al., 1993), preventing the uptake of diaminopimelate essential for the growth of asd mutants from the medium.

D. Biosynthesis of diaminopimelate and lysine

1. Biosynthetic reactions

Intermediates and the biosynthetic route in the lysine-specific pathway in E. coli are shown in Fig. 4. The first specific reaction is the condensation of aspartate ß-semialdehyde and pyruvate to yield a cyclic compound: dihydrodipicolinate. The reaction is catalyzed by dihydrodipicolinate synthase (Bukhari and Taylor, 1971). Tetrahydrodipicolinate is formed through a reduction catalyzed by the NADPH-dependent dihydrodipicolinate reductase (Farkas and Gilvarg, 1965). The cyclic structure is opened by concerted hydrolysis and succinylation, with succinyl-CoA as the donor, to form N-succinyl-epsilon-keto-alpha-aminopimelate. The succinylation is catalyzed by tetrahydrodipicolinate succinylase (Gilvarg, 1961). Aminotransferase aminates the keto group to yield succinyldiaminopimelate (Peterkofsky and Gilvarg, 1961). The succinyl group is then cleaved by succinyl diaminopimelate desuccinylase

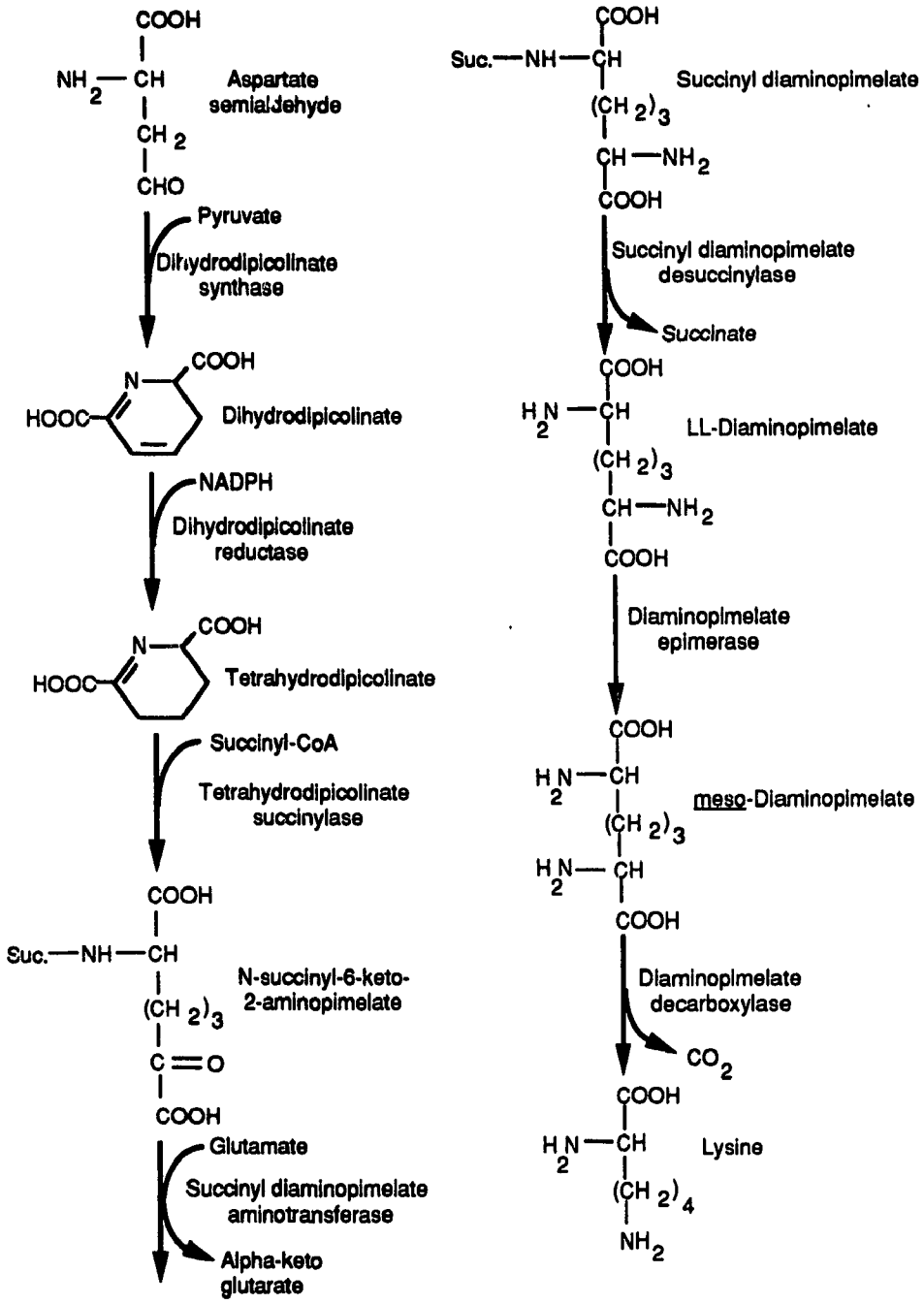


Figure 4. The biosynthetic pathway to lysine.

to yield LL-diaminopimelate (Gilvarg, 1959). Conversion of LL-diaminopimelate to meso-diaminopimelate is catalyzed by an epimerase (Work, 1962a). Diaminopimelate decarboxylase catalyzes the conversion of meso-diaminopimelate to lysine (Work, 1962b).

2. Alternative routes for diaminopimelate biosynthesis

Diaminopimelate is a component of bacterial cell walls and is required for growth of most eubacteria. The E. coli cell wall contains meso-diaminopimelate (Mengin-Lecreulx et al., 1982). The presence of only LL-diaminopimelate has been considered a characteristic of Streptomyces cell walls (Dietz, 1986); in contrast meso-diaminopimelate is found as a major component in other actinomycetes, such as Nocardia. Instead of diaminopimelate, some bacilli use lysine in their cell walls (Bartlett et al., 1985).

In addition to the pathway described above, two alternative pathways to diaminopimelate have been found in bacilli (Bartlett and White, 1985), brevibacteria and corynebacteria (Misono et al., 1979). [illegible] diaminopimelate dehydrogenase in those organisms can catalyze the reversible conversion of tetrahydrodipicolinate to meso-diaminopimelate (Misono et al., 1979). Bacillus macerans has another route (Bartlett and White, 1985). This synthesizes diaminopimelate via an acetylated intermediate. The organisms having such by-passes are able to grow on a medium without diaminopimelate, even if one set of genes responsible for the conversion from tetrahydrodipicolinate to meso-diaminopimelate is inactivated, but the physiological significance of having by-passes in the formation of diaminopimelate is not clear. The presence of such by-passes may, at least in part, explain the difficulty in obtaining dap mutants in corynebacteria (Yeh et al., 1988). The diaminopimelate dehydrogenase gene from Corynebacterium glutamicum is able to complement an E. coli dap mutant (Ishino, et al., 1988).

3. Regulation of lysine- and diaminopimelate-specific biosynthesis

In E. coli, the targets for regulation of diaminopimelate and lysine biosynthesis are aspartate kinase, aspartate semialdehyde dehydrogenase, dihydrodipicolinate synthase, dihydropicolinate reductase and diaminopimelate decarboxylase. Dihydrodipicolinate synthase is inhibited by lysine (Yugari and Gilvarg, 1962), and some evidence also suggested that this is the rate-limiting reaction after aspartate kinase (Patte, 1983). This enzyme is also a regulatory enzyme in bacilli (Bartlett and White, 1986), but in corynebacteria dihydrodipicolinate synthase is not inhibited by lysine (Cremer et al., 1988). Dihydrodipicolinate reductase and succinyl diaminopimelate desuccinylase are repressed by lysine (Boy et al., 1978; Patte, 1983). Diaminopimelate decarboxylase is repressed by lysine but induced by diaminopimelate in E. coli (Patte, 1962; White, 1976). This enzyme, is not repressed by lysine in bacilli (Bartlett and White, 1986). Lysyl-tRNA synthase, the catabolic enzyme lysine decarboxylase, and the enzymes involved in incorporating diaminopimelate into cell walls may also play a role in regulating lysine metabolism (Patte, 1983).

E. Biosynthesis of methionine

1. The biosynthetic pathway

The specific pathway to methionine (Fig. 5) begins with succinylation of homoserine to form O-succinylhomoserine catalyzed by homoserine succinyltransferase with succinyl-CoA as the donor (Rowbury and Woods, 1966). Cystathionine synthase converts O-succinylhomoserine to cystathionine; a cysteinyl group is exchanged with the succinyl group in the reaction (Flavin et al., 1964). Homocysteine is synthesized by cystathionine lyase (Rowbury and Woods, 1964). The last step in methionine formation is the methylation of homocysteine catalyzed by a homocysteine methylase (Rowbury, 1983). The methyl donor,

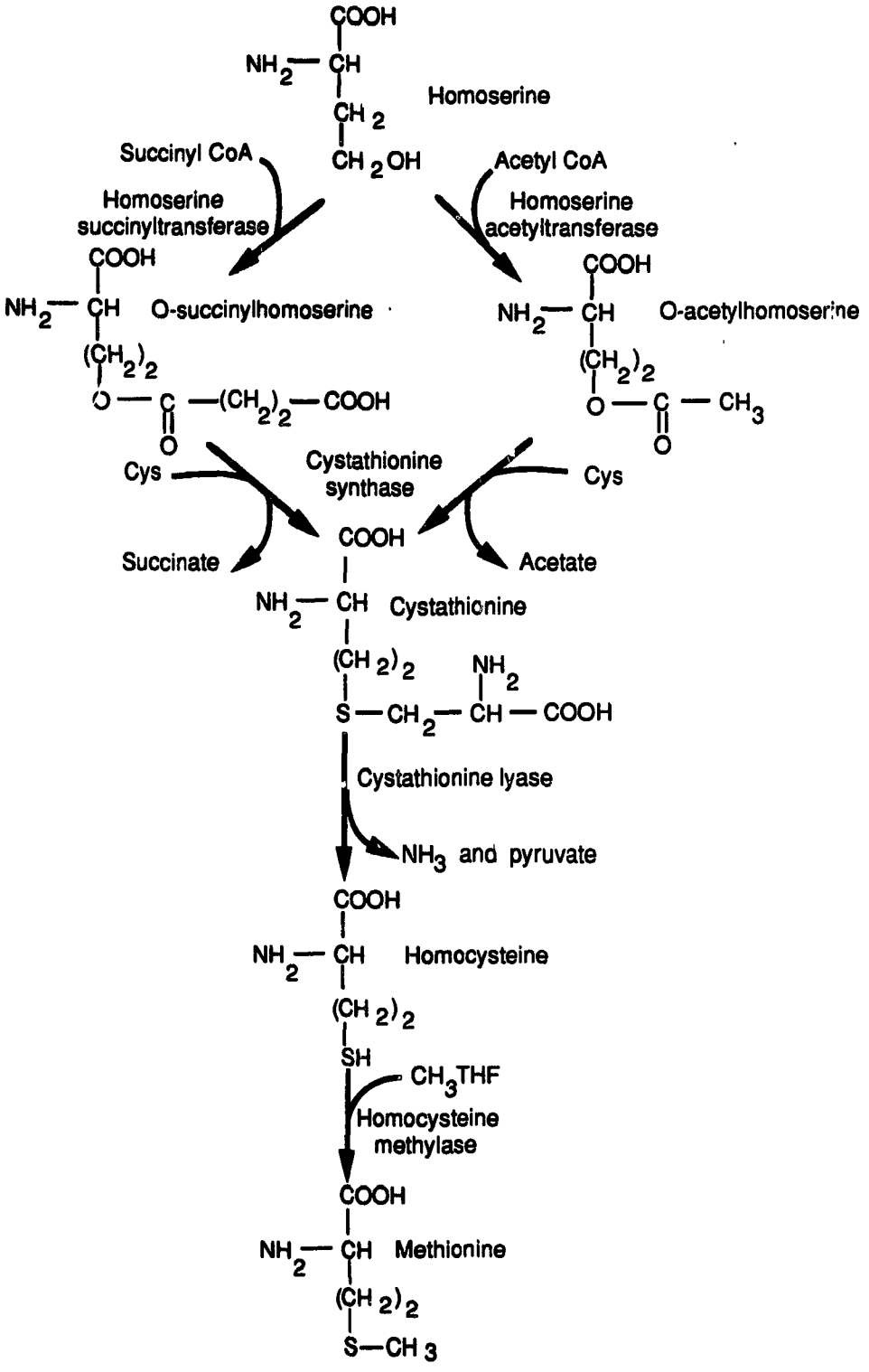


Figure 5. Biosynthesis of methionine.

5-methyltetrahydrofolate, is synthesized from serine in two steps via 5, 10-methylenetetrahydrofolate. The second step is a methionine-specific reaction catalyzed by 5,10-methylenetetrahydrofolate reductase.

2. An alternative biosynthetic route

The first specific reaction from homoserine during methionine biosynthesis in bacteria, such as bacilli, corynebacteria and brevibacteria, uses a route similar to that used in fungi. Instead of succinyl-CoA, acetyl-CoA is used to form O-acetylhomoserine in a reaction catalyzed by homoserine acetyltransferase (Rowbury, 1983; Wyman and Paulus, 1975). The methionine biosynthesis genes in all organisms so far examined are scattered on the chromosome.

3. Regulation of methionine-specific biosynthesis

Besides steps in the core pathay, some steps in the specific pathway are feedback regulated by methionine. In E. coli, this includes the first step from homoserine in the specific pathway; it is feedback regulated by methionine and coordinately regulated by S-adenosylmethionine, a methyl donor in vivo (Lee et al., 1966). Such coordination is achieved via synergistic feedback inhibition. In Bacillus polymyxa, the enzyme responsible for the first methionine-specific reaction is also inhibited in a concerted manner by methionine and S-adenosylmethionine (Wyman and Paulus, 1975). Enzyme synthesis in the methionine pathway is subject to repression by methionine (Rowbury, 1983). In E. coli, the strongest repression is observed in the synthesis of homocysteine methylase; other enzyme syntheses are repressed by methionine to different degrees.

F. Biosynthesis of threonine

Homoserine kinase and threonine synthase catalyze the two specific reactions (Fig. 6)

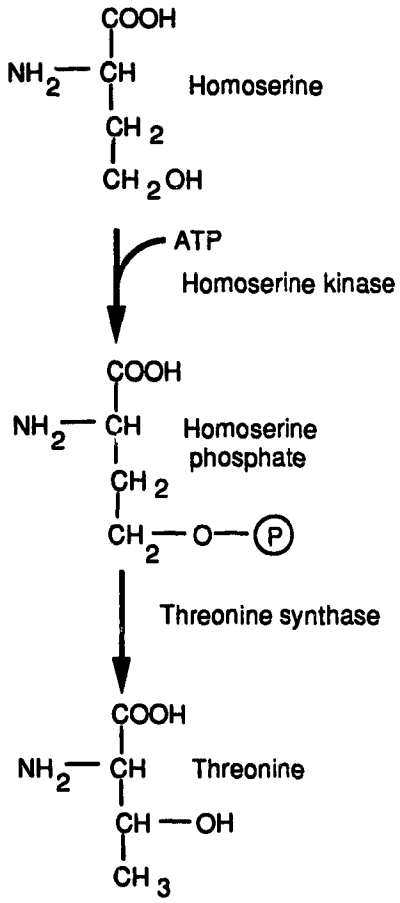


Figure 6. Biosynthesis of threonine.

from homoserine to threonine via homoserine phosphate (Burr et al., 1976; Szczesiul and Wampler, 1976). The first step requires ATP and the activity is inhibited by threonine and isoleucine (Burr et al., 1976). Synthesis of these two enzymes is subject to repression by threonine and isoleucine (Freundlich, 1963).

G. Regulation of carbon flow in the aspartate pathway

In a complex branching pathway, sophisticated mechanisms must be in place to ensure that the appropriate amount of each end-product is made. Coordination is achieved by end product inhibition and feedback repression of key enzymes in the pathway. The presence of more than one aspartate kinase, and their association with homoserine dehydrogenase in E coli, are likely due to the need to regulate a pathway that has acquired increasingly complex functions during its evolution. In E. coli, the control of carbon flow in the early pathway is coordinated by three aspartate kinases, one aspartate semialdehyde dehydrogenase and two homoserine dehydrogenases. This type of regulation has been observed only in coliform bacteria, indicating that these organisms are evolutionarily closely related.

With the regulation of late steps coordinated by key enzyme(s) in the specific pathways, bacteria are able to balance their physiological requirements. By themselves three aspartate kinases in E. coli do not provide adequate regulation of three specific branching pathways. For example threonine in excess represses and inhibits only aspartate kinase I. The consequence is that less aspartyl phosphate is provided to make each of the end products. An overall decrease of this kind does not ensure that aspartyl phosphate supplies the other specific pathways, and not threonine synthesis; additional control mechanisms are necessary. By also affecting homoserine dehydrogenase I, excess threonine limits but does not stop the flow of intermediates leading to threonine synthesis. Therefore, it is necessary to add

selective end-product regulation by inhibition or repression of the specific threonine branch pathway to stop synthesis of that amino acid.

Concerted feedback inhibition of aspartate kinase by lysine and threonine is another example of a mechanism that can effectively regulate enzyme activity. In some organisms, lysine or threonine alone has little effect on aspartate kinase (Cohen et al., 1969). Since lysine and threonine inhibit and repress the enzymes in their specific pathways, and a high concentration of only one of them has no significant influence on the synthesis of aspartyl phosphate, synthesis of the other amino acid continues. Only if the concentrations of both amino acids reach a threshold level will aspartate kinase activity decrease dramatically, and less intermediate be made. A modification of this regulatory pattern is also effective in controlling the early steps of the pathway. In S. clavuligerus, lysine alone activates aspartate kinase significantly (Mendelovitz and Aharonowitz, 1982), allowing more intermediates to be made. A high concentration of lysine inhibits and represses later enzymes in its own biosynthetic pathway, so the additional early intermediates allow more of the other end-products to be made; in this way the cytoplasmic pool of aspartate family amino acids is balanced.

H. Gene organization in the aspartate pathway

Generally, genes involved in the biosynthesis of aspartate family amino acids are scattered around the chromosomes in all bacteria so far examined. However, the genes responsible for threonine biosynthesis, including a gene for the threonine-specific aspartate kinase in E. coli, are clustered at 0 min on the linkage map and form an operon. The thr genes in other bacteria, such as B. subtilis and Serratia marcescens, are also clustered (Parsot, 1986; Sugita et al., 1987). Two threonine mutations have been mapped to the same location

of S. coelicolor A3(2) (Hopwood and Kieser, 1990).

Although, the genes for three aspartate kinases and aspartate semialdehyde dehydrogenase are not linked in E. coli (Theze et al., 1974), asd in a few Gram-positive bacteria is clustered with the aspartate kinase genes. In corynebacteria, asd is clustered with the genes (akα and akß) for the α- and ß-subunits of aspartate kinase (Kalinowski et al., 1990; 1991; Follettie et al., 1993). The order of these genes in respect to the direction of transcription are: akα --> akß --> asd. The C-terminal part of akα overlaps in frame the N-terminal region of akß. Promoter-like sequences are located upstream of akα, and also of akß but no such sequence was identified between akß and asd. Follettie et al. (1993) suggested that the three genes were expressed from the same promoter located upstream of akα; alternatively, or in addition, the promoter upstream of akß might be responsible for expression of the two downstream genes. The nucleotide sequence of asd and its adjacent region in mycobacteria suggested that the gene is preceded by two ORFs encoding proteins similar to α- and ß- subunits of aspartate kinase (Cirillo et al., 1994). Presumbly, asd and ask genes in mycobacteria are organized in the same way as those in corynebacteria. In bacilli, the diaminopimelate operon contains the genes for aspartate kinase (dapG), aspartate semialdehyde dehydrogenase (asd) and dihydrodipicolinate synthase (dapA), the first three enzymes of diaminopimelate synthesis (Chen et al., 1993). The genes are transcribed in the order asd --> dapG --> dapA.

II. Metabolites biosynthesized from aspartate family amino acids

A. Primary metabolites

Primary metabolism consists of the pathways that provide energy, cofactors, and precursors for the biosynthesis of essential molecules such as proteins, nucleic acids, lipids

and polysaccharides. Aspartate family amino acids are involved in many aspects of primary and secondary metabolism. As components of proteins they participate in virtually all biochemical processes. Aspartate family amino acids count for more than a quarter of the twenty protein amino acids. Although the route for incorporation of amino acids into polypeptides follows a uniform general pathway, it is worthwhile to note that methionine plays a special role in bacterial protein synthesis. The initiation of protein synthesis requires a unique tRNA ($tRNA^{fMet}$) carrying N-formylmethionine, which is formylated after methionyl-$tRNA^{fMet}$ has been formed.

Methionine is also the precursor of S-adenosylmethionine which, as a methyl donor in many biochemical reactions, has a far-reaching involvement in metabolism. Aspartate, besides having an essential role in protein biosynthesis, is also the precursor of pyrimidines, which are ultimately used in the biosynthesis of DNA and RNA. Diaminopimelate is not only an intermediate in lysine biosynthesis but is also a component of many bacterial cell walls.

B. Secondary metabolites

Secondary metabolism is involved in the formation of substances that are not essential to the growth processes. Antibiotics are typical secondary metabolites. Their role is related to the interactions between organisms that occur in natural environments where competition is an important factor in survival. They can often act as antimetabolites inhibiting DNA, RNA, protein, or cell wall synthesis, and membrane function. The majority of microbial secondary metabolites investigated belong to groups of structurally similar compounds, such as polyketides, polypeptides, beta-lactams, aminoglycosides and nucleosides. These groups are clearly related to the major primary macromolecular cell components. The nucleoside secondary metabolites, like the nucleosides of primary metabolism, contain pyrimidine

derivatives that are derived from aspartate. Many polypeptide secondary metabolites contain amino acids belonging to the aspartate family, and there are also many secondary metabolites that are formed by modification of individual aspartate family amino acids.

1. Peptide antibiotics

A large number of the secondary metabolites containing aspartate family amino acids are peptide antibiotics. They are biosynthesized by a mechanism different from ribosomal protein synthesis in that the amino acid sequence is determined by a multifunctional protein possessing catalytic sites for peptide bond formation. Many aspartate family amino acids are incorporated into such peptides without modification. However, some changes can occur. In bacitracin A, isoleucine, lysine and asparagine are not modified, but aspartate is epimerized to the D-configuration (Kleinkauf and von Dohren, 1983). In the peptide part of actinomycins, most of the threonine incorporated is not modified, but in some actinomycins threonine is hydroxylated (Okmura, 1983).

2. ß-lactam antibiotics

Nocardicins, penams and cephems constitute three groups of ß-lactam antibiotics. Many of which are medically important because they inhibit bacterial cell wall synthesis. Methionine is incorporated into nocardicin A (Townsend and Brown, 1981), and lysine is a precursor for all penam and cephem derivatives produced by actinomycetes, where the aminoadipyl component of the tripeptide L-α-aminoadipyl-L-cysteinyl-D-valine intermediate is derived from this amino acid (Madduri et al., 1989). Aminoadipate is biosynthesized from an intermediate in the lysine biosynthetic pathway in fungi (Umbarger, 1978). Mendelovitz and Aharonowitz (1982) observed that lysine or diaminopimelate added to a chemically defined medium significantly increased production of cephamycins in Streptomyces clavuligerus. The yield increased sixfold when both lysine and diaminopimelate were added.

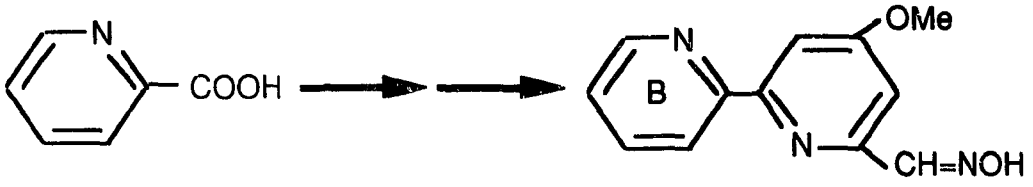
Moreover, mutants impaired in feedback regulation of aspartate kinase gave up to eightfold increases in antibiotic production (Demain et al., 1983). Two of the mutants were capable of increasing their diaminopimelate pool concentration dramatically. Since diaminopimelate induces diaminopimelate decarboxylase and lysine represses the enzyme, it is likely that this enzyme regulates the formation of cephamycins in S. clavuligerus; thus aspartate pathway functions can influence the carbon flow in cephamycin biosynthesis.

3. Secondary metabolites derived from picolinic acid

Caerulomycin A (Fig. 7) is a member of the 2,2'-dipyridyl group (Vining et al., 1988) of antibiotics produced by a number of actinomycetes. Early studies suggested that lysine was the precursor for its B ring (Fig. 7) (McInnes et al., 1979); this was confirmed by incorporation of isotopically labelled lysine. Picolinic acid (Fig. 7) was incorporated into the antibiotic even more efficiently (Vining et al., 1988), suggesting that picolinic acid was formed as a free intermediate from lysine. Other studies have suggested that proferrorosamine A is also derived from picolinc acid (Pouteau-Thouvenot et al., 1976). A derivative of picolinic acid (3-hydroxypicolinic acid), is found in virginamycin antibiotics (Cocito, 1979).

4. 3-Methylthioacrylic acid

3-Methylthioacrylic acid (Figure 8) is a secondary metabolite found in some streptomycetes (Visser and Merer, 1969; Arima et al., 1970; Surette and Vining, 1976). Its synthesis is stimulated by methionine. The biosynthesis of 3-methylthioacrylic acid was established by isolation of intermediates from the cultures, by isotopic feeding experiments and by isolation of a key enzyme. With isotopically labelled methionine, Surette and Vining (1976) showed that in Streptomyces lincolinesis 3-methylthioacrylic acid is formed specifically from methionine with loss of the carboxyl group. Moreover, they isolated a peroxidase that catalyzed the oxidative decarboxylation of methionine to 3-methythiopropionamide. Surette



Picolinic acid

Caerulomycin A

Figure 7. Biosynthetic route for caerulomycin A.

and Vining (1976) suggested two alternative routes (Figure 8) depending on the specificities and relative activities of the required amidases and dehydrogenases in different organisms.

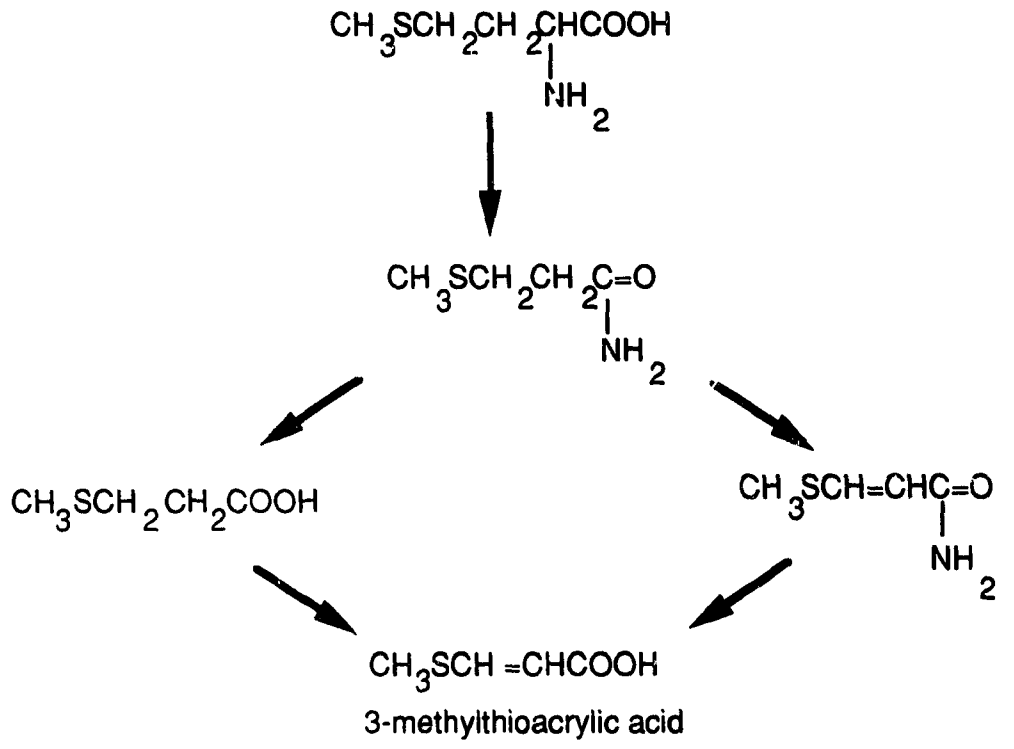


Figure 8. Biosynthetic route for 3-methylthioacrylic acid.

MATERIALS AND METHODS

I. Bacteria, plasmids and Phage

Bacterial strains, plasmids and the helper phage used in this study are listed in Table 1.

II. Chemicals and biochemicals

Reagent grade or analytical grade solvents and chemicals were used unless otherwise indicated. The solvents used in high performance liquid chromatography (HPLC) were HPLC grade. Thiostrepton was a gift from S. J. Lucania of E. R. Squibb and sons, New Brunswick, NJ. 5-Hydroxy-4-oxonorvaline (HON) was a gift from R. L. White, Chemistry Department, Dalhousie University. Ampicillin, kanamycin, tris-(hydroxymethyl) aminomethane (Tris), N-tris-(hydroxymethyl) methyl-2-aminoethane sulphonic acid (TES), bovine serum albumn (BSA), acetyl coenzyme A, NADP, isopropyl-ß-D-thiogalactopyranoside (IPTG) and polyethylene glycol (PEG) 8,000 were purchased from Sigma Chemical Company, St. Louis, Mo. PEG 1,000 was from Koch-Light, Haverhill, UK. Morpholinopropane sulfonic acid (MOPS) was from ICN, Montreal, PQ. 5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) was purchased from Diagnostic Chemicals Limited, Charlottetown, P. E. I. Lysozyme, pronase, proteinase K, DNase I, RNase A, dNTPs, ethidium bromide, phenylmethylsulfonyl fluoride (PMSF), the nylon membrane for hybridization and the Dig-DNA labelling and detection kit were purchased from Boehringer-Mannheim, Montreal, PQ. Ultrapure grade agarose for DNA elution or Southern blotting, electrophoresis-grade acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylenediamine (TEMED), urea and ammonium persulphate were from Bio-Rad Laboratories, Mississauga, Ont.

Restriction enzymes, T_4 DNA ligase, Klenow fragment and lambda DNA were from

Table 1. List of bacterial strains, plasmids and phage used in this study.

Strains, plasmids and phage	Genotype or phenotype	Source or reference
Streptomyces akiyoshiensis		
ATCC 13480	Wild-type	R. L. White, Chem. Dept., Dalhousie Univ.
L16	HON$^-$	this study
L21	HON$^-$	this study
L86	HON$^-$	this study
L121	HON$^-$	this study
L127	HON$^-$	this study
L138	HON$^-$	this study
L141	HON$^-$	this study
L156	HON$^-$	this study
L159	HON$^-$	this study
L174	HON$^-$	this study
L195	HON$^-$	this study
LC153C	requires lys, met, thr	this study
LC175Z	requires lys	this study
LC224X	requires met, thr	this study
LC242B	requires lys, met, thr	this study
LC263Z	requires lys, met, thr	this study
LC263R	revertant of LC 263Z	this study
LC296Y	requires met, thr	this study
LC296R	revertant of LC 296Y	this study

(Table 1. continued)

LC367Y	requires lys, met, thr	this study
LC367R	revertant of LC367Y	this study
LC461A	requires lys	this study
Escherichia coli		
CGSC 5074	F⁻thrA1101,supE44,λ⁻,rpsL9, malT1,(λ^R),xyl-7,mtl-2, ivlA296,metL1000,arg-1000, thi-1,lysC1001	E. coli Genetic Stock Center
CGSC 5080	Hfr PO101λ⁻,hisA323, Δ(bioH-asd)29.	E. coli Genetic Stock Center
CGSC 6212	F⁻ϕ80dlacZΔ(lacZYA-argF)4169 supE44,λ⁻gyrA,recA1,relA1, endA1,Δasd4,Δ[zhf-2::Tn10], hsdR17	R. Curtiss III, Biology Dept. Washington Univ.
DH5α	F⁻ϕ80d,lacΔM15Δ(lacZYA-argF)U169,supE44,λ⁻thi-1,gyrA, recA1,relA1,endA1,hsdR17	Sambrook, et al., 1989
TG1	Δ(lac,pro) supE,thi,hsdΔ5, F'(traD36,proAB⁺,lacI^q, lacZΔM15)	Carter et al., 1985
Saccharomyces cerevisiae		
A-4840A	Commercial baker yeast	Bhanot and Brown, 1980
pBluescript II SK (+)	amp^r,lacZ	Stratagene
pBluescript II SK (-)	amp^r,lacZ	Stratagene
pHJL400	amp^r,lacZ,tsr	Larson and Hershberger, 1986

(Table 1. continued)

pIJ702	tsr,mel$^+$	Katz et al., 1983
pTZ18R	amp^r,lacZ	Mead et al., 1988
pTZ19R	amp^r,lacZ	Mead et al., 1988
pJV21	amp^r,asd$^+$,tsr	this study
pJV24	amp^r,asd$^+$	this study
pJV25	amp^r	this study
pJV26	amp^r	this study
pJV27	amp^r	this study
pJV28	amp^r	this study
pJV29	amp^r	this study
pJV30	amp^r	this study
pJV31	amp^r	this study
pJV32	amp^r,asd$^+$	this study
pJV33	amp^r,asd$^+$	this study
VCSM13	kan^r	Stratagene

Bethesda Research Laboratories, Burlington, Ont. The GENECLEAN kit was from BIO 101 Inc., LaJolla, CA. The DNA sequencing kit was purchased from United States Biochemical Co., Cleveland, OH. ^{35}S-ATP was from ICN Biochemicals, Montreal, PQ.

Bacto-agar, Bacto-tryptone, Casamino acids, Bacto-yeast nitrogen base without amino acids, nutrient agar, yeast extract, malt extract, and nutrient broth were purchased from Difco Laboratories, Detroit, MI. Trypticase Soy Broth and Potato Dextrose Agar were from BRL. Purified casein was from Anachemia Chemicals Limited, Toronto, Ont.

III. Media, stock solutions and buffers

A. Media

All media were sterilized in an autoclave for 20 min at 121°C and 15 psi. Unless otherwise indicated, semisolid media contained 15% (w/v) agar.

MYM medium

Maltose	4.0 g
Yeast extract	4.0 g
Malt extract	10.0 g
Distilled water to	1000 ml

pH adjusted to 7.0.

Starch-casein (SC) medium

Starch	30.0 g
Purified casein	9.0 g
Na_2HPO_4	7.4 g
KH_2PO_4	10.9 g
$MgSO_4 \cdot 7H_2O$	30.0 g

Distilled water to..... 1000 ml

pH adjusted to 6.3.

Modified starch-casein (MSC) medium

Starch..... 30.0 g

Purified casein..... 4.00 g

K_2HPO_4..... 1.75 g

KH_2PO_4..... 0.75 g

$MgSO_4 \cdot 7H_2O$..... 0.20 g

Trace element solution A..... 4.5 ml

$FeSO_4 \cdot 7H_2O$ solution..... 4.5 ml

Salt solution..... 1.0 ml

Distilled water to..... 1000 ml

pH adjusted to 6.5.

Starch-nitrate (SN) medium for HON

Starch..... 30.0 g

KNO_3..... 3.00 g

K_2HPO_4..... 1.75 g

KH_2PO_4..... 0.75 g

$MgSO_4 \cdot 7H_2O$..... 0.20 g

MOPS..... 21.00 g

Trace element solution A..... 4.5 ml

$FeSO_4 \cdot 7H_2O$ solution..... 4.5 ml

Salt solution..... 1.0 ml

Distilled water to..... 1000 ml

The pH of the medium was adjusted according to the needs of the experiment.

Modified starch-nitrate (MSN) medium

Starch	30.0 g
KNO_3	3.00 g
K_2HPO_4	1.75 g
KH_2PO_4	0.75 g
$MgSO_4\ 7H_2O$	0.20 g
Trace element solution A	4.5 ml
$FeSO_4\ 7H_2O$ solution	4.5 ml
Salt solution	1.0 ml
Distilled water to	1000 ml

The pH was adjusted according to the needs of the experiment. The medium was supplemented to 0.3 mM with the required amino acid(s) for auxotrophs.

Minimal medium (MM) for isolation auxotrophic mutants

Glucose	5.0 g
KNO_3	3.00 g
K_2HPO_4	1.75 g
KH_2PO_4	0.75 g
$MgSO_4\ 7H_2O$	0.20 g
Trace element solution A	4.5 ml
$FeSO_4\ 7H_2O$ solution	4.5 ml
Salt solution	1.0 ml
Distilled water to	1000 ml

The pH was adjusted according to the needs of the experiment. The medium was

supplemented to 0.3 mM with the required amino acid(s) for auxotrophs.

Minimal medium (MME) for cultures in which enzymes were assayed

K_2HPO_4	4.4	g
$MgSO_4 \cdot 7H_2O$	0.6	g
MOPS	21.0	g
Trace element solution B	1.0	ml
Distilled water to	1000	ml

Either glycerol (5.0 g/l) or starch (30 g/l) was used as the carbon source. Ammonium sulphate (2.0 g/l) or an amino acid (30 mM) was used as the nitrogen source. The pH was adjusted to 6.8 with NaOH. The medium was supplemented to 0.3 mM with the required amino acid(s) for auxotrophs.

M9 minimal medium

5X M9 salts	200	ml
20% (w/v) glucose	20	ml
Distilled water to	1000	ml

The components were autoclaved separately before they were mixed together.

TSB A medium

Trypticase Soy Broth	30	g
Glycerol	40	g
Distilled water to	1000	ml

pH adjusted to 7.0.

TSB B medium

Trypticase Soy Broth	30	g
Glucose	20	g

Distilled water..... 1000 ml

pH adjusted to 7.0.

TSB C medium

Trypticase Soy Broth..... 30 g

Yeast extract..... 10 g

Sucrose..... 100 g

$MgCl_2.6H_2O$..... 10.12 g

Distilled water to..... 980 ml

$CaCl_2$ (7.36%) 20 ml was added immediately before use. The pH was adjusted to meet the needs of the experiment.

R2YE Medium (R5)

Sucrose..... 103 g

K_2SO_4..... 0.25 g

$MgCl_2 6H_2O$..... 10.12 g

Glucose..... 10.00 g

Casamino acids..... 0.10 g

Yeast extract..... 5.00 g

TES buffer..... 5.73 g

Distilled water to..... 1000 ml

The pH was adjusted to 7.0 with 1 M NaOH and agar was added before autoclaving. At the time of use, the following were added per 1000 ml:

KH_2PO_4 (0.5%)..... 10 ml

$CaCl_2$ (5 M)..... 4 ml

L-Proline (20%)..... 15 ml

Trace element solution C..... 2 ml

Soft nutrient agar

Nutrient Broth..... 8.0 g

Agar..... 3.0 g

Distilled water to..... 1000 ml

Potato dextrose agar

Potato Dextrose Agar..... 39 g

Distilled water to..... 1000 ml

Bioassay medium

Glucose..... 1.0 g

Yeast nitrogen base without amino acids..... 6.7 g

Distilled water..... 1000 ml

Semi-solid medium used in bioassays included 1.2% (w/v) agar. Top layer soft agar contained 0.5% (w/v) agar.

L-broth (LB)

Tryptone..... 10.0 g

Yeast extract..... 5.0 g

NaCl..... 5.0 g

Glucose..... 1.0 g

Distilled water to..... 1000 ml

2X YT medium

Tryptone..... 16.0 g

Yeast extract..... 10.0 g

NaCl..... 5.0 g

Distilled water to..... 1000 ml

Adjust pH to 7.0.

B. Stock solutions and buffers

<u>$FeSO_4$ solution</u>

$FeSO_4\ 7H_2O$..... 1.0 g

Distilled water to..... 1000 ml

<u>Salt solution</u>

NaCl..... 10 g

$CaCl_2$..... 10 g

Distilled water to..... 1000 ml

<u>Trace element solution A</u>

H_3BO_3..... 1.6 mg

$(NH_4)_6Mo_7O_{24}\ 4H_2O$..... 10 mg

$CuSO_4\ 5H_2O$..... 10 mg

$MnCl_2\ 4H_2O$..... 10 mg

$ZnCl_2$..... 40 mg

$FeCl_3\ 6H_2O$..... 120 mg

Distilled water to..... 1000 ml

<u>Trace element solution B</u>

$ZnSO_4\ 7H_2O$..... 1.0 g

$CaCl_2$..... 1.0 g

$FeSO_4\ 7H_2O$..... 1.0 g

$MnCl_2\ 4H_2O$..... 1.0 g

Trace element solution C

$ZnCl_2$	40 mg
$FeCl_3 \cdot 6H_2O$	200 mg
$CuCl_2 \cdot 2H_2O$	10 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	10 mg
$MnCl_2 \cdot 4H_2O$	10 mg
$Na_2B_4O_7 \cdot 10H_2O$	10 mg
Distilled water to	1000 ml

P buffer

Basal solution:

Sucrose	103 g
K_2SO_4	0.25 g
$MgCl_2 \cdot 6H_2O$	2.02 g
Trace element solution C	2 ml
Distilled water to	800 ml

The basal solution was dispensed in 80 ml aliquots and autoclaved. Before use, each aliquot was supplemented with following:

KH_2PO_4 (0.5% w/v)	1 ml
$CaCl_2 \cdot 2H_2O$ (3.68% w/v)	10 ml
TES buffer (5.73%), adjusted to pH 7.2	10 ml

5X M9 salts

$Na_2HPO_4.H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g

NH_4Cl..... 5.0 g

Distilled water to..... 1000 ml

The solution was divided into 200-ml aliquots before it was sterilized.

HON stock solution used for making standard solution was prepared in distilled water. Ampicillin stock solution was prepared in distilled water at 100 mg/ml (w/v), and filter sterilized through a Millipore membrane (pore size 0.22 μm). Thiostrepton stock solution was made in DMSO at 50 mg/ml (w/v). Buffers for restriction endonucleases, T_4 DNA ligase, enzymes used in DNA sequencing and Southern hybridization were supplied by the manufacturers. TE (Tris-HCl-EDTA) buffer, Tris-maleic buffer, lysozyme buffer, TES buffer, Lysis (L) buffer, protoplasting (P) buffer, buffers and phenol solutions for DNA isolation were prepared as described by (Hopwood et al., 1985). TAE (Tris-acetate-EDTA) buffer for agarose gel electrophoresis, TBE (Tris-borate-EDTA) buffer for polyacrylamide gel electrophoresis, 30% (w/v) acrylamide stock solution, standard sequencing gel solution and solutions used for hybridization were prepared using the procedures described by Sambrook et al. (1989). Buffers and solutions for cell extracts, enzyme assays and HPLC were prepared according to the methods described in the corresponding references.

IV. Maintenance of stock cultures

A. Escherichia coli

Stock suspensions of E. coli cultures were prepared by mixing 0.9 ml of an overnight culture with 0.6 ml of 50% (v/v) glycerol. They were stored at -70°C.

B. Streptomyces akiyoshiensis

Spore suspensions were prepared from surface cultures grown at 30°C on MYM agar

plates (100 mm) for 7 to 10 days. Sterile water (10 ml per plate) was added, and spores were dislodged from the surface growth with an inoculating loop. The suspension was filtered in a sterile syringe through nonabsorbent cotton and the filtrates were centrifuged at 5000 rpm for 10 min in a bench top centrifuge. The spore pellet was washed by centrifugation with 5 ml sterile water and resuspended in 20% (v/v) sterile glycerol. It was then stored at -20°C.

C. Saccharomyces cerevisiae

Stock cell suspensions were prepared by growing S. cerevisiae on Potato Dextrose Agar overnight, and were maintained at 4°C.

V. Culture conditions

A. Escherichia coli

1. For preparation of competent cells

A single colony grown on L-broth agar was used to inoculate 10 ml of L-broth. The culture was shaken at 250 rpm and 37°C overnight, then used (0.5 ml) to inoculate 50 ml of L-broth. This culture was shaken (250 rpm) at 37°C for 2.5 h, and then used to prepare competent cells.

2. For single-strand DNA isolation

The procedures of Sambrook et al. (1989) were used. A fresh single colony was suspended in 2 ml 2X YT medium, and helper phage VCSM13 was added to a final concentration of $2X10^7$ pfu/ml. The culture was incubated at 37°C for 1.5 h with vigorous shaking. Kanamycin was added to a final concentration of 75 μg/ml. The culture was then shaken at 250 rpm and 37°C for 16 h.

3. For isolating plasmids

For small scale isolation, a single colony with the desired plasmid was used to inoculate 2 ml of L-broth containing 100 μg/ml of ampicillin; the culture was shaken at 250 rpm and 37°C for 12 h. For large scale isolation, the same medium was used; the culture was scaled up to 50 ml and 500 μl of overnight culture was used as the inoculum.

B. Streptomyces akiyoshiensis

1. For isolating total DNA

A 125-ml flask containing 20 ml of TSB C medium (pH 6.0) was inoculated with a piece of agar (approximately 1 cm^2) from a confluent culture of S. akiyoshiensis on MYM agar. The flask was shaken (250 rpm) for 48 h at 27°C. As an equivalent inoculum, 1.5% (v/v) of a spore suspension ($O.D._{640}$ = 10; Glazebrook et al., 1992) was also used.

2. For isolating plasmids

TSB C medium supplemented with thiostrepton (10 μg/ml) was used. Other conditions (inoculum, aeration and temperature) were the same as for cultures used to isolate total DNA from S. akiyoshiensis. Usually more than 48 h was needed to grow enough mycelium for an extraction.

3. For protoplasting

TSB C medium was used. The inoculum, aeration, temperature and incubation time were the same as used for cultures from which total DNA was isolated.

4. For HON production

a. In MSC medium

A 125-ml flask containing 10 ml of MSC medium was inoculated with washed mycelium obtained from 0.1 ml of a 48-h culture in TSB C medium. Alternatively, 1.5%

(v/v) of spores (OD_{640} = 10; Glazebrook et al., 1992) was used as the inoculum. The flask was shaken at 250 rpm and 27°C. The culture medium was supplemented to 3 mM with sterile solutions of amino acid(s) when amino acid repression of HON production was tested. Samples (50-100 μl) were taken at 24-h intervals unless otherwise indicated.

b. In MSN medium

The inoculum, aeration and temperature were the same as used for HON production in MSC medium. When HON production by auxotrophs was examined, the required amino acid(s) (0.3 mM) were added. Samples (100 μl) were usually taken at 24-h intervals.

5. For in vitro HON synthesis

A 1.5% (v/v) of spore inoculum (OD_{640} = 10; Glazebrook et al., 1992) was introduced into 100 ml of SC medium in a 2-l flask. The culture was incubated at 250 rpm and 27°C for 48 h, or until the onset of HON production.

6. For assaying aspartate kinase and homoserine dehydrogenase activity

A 1000-ml flask containing 100 ml of MME medium and supplements was inoculated with the washed mycelium from 1 ml of a 48-h culture grown in TSB C medium. The culture was shaken at 250 rpm and 27°C.

C. Saccharomyces cerevisiae

Cultures of S. cerevisiae are described in the section on bioassays.

VI. Measurement of growth

Growth of S. akiyoshiensis cultures was determined by measuring optical density (OD) or dry weight. Culture broth (0.5 ml) was aseptically removed from a production culture and mixed with 0.45 ml of distilled water in a round cuvette 12 mm in diameter. The OD_{640} of

the sample was measured against a water blank with a Bausch & Lomb Spectronic 21 spectrophotometer. Dry weight values are the average from three replica cultures. Each culture (10 ml) was filtered under vacuum through a preweighed dry (after 2 h at 75°C) Whatman No. 5 filter paper disk. The disk and mycelium were then washed with distilled water, dried for 2 h at 75°C, and weighed.

VII. Isolation of HON-nonproducing mutants, auxotrophic mutants and revertants of S. akiyoshiensis

A. Mutagenesis

The method described by Hopwood et al. (1985) was used to obtain mutants of S. akiyoshiensis. A spore suspension (1.5 ml) was centrifuged for 2 min at 13,000 g, and the pellet was resuspended in 1.5 ml of TM buffer (0.05 M Tris and 0.05 M maleic acid, pH 8.10) containing N-methyl-N'-nitro-N-nitrosoguanidine (NTG; 2 mg/ml). The spore suspension was incubated at 30°C in a fume hood; samples were removed at 30-min intervals for 3 h. The samples were centrifuged for 2 min at 13,000 g and the pelleted spores, twice washed with sterile distilled water, were resuspended in 20% glycerol. Colony numbers were estimated by diluting spore suspensions serially and plating them on MYM agar. The survival rate was calculated by dividing the cfu (colony forming units)/ml of NTG-treated spores with the cfu/ml of untreated spores. Spores from NTG treatments that gave survival rates of less than 2% were screened for mutants.

B. Screening for mutants blocked in HON production

Mutagenized spores were plated on MSC agar. After 2-3 days at 30°C, 7-mm diameter plugs containing a single colony were cut aseptically from the agar, and incubated

at 30°C in Petri dishes. Dehydration of these independent "minicultures" was avoided by placing 50-80 plugs in each 100-mm Petri dish. After the colonies on most plugs were well sporulated and pigmented, the plugs were transferred individually to bioassay agar and incubated at 27°C for 18 h. The colonies on plugs that failed to give an inhibition zone were considered to be potentially blocked mutants. They were tested by two rounds of single colony isolation and phenotype confirmation by the agar plug procedure as above. Those that gave 100% noninhibitory plugs in the second round were then examined for HON production by HPLC analysis of samples taken daily from cultures grown in liquid MSC medium. Spore suspensions of each blocked mutant were stored in 20% glycerol at -20°C.

C. Screening for auxotrophs

Mutagenized spores were diluted to give about 150 colonies per 100-mm Petri plate containing MYM agar supplemented with diaminopimelate. Colonies were allowed to grow at 30°C for 7 days, when most were well sporulated. They were then replicated on MSN agar medium supplemented to 0.3 mM with each amino acid or combination for which auxotroph was examined. The final replication was to MYM agar; this indicated whether enough spores to allow growth had been transferred to each replica plate. Potential auxotrophs were retested by plating for single colony isolation and replica plating to diagnostic media. Spore suspensions of strains with the desired phenotypes were stored in 20% glycerol at -20°C.

D. Selection of revertants

Spores of auxotrophic mutants were plated on MM agar medium and incubated at 27°C for 2-3 days. Fast growing colonies, considered to be potential revertants, were retested twice by single colony isolation and phenotype confirmation, then stored as spore suspensions.

The rate of reversion was calculated by dividing the cfu/ml of a spore suspension plated on minimal medium with the cfu/ml after plating on complex (MYM) medium. Spore suspensions of revertants were stored in 20% glycerol at -20°C.

VIII. Assays and bioassays

A. Measurement of HON concentration

The concentration of HON was measured by a reversed phase HPLC method after precolumn derivatization of amino acids in the culture broth (White et al., 1989).

1. HPLC system

The Beckman HPLC system with System Gold software, which programmed the solvent gradient and analyzed the chromatographic data, was used. The software was entered in an IBM-compatible personal computer (Jemini 286). A Beckman analogue interface module 406 directed the activity of pumps, detectors and the injector. The Beckman HPLC components consisted of model 110A and model 112 pumps connected to the chromatographic column through a gradient mixer. The amount of sample loaded into the system was controlled by a 20-μl sample loop connected to a model 210 injection valve. Fluorescence in the column eluate was measured with a model 157 fluorometer, equipped a 9-μl flow cell, which allowed excitation at 305-395 nm and detected emission at 420-650 nm.

2. Column and solvent system

Derivatized amino acids were separated on a Beckman reversed phase column (45 X 4.6 mm) packed with ODS Ultrasphere C_{18} silica of 5 μm particle size. This was preceded by a direct connect cartridge guard column (Alltech-Applied Science, Deerfield, IL). The eluting solvent was a gradient of methanol in an aqueous buffer consisting of tetrahydrofuran

(0.5%), methanol (9.5%) and 0.1 M sodium acetate (90%), pH 6.2 (Fig. 9). The 0.1 M sodium acetate was filtered through a cellulose acetate membrane (0.45-um pore size, Millipore Corporation) and degassed under aspirator vacuum before mixing with other components of the buffer. The solvent flow rate in the column was 2.5 ml/min.

3. Derivatization of amino acids

Fluor-R (o-phthaldiadehyde) Reagent (40 ul) was mixed with 20 μl of sample and 20 μl of internal standard solution (cysteic acid 8.8 μg/ml). After 60 s the reaction was quenched with 120 μl of 0.1 M sodium acetate, and the mixture was immediately injected on to the HPLC analysis column. When culture supernatant was analyzed, it was first clarified by centrifugation at 12000 g for 10 min. If the concentration of HON exceeded the range for an accurate flurorescence response, the sample was diluted appropriately and re-analyzed.

4. Calibration

To determine the HON concentration in samples, purified HON (a gift from R. L. White) was used as a standard. The concentrations giving proportional (linear) fluorescence responses, as measured by peak area after chromatography, were determined and the data was entered into the analytical software. The average response (peak area) of three injections was used in these calculations. The fluorescence response was related to that of the internal standard (8.8 μg/ml, cysteic acid) present in each sample. The response for the internal standards was determined from the average peak area of 20 injections of cysteic acid. The ratio of average peak areas for HON and the internal standard of each sample was adjusted to the average response of cysteic acid to correct for variations in instrument response.

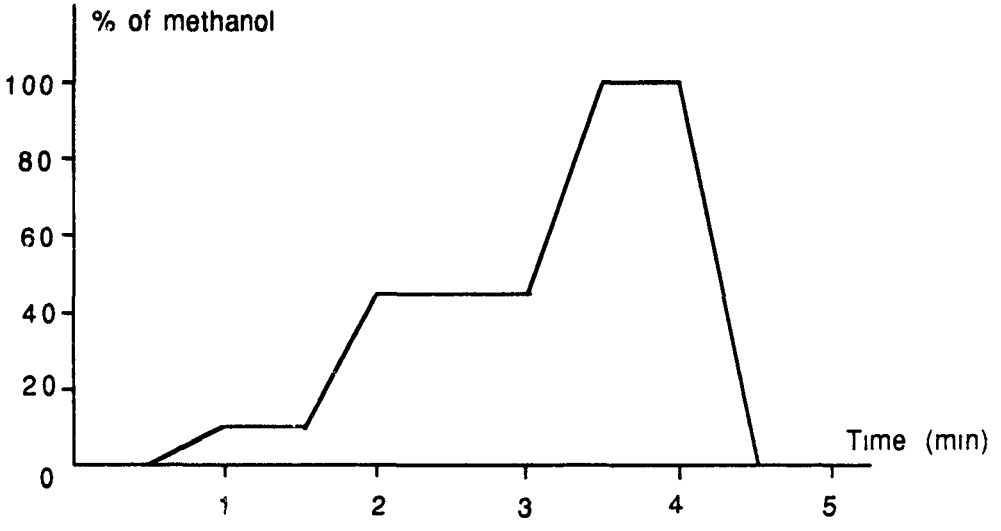


Figure 9 Percentage of methanol in the gradient for HPLC separation of derivatized amino acids

B. Enzyme assays

1. Preparation of cell extracts

Mycelium was collected by vacuum filtration through a Whatman No. 5 paper in a Buchner funnel, and washed three times with phosphate buffer (20 mM potassium phosphate pH 7.0, 30 mM mercaptoethanol and 1 mM PMSF). The washed mycelium was scraped from the paper and immediately frozen at -20^{0}C. To disrupt cells for aspartate kinase or homoserine dehydrogenase assays, 4 g of frozen mycelium was thawed in 8 ml of ice-cold phosphate buffer. In assays for in vitro HON synthesis, MOPS (pH 7.2, 100 mM) was replaced with phosphate buffer and fresh mycelium was used. Extracts were prepared by disruption of the thawed mycelial suspension with a Branson Sonifier (model 210) for 6 X 15 s at 4^{0}C or with an AMINCO French Pressure Cell (model J5-598A) with the press at a medium power setting. The homogenized solution was then centrifuged (10,000 X g) for 10 min at 4^{0}C. The supernatant solution was collected and used as a crude extract.

In experiments where solutes were first removed, the crude extract was loaded on a Sephadex G-25 column and eluted with the same buffer as was in the extract. Fractions containing proteins were collected. Alternatively, the protein fraction was precipitated by slowly adding solid ammonium sulfate with stirring to 65% saturation, and collected by centrifugation. To obtain partially purified protein preparations, the fraction precipitated at 35 to 55% saturation with amonium sulfate was collected. The suspension was kept for 20 min at 4^{0}C, and was then centrifuged at 10,000 g for 10 min at 4^{0}C. The pellet was redissolved in ice-cold buffer.

2. Aspartate kinase assay

Aspartate kinase was assayed as described by Black et al. (1955). The reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.6), 0.02 M ATP, 0.02 M L-aspartate, 0.01

M $MgSO_4$, 1.4 mM 2-mercaptoethanol, 0.8 M NH_2OH-HCl (neutralized with KOH). It was mixed with the enzyme preparation to give a final volume of 1.0 ml. The reaction mixture was incubated at 30°C for 15 min, then diluted with 2.5 ml of stop solution (10% $FeCl_3.6H_20$, 3.3% trichloroacetate, 0.7 N HCl) and centrifuged. The supernatant was used to measure the OD at 540 nm.

3. Homoserine dehydrogenase assay

Homoserine dehydrogenase was assayed by measuring the reverse reaction (Datta et al., 1970). The reaction mixture contained 0.4 mM NADP, 10 mM L-homoserine (adjusted to pH 7.0 with KOH), Tris-HCl buffer (100 mM Tris-HCl, 1 mM EDTA, pH 7.0 or 8.4) and 0.1 ml of enzyme preparation to a final volume of 1 ml. The mixture was incubated at 25°C, and the decrease in OD at 340 nm was measured.

4. Assay for in vitro HON production

The procedure used was developed by R. L. White (personal communication). The assay mixture contained 0.06 mM aspartic acid, 0.20 mM ATP, 2.5 mM $MgSO_4$, 0.08 mM NADH, 0.08 mM NAD^+, 0.06 mM acetyl CoA, 2.5 mM HCO_3^- and 260 μl of cell extracts. MOPS (100 mM) at pH 7.2 was added to give a total volume of 400 μl. When the effect of pH was tested, the pH of the MOPS buffer was adjusted. The mixture was incubated at 30°C; formation of HON was tested by HPLC analysis of samples removed at intervals during the incubation.

C. Protein concentration assay

Protein concentrations were determined by the method of Lowry et al. (1951) or with Coomassie blue (Bio-Rad Protein Dye Reagent). Bovine serum albumin was used as a protein standard.

D. Bioassays

1. Bioassay for HON

Bioassay medium (25 ml) containing 1.2% (w/v) agar in a 100-mm Petri dish was carefully overlaid with 2.5 ml of 0.5% (w/v) soft agar seeded with an S. cerevisiae culture. Paper discs (10 mm) were placed on top of the overlay, and up to 80 μl of sample was pipetted on to each disc. The assay plate was incubated at 27°C overnight, and then examined for inhibition zones. Agar plugs with surface growth, as well as pieces of chromatographic paper used in HON analysis, were bioassayed similarly by placing them on top of the overlay. Where a large number of samples was assayed, the method was scaled up by using larger (22.5 X 22.5 cm) square plates.

2. Detection of HON cosynthesis by blocked mutants

Spores of two mutants were mixed, and the mixture was spread on MSC agar. Production of HON was measured by bioassay of agar plugs removed at intervals. Alternatively, the spore mixture was used to inoculate liquid MSC medium, and culture filtrates were analyzed by bioassay and HPLC at 24-h intervals for several days. Cultures inoculated with spores of each mutant alone served as controls.

3. Distinguishing feeder and converter strains by cross-feeding

a. On agar medium

Pairs of nonproducer strains were patched side-by-side (without touching) on MSC agar medium. When the cultures started to show pigmentation, plugs were cut from the agar on each side of the line dividing the patches, and at increasing distances from it. The plugs were bioassayed by placing them on agar seeded with S. cerevisiae. The procedure was repeated daily for 4-7 days, depending on the amounts of HON detected. Agar medium spread with spores from the mutants individually served as controls.

b. In liquid medium

The mycelium and culture supernatant from pairs of mutant cultures were exchanged and the recombined cultures were analyzed for HON production by HPLC analysis. In this procedure, broths (10 ml) from each of two cultures grown in MSC medium for 48 h from spore inocula were centrifuged at 5000 g in sterile tubes. The supernatants were sterile filtered by syringing through a 0.2 μm pore size membrane filter (Nalgene, Rochester, NY), into the centrifuge tube containing mycelium of the another mutant. The recombined filtrates and mycelium were then slurried and transferred to clean and sterile flasks for further incubation. Production of HON in the exchanged cultures was measured by HPLC after 24, 48 and/or 72 h. Cultures in which the mycelium and filtrates were reconstitued without exchange served as controls.

4. Conversion assays for feeder products

a. Small-scale bioassay of small quantities of a feeder cultures

A 100-150 μl sample of solution obtained from a feeder culture by sterile filtration of the broth was supplied to a 10-mm agar plug cut from a 2 - 3 day plate culture of a converter by using a short length of sterile plastic drinking straw inserted as a reservior into the top of the agar plug. Production of HON was examined by bioassaying the plug after incubation for 24 h at 27^{0}C.

b. HPLC analysis of feeder cultures

Filter-sterilized broth (10 ml) from a feeder culture was added aseptically to mycelium from a converter culture of the same age. The recombined culture was incubated at 250 rpm for 24 h at 27^{0}C. Samples were taken at intervals for bioassay and HPLC analysis to measure HON production.

IX. Fractionation of HON culture broths

A. Isolation of HON by ion-exchange chromatography

Wild-type culture broth (250 ml) grown in MSC medium for 6 days was centrifuged at 5000 x g for 10 min. The supernatant was applied to column (2 X 30 cm) of Dowex 50X8 (H^+ form; 100-200 mesh particle size) cation exchange resin; the column was washed with deionized water until the effluent was colorless. Amino acids were then eluted with 0.3 M NH_4OH. Fractions (20 ml) were collected, adjusted to pH 7.0 with HCl, and analysed for HON by HPLC. Those containing HON were pooled and concentrated in a rotary evaporator until neutral (excess ammonium removed). The pooled sample was loaded on a column (2 X 6 cm) of Dowex 1 X 8 (acetate form; 200-400 mesh column particle size). Fractions (5 ml) of effluent were collected and assayed by HPLC. Those containing HON were pooled and evaporated in vacuo.

B. Paper chromatography of HON

Samples (10 X 10 μl) of crude HON obtained by ion-exchange chromatography and redissolved in water were applied ever[illegible] as a 50 mm band on Whatman (3MM) filter paper (10 X [illegible]ned n-butanol, acetic acid and water (12:3:5). After [illegible], and two narrow strips (10 mm) were removed. Or[illegible]tion [0.2% (w/v) in 1% (v/v) acetic acid and 99% acet[illegible]detect amino acids. Another strip was bioassayed by placing it [illegible] cerevisiae. The region of the remaining strip corresponding to the zone of antibiotic activity was cut and soaked in 1.0 ml deionized water for 5 min. The extracts was analyzed for the presence of amino acids by HPLC.

X. Examination of blocked mutants

A. Treatment of broths with sodium borohydride

Broth (200 μl) collected by centrifugation of cultures grown in MSC medium was mixed with 40 μl of sodium borohydride solution (10 mg/ml). After 5 min at room temperature, the amino acid profile was compared by HPLC with that of untreated broth.

B. Ethanol extraction of the mycelium

Mutant culture broth (0.5 ml) was centrifuged at 12,000 x g for 5 min. The pellet was mixed with 0.5 ml of 75% (v/v) ethanol and, after 5 min at room temperature, centrifuged at 12000 x g for 10 min. The amino acid profile of the supernatant was examined by HPLC.

C. Examination of mutant L138

Supernatant solutions from 48-h cultures of mutant L138 grown in MSC medium were treated in various ways before recombination with the mycelium of the converter mutant L127. Treated samples were always adjusted to their original pH (6.5), and sterilized by filtration through a 0.2 μm syringe filter (Nalgene, Rochester, NY) before their ability to restore HON production in the converter was tested. Procedures used for the conversion assays were as described in the section VIIID 3b of MATERIALS AND METHODS. Conversion assay cultures were analyzed for HON production by HPLC after 24 h. Cultures of L127 grown in MSC medium served as negative controls. Replica cultures fed untreated L138 broth served as positive controls.

1. Ultrafiltration of mutant L138 broth

Culture supernatants were passed through a cellulose acetate membrane with 10-kDa

cut-off (Millipore) by centrifugation at 5000 rpm in a bench top centrifuge for 10 min.

2. Solvent extraction of mutant L138 broths

Samples of the filtered broth (10 ml) were extracted with ethyl acetate or chloroform. The extracted broth was aspirated to remove solvent.

3. Stability of the product in L138 broths

a. Effect of pH

Culture supernatant was adjusted to acidic pH with hydrogen chloride, and to alkaline pH with sodium hydroxide. The samples were then kept at various temperatures for the designated time.

b. Effect of heat

The culture supernatant was maintained at various temperatures for the designated periods of time.

XI. DNA manipulation

Unless otherwise specified, all centrifugations were at 12,000 x g and room temperature for 10 min. Incubations with restriction enzymes, T_4 DNA ligase, calf alkaline phosphatase and DNA polymerase Klenow fragment followed procedures specified by the suppliers.

A. Isolation of plasmid DNA

Plasmids from E. coli and S. akiyoshiensis were isolated by the method of Lee et al. (1990). Cells were collected by centrifugation and resuspended in 200 μl of freshly made lysis buffer containing 2 mg/ml of lysozyme in 50 mM sucrose, 25 mM Tris-HCl, pH 8.0 and 25 mM EDTA. The suspension was kept at room temperature for 5 min for E. coli, and

20 min for S. akiyoshiensis; it was mixed with 100 μl of freshly prepared alkaline lysis buffer (0.3 M NaOH, 2% SDS). After 5 min at 0°C, 225 μl of 7.5 M ammonium acetate (pH 7.6) was added. After another 5 min at 0°C, the mixture was centrifuged. The supernatant was transferred to a clean tube and 0.6 volume of isopropanol was added. After 10 min at room temperature, the sample was centrifuged at 12,000 g for 10 min at room temperature. The precipitated DNA, after being washed with 70% ethanol and dried, could be digested with restriction enzymes for screening.

For further purification, the DNA pellet in the tube was dissolved in 100 μl of 2.0 M ammonium acetate (pH 7.5), kept on ice for 10 min, and then centrifuged. The supernatant was transferred to a clean tube and the DNA was precipitated by adding an equal volume of isopropanol. After 10 min at room temperature, the sample was centrifuged. The DNA pellet was dissolved in 50 ml TE buffer and RNase was added to a final concentration of 100 μg/ml. After incubation at 37°C for 15 min, the sample was extracted with an equal volume of neutral phenol/chloroform (previously equilibrated with 1 M Tris-HCl pH 8.0) and then chloroform. The DNA was precipitated by adding 0.1 volume of 3.0 M sodium acetate and 2.5 volumes of 95% ethanol. After 10 min at room temperature, the sample was centrifuged; the DNA pellet was washed with cold 70% ethanol and dried. The amount of DNA was determined by agarose gel electrophoresis using lambda DNA digested with HindIII as the standard. For larger scale preparations, the same procedure was scaled up.

B. Isolation of genomic DNA from S. akiyoshiensis

Genomic DNA was isolated as described by Hopwood et al. (1985). Cultures were centrifuged at 4°C, and the mycelium pellet was washed twice with 10.3% sucrose. A 1-g portion of the pellet was suspended in 5 ml freshly prepared lysozyme solution (2 mg/ml

lysozyme and 50 μg/ml RNase in 0.3 M sucrose, 25 mM Tris-HCl, pH 8.0 and 25 mM EDTA). The suspension was kept at 37°C for 30 min and was gently swirled every 15 min. In this and all following steps, vigorous shaking was avoided to prevent shearing the DNA. The suspension was mixed with 1.2 ml of 0.5 M EDTA, 0.13 ml of pronase (2 mg/ml) and 0.7 ml of 10% (w/v) SDS, and was incubated at 37°C for 1.5 h. Neutral phenol/chloroform (6 ml) was then mixed in thoroughly by shaking gently. After centrifugation, the aqueous phase was transferred to a new tube with a wide-mouth pipette and extracted again with 6 ml of neutral phenol/chloroform. After centrifugation, the aqueous phase was transferred to a beaker and mixed with 0.1 volume of 3 M sodium acetate. Ice-cold absolute ethanol (2 volumes) were layered on top of the aqueous solution. The DNA was collected by gently stirring with a glass rod, air dried for 10 min and redissolved in 5 ml TE buffer.

C. Preparation of single-strand DNA

Isolation of single-strand DNA followed the protocol described by Sambrook et al. (1989). The culture (1.5 ml) containing single-strand phagemid was centrifuged. The supernatant was recentrifuged and a sample (1.3 ml) was then mixed with a solution (200 μl) containing 2.5 M NaCl and 20% (w/v) PEG 6,000. After 15 min at room temperature, the mixture was centrifuged. The phagemid pellet was resuspended in 100 μl TE buffer and mixed with 50 μl phenol previously equilibrated with 1 M Tris-HCl, pH 8.0. The mixture was vortexed for 15 s to mix, then after 15 min at room temperature, vortexed again for 15 s and centrifuged. The aqueous top layer was transferred to a new tube and 4 M LiCl (10 μl) and 95% ethanol (250 μl) were added. The tube was kept at -20°C for 1 h and was then centrifuged at 12,000 g for 10 min at room temperature. The DNA pellet, after being washed with 70% ethanol and dried, was dissolved in 20 μl TE buffer. The quality and

quantity of DNA were examined by gel electrophoresis using single-strand M13mp18 DNA as a standard.

D. Elution of DNA from agarose gels

The GENECLEAN kit from BIO 101 Inc. was used to elute DNA from agarose gels. In a microcentrifuge tube, a piece of agarose gel (less than 0.5 g) containing the DNA fragment was incubated with 3 volumes of DNA binding solution (saturated sodium iodide) at 50°C for 3 to 5 min, until the gel had melted. Glass Milk (5 μl) was mixed in, and the mixture, after cooling on ice for 5 min, was centrifuged for 20 s. The Glass Milk pellet and its bound DNA was washed three times by resuspending it in 0.5 ml of ice-cold New Wash solution, and pelleting by centrifugation for 20 s. The DNA was eluted by suspending the pellet in 20 μl of water at 55°C for 5 min. The suspension was clarified by centrifugation for 20 s; the supernatant, containing the DNA, was transferred to a new tube.

E. Size fractionation of DNA

Sucrose gradients were as described by Hopwood et al., (1985). To a 14 X 89-mm polyallomer tube, 6 ml of buffered 40% sucrose solution (40% w/v sucrose, 1 M NaCl, 20 mM Tris-HCl, pH 7.5, 5mM EDTA) were added. Another 6 ml of buffered 10% sucros solution (10% w/v sucrose, 1 M NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA) were carefully placed on top. A 10-40% sucrose gradient was generated by centrifugation at 25,000 rpm and room temperature in a Beckman SW-41 (swinging bucket) rotor for 5 h without acceleration or deceleration. The DNA sample was carefully layered on top of the gradient, and centrifugation was resumed at 35,000 rpm for 16 h at 20°C. Fractions (0.4 ml) were collected from a hole punched into the bottom of the centrifugation tube. Samples (5

μl) from each fraction were examined by gel (0.8% agarose) electrophoresis using lambda DNA digested with HindIII as size markers. The fractions containing DNA of the desired size range were individually diluted with 200 μl TE buffer and 600 μl isopropanol to precipitate DNA. DNA was pelleted, washed with 70% ethanol, dried, and then redissolved in 50 μl of TE buffer.

XII. Transformation

A. Transformation of E. coli

1. Preparation of competent cells

Competent cells of all E. coli strains were prepared by the following procedure (Sambrook, et al, 1989). A fresh culture (25 ml) grown from a single colony was chilled on ice for 10 min and kept at 4°C throughout the preparation. The cells were collected at 5,000 rpm for 15 min in a bench-top centrifuge. The cell pellet was mixed with chilled 0.1 M $CaCl_2$ (10 ml) and was kept on ice for 20 min. The mixture was centrifuged at 5,000 rpm for 15 min and the pellet was resuspended in 0.1 M $CaCl_2$ (1 ml). This suspension of competent cells was divided into 60 μl aliquots and was kept on ice for 2 h. The cells were either used in transformations immediately, or were mixed with 40 μl of sterile 50% glycerol and stored at -70°C for future experiments. They gave more than 10^7 transformants/μg of ccc pHJL400 when used in shot-gun cloning experiments.

2. Transformation

Up to 5 μl of plasmid DNA solution or ligation mixture was mixed with 100 μl competent cells and kept on ice for 20 min. The mixture was heat shocked at 42°C for 1.5 min, and then was cooled again on ice for 2 min before L-broth (0.5-0.9 ml) was added.

After incubation at 37°C for 60 min, the mixture was plated on LB agar containing 100 μg/ml ampicillin. Diaminopimelate was added when the asd⁻ strain was used. The transformed cells were incubated at 37°C for less than 16 h.

B. Transformation of S. akiyoshiensis

Protoplasting, transformation and regeneration of S. akiyoshiensis protoplast were carried out by the procedures described by Hopwood et al. (1985) with some modifications.

1. Preparation of protoplasts

Mycelium from 10 ml of culture was collected by centrifugation in a bench top centrifuge (3000 rpm for 10 min) at room temperature and washed twice with 0.3 M sucrose. The mycelium was resuspended in 4 ml lysozyme solution (5 mg/ml in P buffer, filter sterilized) and incubated at 30°C for 30 min. After 5 ml of P buffer had been added, the suspension was mixed by trituration and filtered through cotton wool into a new tube. The protoplasts were pelleted by centrifugation at 3000 rpm for 10 min and resuspended in 10 ml of P buffer. Aliquots (50 μl) were placed in microcentrifuge tubes and stored at -70°C.

2. Transformation and regeneration of protoplasts

A 1.5 ml microcentrifuge tube containing 50 μl of protoplast suspension was thawed quickly in warm water. Up to 5 μl of DNA was added to the tube and mixed in by tapping; 200 μl of 25% (w/v) PEG 1000 in P buffer was mixed in, and the suspension was spread on partially dried R2YE agar contained in two 100-mm Petri plates. After incubation at 30°C for 24 h to allow regeneration of the protoplasts, the agar was overlayed with 2.5 ml of soft agar containing thiostrepton (50 μg/ml) for selection. Resistant colonies were scored after another three days' of incubation.

XIII. Preparation of an S. akiyoshiensis genomic library

A. Partial digestion of genomic DNA with Sau3AI

Genomic DNA (100 μg) from S. akiyoshiensis was digested with 10 units of Sau3AI at 37°C. Portions of the reaction mixtures were removed at 5, 10, 15, 20, 30, 40, 50 and 60 min. Each portion was mixed with EDTA to a final concentration of 10 mM and then incubated at 65°C for 10 min to stop the reaction. The size of the DNA fragments was assayed by gel electrophoresis; portions containing a majority of fragments in the 6 to 15-kb range were collected for size fractionation described in the section XI. E.

B. Ligation

The vector pHJL400 was digested with BamHI and treated with calf intestine alkaline phosphatase. The ligation system (25 μl) contained the dephosphorylated pHJL400 (0.1 μg), partially digested and size-fractionated genomic DNA (0.5 μg), T_4 DNA ligase (0.5 Weiss unit), buffer and deionized water. The reaction mixture was incubated at 16°C for 8 to 12 h or at room temperature for 5 h.

XIV. DNA sequencing

A. Generation of nested sets of deletions

The pancreatic DNAase I method described by Sambrook et al. (1989) was used. DNase I stock solution (1 mg/ml in 0.01 M HCl) was divided into 10 μl aliquots and stored in -70°C. An aliquot of the enzyme was diluted (1:100,000) with ice-cold digestion buffer containing 50 mM Tris-HCl (pH 7.6), 10 mM $MnCl_2$ and 1 mg/ml bovine serum albumin. Dilution was adjusted to give a working concentration of the enzyme yielding 50% of the original cccDNA after digestion for 5 min. The recombinant phagemid (5 μg) containing the

DNA fragment to be sequenced was digested at room temperature with 5 μl of diluted DNase I in a total volume of 50 μl including the digestion buffer. Samples (10 μl) were removed at 1.5-min intervals and placed in ice-cold microfuge tubes containing 2 μl of 0.5 M EDTA (pH 8.0). The degree of digestion was analysed by agarose gel electrophoresis of each sample (3 μl). Those in which linear DNA represented 30-40% of the total DNA were pooled, extracted with an equal volume of phenol:chloroform, and collected by ethanol precipitation. The DNA was digested with a restriction enzyme that cleaved only in a nonessential region of the vector, upstream of the sequences to be deleted. To generate a blunt end, the digestion mixture (20 μl) was incubated at room temperatue for 15 min with 3 μl of the four dNTPs (each 0.5 mM), 4 μl of deionized water and 1 μl (5 units) of the Klenow fragment of E. coli DNA polymerase I. The quality and quantity of DNA was examined by agarose gel electrophoresis after ethanol precipitation. The DNA molecules (50 μg) were recircularized by ligation for 12 h at 16^{0}C with 0.5 Weiss unit of T_4 DNA ligase, 1 X blunt-end ligation buffer or 0.3 X normal ligation buffer in a total volume of 40 μl. After inactivation at 68^{0}C for 10 min, the ligation mixture was digested with a restriction enzyme cleaving only the vector sequence downstream of the restriction site cut in the previous step. The restriction digest was then used in transformations.

B. Sequencing reaction

The dideoxy-mediated chain-termination method (Sambrook et al., 1989) and a Sequenase version 2.0 kit (from U.S. Biochemical Corp., Cleveland, OH) were used. There were three steps in the sequencing procedure: annealing the template and primer, labelling the DNA, and terminating the reaction. All steps were carried out according to the instructions supplied with the kit. For sequencing single-strand DNA, 0.5 μg of the template and 3 ng

of primer were used in the first step. [α-^{35}S]-dATP was used for labelling. Reaction mixtures after termination were denatured at 95°C for 2 min and placed on ice until they were fractionated by gel electrophoresis.

C. Preparation of polyacrylamide gel

Gel solution was prepared by mixing 63 g of urea, 15 ml of 10X TBE (0.45 M Tris-borate, pH 8.0, 0.01 M EDTA), 25 ml of 30% acrylamide (28.5% w/v acrylamide and 1.5% w/v bis-acrylamide) and distilled water to a final volume of 150 ml. The gel solution was filtered and degassed. The 6% acrylamide/urea solution used to seal the bottom part of the gel was made by mixing 10 ml of gel solution , 50 μl of freshly made 25% (w/v) ammonium persulphate and 50 μl of TEMED. The solution was immediately poured along the entire length of the "sealing strip" in the "casting tray" in the Sequi-gen sequencing apparatus (Bio-Rad Laboratories, Richmond, CA.). The plate-chamber assembly was immediately placed on top of the "sealing strip" and pressure was maintained for 5 min to allow the solution to move upward in the plate-chamber assembly by capillary action and solidify to form a seal in the bottom of the assembly. The 6 % acrylamide/urea top solution was prepared by mixing 40 ml of gel solution, 40 μl of freshly made 25% (w/v) ammonium persulphate and 40 μl of TEMED. The solution was injected from a 50-ml syringe between the two (21 X 40 cm) glass plates in the plate-chamber assembly. The comb was placed on top of the gel with the flat side towards the solution. The solution was allowed to polymerize for at least 3 h.

D. Gel electrophoresis and radioautography

The buffer chamber was filled with TBE buffer to 1.5 cm above the top of the gel. The gel was then prewarmed to 50°C by applying 1,900 v (from a 3,000 Xi power source,

Bio-Rad Laboratories, Richmond, CA.) for 30 min. The comb was reinserted with the sharp end touching the surface of the gel. The wells were washed with TBE buffer. Sequencing reaction mixtures (1.5 μl) were loaded in the wells with a micropipette. Electrophoresis was carried out at approximately 1600 v (50°C) for 1.5 to 3.5 h, depending on the region to be sequenced. After power had been disconnected, the glass plate was carefully separated from the plate-chamber assembly. The gel adhering on the outer plate was fixed with 10% methanol and 10% acetic acid for 20 min, transferred to Whatman 3 MM chromatography paper, covered with a plastic film (Saran Wrap) and then dried at 80°C for 1 h under a vacuum of 25 mm-Hg. The plastic film was removed, and the gel was exposed to Kodak X-Omat film for 20 h at room temperature.

E. Sequence analysis

DNA sequence data were analyzed with various programs of the Genetics Computer Group (GCG, University of Wisconsin Biotechnology Center, Madison, Wisconsin; version 7.1; Devereux et al., 1984), and "DNA trider" (Marck, 1988) programmes. Deduced amino acid sequences encoded by open reading frames (ORFs) were compared with the sequences of other proteins in the GenBank database using BLAST (Altschul et al., 1990). Alignment of amino acid sequences was carried out using CLUSTAL software (Higgins et al., 1988).

XV. Southern hybridization

A. Transfer of DNA from agarose gels to nylon membranes

After electrophoresis, the agarose gel was stained with ethidium bromide and photographed. It was then soaked in 0.25 M HCl for 10 min at r om temperature to depurinate the DNA, and rinsed with distilled water before submersion in denaturing solution

(0.5 N NaOH, 1.5 M NaCl) for 30-60 min at room temperature with gentle shaking. After rinsing with distilled water, the gel was immersed in neutralization solution (1.0 M Tris-HCl, pH 8.0; 1.5 M NaCl) by for 30-60 min at room temperature. The DNA was blotted to a positively charged nylon membrane (Boehringer Mannheim) by capillary transfer using 20X SSC buffer. To ensure efficient transfer, blotting was carried out overnight. To fix the DNA to the membrane, the nylon was washed with 5X SSC buffer at room temperature for 1 min, placed on a piece of filter paper (Whatman 3 MM) and baked at 80°C for 1 h.

B. Labelling of the DNA probe

DNA was labelled with digoxigenin-11-dUTP by a random primer method using a kit and a procedure supplied by Boehringer Mannheim. The DNA sample (10 ng to 3 μg) was heat-denatured in a boiling water bath for 10 min and chilled in dry ice/ethanol. It was then mixed with 2 μl of a hexanucleotide mixture (10X), 2 μl of dNTP labeling mixture (10X) and 1 μl of labelling grade Klenow enzyme at a final concentration of 100 units/ml. The total volume of the reaction was adjusted to 20 μl using distilled water. The reaction mixture was incubated at 37°C for 20 h to achieve efficient labelling. The reaction was terminated by adding 2 μl of 0.2 M EDTA (pH 8.0) and 1 μl of glycogen solution (20 mg/ml). The labelled DNA was precipitated by adding 0.1 volume of LiCl and 3.0 volumes of chilled absolute ethanol, mixed and kept at -20°C for 30 min. The precipitated DNA was then centrifuged at 12,000 g for 10 min. The DNA pellet was washed with 100 μl of chilled 70% ethanol, dried, resuspended in 50 μl of TE/SDS buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.0-8.0; containing 0.1% SDS) and stored at -20°C.

C. Prehybridization and hybridization

The nylon membrane carrying transferred DNA was placed in a polyethylene hybridzation bag containing prehybridization solution [5X SSC, 1.0% (w/v) blocking reagent, 0.1% N-laurylsarcosine and 0.02% SDS] in a volume corresponding to 20 ml per 100 cm^2 of membrane surface area. The bag was sealed and incubated at 65^{0}C for 2 h. The membrane had to be kept wet until the end of hybridization. The prehybridization buffer was replaced with hybridization buffer (10 ml per 10 cm^2 of membrane). The hybridization buffer consisted of prehybridization buffer and a DIG-labelled DNA probe (5-20 ng/ml) that had been denatured by heating in a boiling water bath for 10 min. The hybridization bag was resealed and incubated at 65^{0}C for 6 to 18 h. The membrane was then washed twice, 5 min per wash, with 2X wash solution (2X SSC containing 0.1% SDS) at room temperature to remove unbound probe. The membrane was again washed twice, 15 min per wash, with 0.1X wash solution (0.5X SSC containing 0.1% SDS) at 65^{0}C to remove unspecific hybridization signals.

D. Detection of hybridization signals

The procedure involved binding an anti-DIG antibody to the DIG-labelled DNA probe, adding the Lumigen-PPD substrate (Lumi-phos 530) and detecting the emitted visible light with a photographic film. Most of the reagents used were provided in the DNA labelling kit supplied by Boehringer Mannheim. All steps were carried out at room temperature. The membrane, after post-hybridization washing, was equilibrated for 1 min with 50 ml of buffer 1 containing 100 mM Tris-HCl, 150 mM NaCl; pH 7.5 (filtered through a 0.45 μm membrane filter before use). It was then gently agitated in a freshly washed dish containing buffer 2 (2% w/v blocking reagent dissolved in buffer 1) for 1 h. The anti-DIG-alkaline phosphatase was diluted 1:5,000 with buffer 2 to a working concentration of 150 mU/ml. The

membrane was incubated with this solution for 30 min, washed twice (15 min per wash) with buffer 1, and equlibrated with buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9 5) for 2 min The membrane was then placed between two plastic sheets, and 1 ml of Lumigen-PPD was introduced. The reagent was applied evenly to the membrane surface by gentle pressure across the top plastic sheet. After the two plastic sheets had been sealed, the membrane was exposed to a Kodak XAR film for 7-8 h. The film was developed according to the manufacturer's instruction

RESULTS

The first approach to establishing the reaction sequence for HON biosynthesis was to examine whether HON could be formed in crude cell-free extracts. If S. akiyoshiensis cell extracts contain all the enzymes in the HON biosynthetic pathway, addition of the initial substrates along with all necessary cofactors should allow HON to be synthesized.

I. In vitro production of HON

Aspartic acid and ATP were used to generate aspartyl phosphate, one of the postulated substrates, and acetyl coenzyme A was added as the second substrate. To obtain cell extracts, two alternative procedures were used; in one the cells were disrupted with a French pressure cell, and in the other by sonication. The possible influence of culture conditions was tested by growing cultures in two different media and for two lengths of time. The enzyme preparation without added substrates served as the control. HON production was measured by HPLC.

A. With mycelium grown in complex SC medium

Cultures were grown in SC medium buffered at pH 6.3 with MOPS and supplemented with a high concentration of magnesium sulphate. When the culture started to accumulate HON (culture supernatants were analyzed for HON by HPLC) at 44 h, the mycelium from one of the replica cultures was pelleted by centrifugation. The remaining cultures were harvested in the same way 24 h later. Cell-free extracts made immediately after the mycelium had been pelleted were used to test for in vitro HON synthesis under a variety incubation conditions (Table 2). The results showed no net HON synthesis occurred during the 8-h incubation period. Reanalysis of the samples with the HPLC detector at highest usable

Table 2. Reaction conditions used to test for in vitro synthesis of HON in cell-free extracts prepared from S. akiyoshiensis mycelium grown in complex SC medium.*

	Conditions			
	Culture age (h)	Cell disruption	Treatment of extracts	Reaction temperature
A.	+4	Sonication	Desalted	27°C
B.	44	Sonication	No	27°C
C.	44	French presure cell	Desalted	27°C
D.	44	French presure cell	No	27°C
E.	44	Sonication	Desalted	15°C
F.	72	Sonication	Desalted	27°C
G.	72	Sonication	No	27°C
H.	72	French presure cell	Desalted	27°C
I.	72	French presure cell	No	27°C
J.	72	Sonication	Desalted	15°C

* Each incubation mixture was carried out at pH 6.2, 6.7 and 7.2. The amount of HON present in incubation samples was measured in duplicate by HPLC. Samples were taken from the reaction mixture at 0, 15, 30, 60 min, and 2, 3, 4, 6 and 8 h.

sensitivity did not alter this conclusion.

B. With mycelium grown in defined medium

Cultures were grown in SN medium, which contained a low phosphate concentration and was buffered with MOPS at pH 5.5. This medium gives an abrupt onset of HON biosynthesis (Glazebrook, 1990). In vitro synthesis of HON was examined with mycelium extracts from cultures just starting to accumulate HON as shown by HPLC; this was usually around 48 h. The incubation conditions tested are shown in Table 3. The results again showed that no net HON synthesis during the 8-h incubation period, even when the HPLC detector was adjusted for high sensitivity.

C. Conclusions

Since the results indicated that HON was not synthesized under any of the condition tested, either all of the enzymes necessary were not present, or the reaction conditions were not suitable. It was noteworthy that HON was not found in the cell extracts at any time during the incubation, but was stable when added to the reaction mixture (condition B and G in Table 2 and condition B in Table 3) during incubation. From this, it was concluded that HON does not accumulate inside cells during the production phase, and is probably exported by a membrane protein. Transportation of the end product might also be coupled with the final step in the biosynthesis of HON.

II. Aspartate kinase activity in S. akiyoshiensis

The initial step leading from aspartate to lysine, methionine, threonine and isoleucine is catalyzed by aspartate kinase. The product of the reaction is aspartyl phosphate which,

Table 3. Reaction conditions used to test for the in vitro synthesis of HON in cell-free extracts prepared from S. akiyoshiensis mycelium grown in SN medium.*

	Conditions		
	Cell disruption	Treatment of extracts	Reaction temperature
A.	Sonication	Desalted	27°C
B.	Sonication	No	27°C
C.	French presure cell	Desalted	27°C
D.	French presure cell	No	27°C
E.	Sonication	Desalted	15°C

* Each incubation mixture was carried out at pH 5.1, 5.8, 6.5, 7.0. The amount of HON present in incubation samples was measured in duplicate by HPLC. Samples were taken from the reaction mixture at 0, 15, 30, 60 min, and 2, 3, 4, 6 and 8 h.

besides being a primary metabolic intermediate, is a candidate for the branchpoint substrate that initiates HON biosynthesis. To determine whether cell extracts prepared for in vitro HON biosynthesis contained aspartate kinase activity, the assay method of Black et al. (1955) was adopted. In this procedure, aspartate phosphate is trapped as it is formed by reaction with hydroxylamine, and the hydroxamate is measured colorimetrically as its ferric chelate.

A. Detection of enzyme activity

Cultures tested for aspartate kinase activity were grown in MSC medium, or in SN medium (pH 6.5) as described for in vitro HON biosynthesis. No aspartate kinase activity was detected. However, by growing S. akiyoshiensis under conditions (glycerol as the carbon source and asparagine as the nitrogen source) where aspartate kinase was detected in Streptomyces clavuligerus (Mendelovitz and Aharonowitz, 1982), it was possible to detect aspartate kinase activity in S. akiyoshiensis. Since the specific activity of the enzyme was constant during the rapid growth phase (data not shown; confirmed by T. Lagace, personal communication), the mycelium was harvested at 48 h when cell yields allowed the conditions for aspartate kinase to be conveniently optimized.

B. Influence of protease and solutes in cell extracts

In a preliminary study, with an extract of mycelium grown in MME medium with glycerol and asparagine as carbon and nitrogen sources, respectively, the specific activity of aspartate kinase in protein precipitated from cell extracts with ammonium sulfate was significantly higher than in crude cell extracts. Since aspartate kinase in the extracts was apparently inhibited by soluble components, routine assays were carried out with the fraction precipitated at 0-65% ammonium sulfate (partially purified enzyme). In a test of the stability

of aspartate kinase (Table 4), a partially purified enzyme preparation lost more than 98% of its activity after storage at 4°C for 24 h. The decrease in activity was reduced to 50% by adding the protease inhibitor phenylmethylsulfonyl fluoride (PMSF, 1mM), indicating that proteolysis was at least partially responsible.

C. Influence of nitrogen and carbon sources in cultures on aspartate kinase in cell extracts

The influence of the nitrogen and the carbon sources on the aspartate kinase activity in mycelium from S. akiyoshiensis cultures harvested after 48 h growth was tested.

With glycerol as the carbon source, cultures supplemented with asparagine, aspartic acid, glutamine or glutamic acid as nitrogen sources all gave aspartate kinase activity in cell extracts (Table 5). Asparagine supported the highest specific activity.

When starch and glycerol were compared as carbon sources in a medium containing asparagine as the nitrogen source, starch gave higher aspartate kinase activity (2.90 u/mg protein) than glycerol (1.87 u/mg protein). Thus the starch-asparagine medium was considered more suitable for producing the enzyme.

D. Aspartate phosphate as a potential substrate for in vitro HON biosynthesis

To determine whether aspartate kinase present in S. akiyoshiensis cell extracts could provide a source of aspartyl phosphate, which could then be used as a substrate in testing for HON synthase, samples of crude and partially purified extracts were incubated with aspartate under assay conditions where hydroxylamine was either present throughout the reaction, or omitted from the reaction mixture until the end of the incubation. The colorimetric assay measures aspartyl hydroxyamate, which forms rapidly by reaction between aspartate phosphate and hydroxylamine, trapping the reaction product in a stable form. When addition of

Table 4. Effect of phenylmethylsulfonyl floride (PMSF) on aspartate kinase activity in a partially purified cell extracts of S. akiyoshiensis.*

PMSF concentration (mM)	Specific activity (U/mg protein) At 0 h	After 24 h
O	1.70	0.034
1	1.87	0.94

*0-65% saturated ammonium sulfate fractions were used in the enzyme assay. The enzyme activity was assayed after 0 h and 24 h at 4°C. One unit (U) of enzyme activity is the amount giving an increase in absorbance at 540 nm of 0.001/min under the standard assay conditions. Protein was determined by the method of Lowry et al. (1951). The enzyme activity reported is the average of three assays.

Table 5. Aspartate kinase activity in partially purified enzyme preparations (0-65% saturated ammonium sulfate fractions) from cells grown on various nitrogen sources.*

Nitrogen Source	Specific activity (U/mg protein)	Relative specific activity to mycelium grown on Asn (%)
Asn	1.87	100%
Glu	1.63	87%
Gln	1.09	58%
Asp	0.29	16%

* One unit (U) of enzyme activity is the amount giving an increase in absorbance at 540 nm of 0.001/min under the standard assay condition. Protein concentrations were determined by the method of Lowry et al. (1951), Enzyme activity reported is the average of three assays.

hydroxylamine was delayed, no aspartate phosphate could be detected. Although aspartate kinase is present, even the partially purified cell extracts (35-55% ammonium sulfate fractions) from S. akiyoshiensis did not accumulate sufficient aspartyl phosphate during an incubation to be useful as an enzyme substrate. Since aspartate phosphate is an intermediate shared by a number of metabolic pathways, it is undoubtedly converted to further intermediates and products if the enzyme preparation is not substantially pure. From this results, it was apparent that, assuming aspartyl phosphate is a precursor of HON, there are other enzymes present in cell extracts that compete strongly for it as a substrate.

III. Bioassay for HON

A. Bioassay of HON produced in MSC medium

Since HON inhibited the growth of a number of fungi in liquid culture (Yamaguchi et al., 1988 and 1990; Yamaki et al., 1988), the possibility of devising a bioassay that could be used to isolate and characterize nonproducing mutants was investigated. The inhibitory activity of a S. akiyoshiensis culture supernatant was tested in a disc assay on bioassay agar seeded with S. cerevisiae. Clear inhibition zones were obtained.

B. Identification of the antibiotic inhibiting S. cerevisae

To be useful, a bioassay for HON must not respond to other products in S. akiyoshiensis broth. Whether HON was the only antibiotic in culture supernatant of S. akiyoshiensis grown in MSC medium to inhibit the growth of S. cerevisiae was determined by paper chromatograph of the supernatant in a solvent system containing n-butanol-acetic acid-water (12:3:5). Bioautography of a strip of the chromatogram gave a single zone of inhibition at R_f 0.13. This corresponded to a greenish yellow color on the second strip reacted

with ninhydrin. When the region that corresponded to both the inhibition zone and the greenish yellow color was excised from the remaining chromatogram, and the substances present that eluted with water were analyzed by HPLC, a chromatographic peak corresponding to HON was obtained. As further evidence that the inhibitory substance was HON, the culture supernatant was fractionated by ion exchange chromatography. It was first applied to a cation exchange column, which retained HON and all other amino acids. As HON is a neutral amino acid, it does not exchange with the acetate form of strong anion exchange resins when applied in 2N acetic acid solution. In contrast, acidic amino acids exchange with acetate on the resin. Thus chromatography under these conditions gave only HON and other neutral amino acids in the effluent. The fractions from both cation and anion exchange columns were compared with unpurified culture broth by paper chromatography. Bioautography indicated that only the samples containing HON purified by the ion exchange method gave an inhibition zone and a greenish yellow color with the same R_f as given by unpurified culture supernatant. Overall, the results indicated that HON was the only substance present that inhibited the growth of S. cerevisiae.

C. Bioassay of HON produced by single colonies

The ability to measure HON production by bioassay potentially offered a means of screening mutagenized strains for mutants blocked in HON biosynthesis. For the method to be successful, adequate amounts of HON must be produced by single colonies to give a measurable inhibition zone. MSC medium and MSN medium were tested for their ability to support HON production. On MSC agar, HON was produced in the agar plugs by single colonies of S. akiyoshiensis in concentrations that gave suitable inhibition zones on bioassay plates seeded with S. cerevisiae. The agar plug colonies were incubated for 5 days.

Presumbly less HON was produced by colonies on the MSN medium, since the bioassay was less sensitive. The results indicated that the bioassay of single colonies on MSC medium was feasible.

IV. Mutants blocked in HON biosynthesis

A. Mutagenesis

Treatment with NTG is effective in creating mutations in streptomycetes (Delic et al., 1970), and was used in this study. Exposure of S. akiyoshiensis spores to NTG (2 mg/ml) for 2-3 h gave a survival rate of 2%.

B. Isolation of blocked mutants

Spores treated with NTG to give 2% survival were screened for HON by bioassay of single colonies grown on MSC agar medium. Of 4230 single colonies tested, 198 gave very small or no inhibition zone in the bioassay. Some of these were obviously impaired in sporulation (i.e., were bald) and secondary metabolism (lacked normal pigmentation) and were not characterized further. The phenotypes of the remainder were checked by two rounds of single colony isolation. This yielded 11 mutants with a confirmed HON^- phenotype, the rest were leaky. The production of HON by cultures of mutants grown in MSC medium is listed in Table 6.

C. Examination of mutants for biosynthetic intermediates

1. Reduction with sodium borohydride

Since postulated amino acid intermediates in HON biosynthesis might possess a keto group that would be reduced by sodium borohydride, culture supernatants were treated with

Table 6. HON production (mM) by blocked mutants in MSC medium.*

Strain	0 h	48 h	72 h	96 h	120 h
L016	0	0	0.12	0.17	0.24
L021	0	0	0	0	0
L086	0	0	0	0	0
L127	0	0.02	0.24	0.25	0.24
L138	0	0	0	0	0
L141	0	0	0.049	0.23	0.36
L156	0	0	0	0	0
L159	0	0	0	0	0
L167	0	0	0	0.025	0.33
L174	0	0	0	0	0
L195	0	0	0	0	0
Wild-type	0	2.00	5.04	10.0	12.1

*Spores were used as the inoculum. The wild-type culture served as a positive control.

this reagent and analyzed by HPLC to detect alterations in the amino acid chromatographic profile. Mutant cultures grown in MSC medium were examined with and without sodium borohydride treatment. If a keto compound were present, the treated sample should show a new peak in the HPLC chromatogram. Because intermediates in HON biosynthesis should have structures similar to HON, they were expected to have similar HPLC retention times, and indeed HPLC analysis of mutant culture supernatants at high detector sensitivity revealed small amino acid peaks with retention times similar to that of HON. However, none of these peaks was affected by sodium borohydride treatment; therefore, these compounds were presumed not to possess a keto group.

2. Ethanol extraction of mutant cells

The intermediate(s) initially postulated in HON biosynthesis were presumed to be amino compounds. Because no such compounds were detected in the supernatants of mutant cultures, their possible accumulation inside the cells was investigated. Mycelium harvested at 48 h, 84, and 120 h was extracted with ethanol and centrifuged. When supernatant solutions were analyzed by HPLC, no peak with a retention time similar to that of HON was identified. In fact, no peaks different from those given by the wild-type culture supernatant were detected.

D. Cross-feeding between mutants

The biosynthetic relationships between pairs of mutants were investigated by cross-feeding experiments. Pairs of mutants were tested by spreading mixtures of spores on MSC agar medium. The culture was allowed to grow for several days, and an agar plug was then removed and bioassayed. Table 7 shows the results of these crossfeedings. The positive results were confirmed by HPLC of mixed cultures grown in MSC medium (Table 8).

Table 7. Bioassay of agar plugs from mixed cultures.*

Strain	(L016	L086	L138	L159	L174)	(L127)	(L167)	(L021	L141	L156	L195)
L016		-	-	-	-	+	+	-	-	-	-
L086			-	-	-	+	+	-	-	-	-
L138				-	-	+	+	-	-	-	-
L159					-	+	+	-	-	-	-
L174						+	+	-	-	-	-
L127							+	-	-	-	-
L167								-	-	-	-
L021									-	-	-
L141										-	-
L156											-
L195											

*Pairs of mutant strains were grown together on MSC agar medium. + indicated that the plug gave an inhibition zone (i.e. represents cross-feeding); - indicated that the plug gave no inhibition zone (i.e. represents no cross-feeding). Strain(s) in the same bracket gave the same cross-feeding phenotype.

To test for the unlikely possibility (since feeder responses between mutants were quite specific) that an autoregulator similar to A-factor (Khokiov et al., 1967) might be resposible for the restoration of HON production, L127 and L167 were supplemented with wild-type culture filtrates. No increase in HON production occurred.

E. Feeder-converter interactions

These were determined by (1) bioassay of agar plugs cut from patched colonies of nonproducers grown side-by-side in pairwise combinations on MSC agar medium, and (2) pairwise exchanges of mycelium and filtrates from shaken cultures grown in MSC medium for 48 h, followed by HPLC analysis of cultures at 24 h intervals. The results of bioassaying agar plugs are listed in Table 8; the HPLC analyses are in Table 9.

The interactions between pairs of mutants blocked in HON production suggested that mutants L016, L086, L127, L138, L159, L167 and L174 could be arranged in three groups, and these groups specified their sequence in the biosynthetic pathway. Figure 10. shows the biosynthetic sequence. Since the remaining mutants, L021, L141, L156 and L195, exhibited no cosynthesis phenotype when mixed with others, they might represent a group with defective in regulatory elements, but they might also be defective in uptake or excretion of intermediates.

F. Growth requirements of blocked mutants

To determine whether any of these mutants were blocked in a primary pathway, their ability to grow on unsupplemented MSN medium was tested. None of the HON mutants was auxotrophic.

Table 8. Summary of bioassay and HPLC analysis of HON production by pairs of blocked mutants that showed cosynthesis in mixed cultures. + represents cross-feeding. - represents no cross-feeding.

Feeder	Convertor						
	L016	L086	L138	L159	L174	L127	L167
L016		-	-	-	-	+	+
L086	-		-	-	-	+	+
L138	-	-		-	-	+	+
L159	-	-	-		-	+	+
L174	-	-	-	-		+	+
L127	-	-	-	-	-		+
L167	-	-	-	-	-	-	

Table 9. Cosynthesis of HON (mM) by the precursor feeding method.*

Cultures	Hours after exchange		
	0	24	48
L016	0	0.08	0.12
L086	0	0	0
L127	0	0	0.07
L138	0	0	0
L159	0	0	0
L167	0	0	0
L174	0	0	0
L016 and L127 mixed cultures	0.17	1.01	1.76
L016 cells + L127 filtrates	0	0.15	0.25
L127 cells + L016 filtrates	0	1.51	3.05
L086 and L127 mixed cultures	0.23	1.02	1.50
L086 cells + L127 filtrates	0	0	0
L127 cells + L086 filtrates	0	0.14	0.35
L127 and L138 mixed cultures	0.10	0.18	0.70
L127 cells + L138 filtrates	0	0.41	2.83
L138 cells + L127 filtrates	0	0	0
L127 and L159 mixed cultures	0.26	0.34	0.46
L127 cells + L159 filtrates	0	0.78	0.94
L159 cells + L127 filtrates	0	0	0
L127 and L174 mixed cultures	0	0.12	0.18

(Table 9 continued)

L127 cells + L174 filtrates	0	0.37	2.44
L174 cells + L127 filtrates	0	0	0
L016 and L167 mixed cultures	0.24	0.82	1.73
L016 cells + L167 filtrates	0	0	0
L167 cells + L016 filtrates	0	0.27	0.33
L086 and L167 mixed cultures	0.24	0.77	1.87
L086 cells + L167 filtrates	0	0	0
L167 cells + L086 filtrates	0	0.35	0.32
L127 and L167 mixed cultures	0.25	0.32	0.36
L127 cells + L167 filtrates	0	0	0.028
L167 cells + L127 filtrates	0	0.26	0.051
L138 and L167 mixed cultures	0.68	1.03	1.43
L138 cells + L167 filtrates	0	0	0
L167 cells + L138 filtrates	0	0.35	0.53
L159 and L167 mixed cultures	0.15	0.63	1.47
L159 cells + L167 filtrates	0	0	0
L167 cells + L159 filtrates	0	0.24	0.12
L167 and L174 mixed cultures	0.23	0.58	0.78
L167 cells + L174 filtrates	0	0.32	0.45
L174 cells + L167 filtrates	0	0	0

* Culture filtrates of a pair of blocked mutants were exchanged at 48 h; HON production in the cultures was followed by HPLC at 24 h intervals. Mutant cultures treated in the same way except that culture filtrates were returned instead of exchanged served as negative controls. Mixed cultures of each pair of blocked mutants served as positive controls.

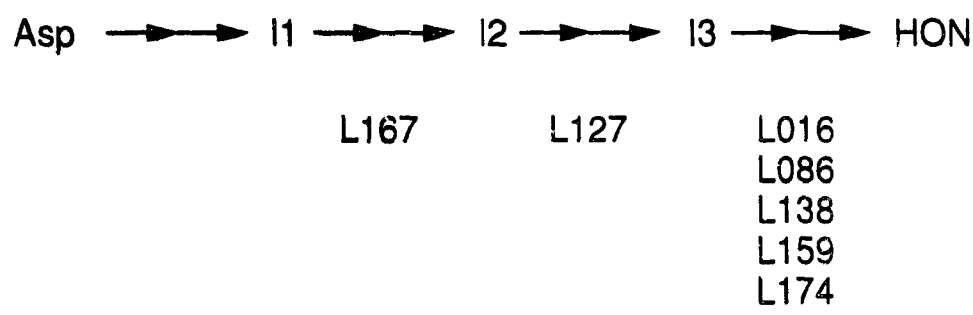


Figure 10. The biosynthetic sequence of three cosynthetic groups: I1, I2 and I3 are postulated intermeidates (I=intermediate).

V. Preliminary studies of mutant L138

Since the culture filtrate of L138 gave the highest HON production for this class of mutant when fed to L127 or L167 mycelium, some preliminary work was done to characterize the stimulatory substance produced. Mutant L127 was used as the converter. Cultures of L138 were grown in MSC medium for 48 h; the filtered broth was then treated in various ways, adjusted to the original pH (6.5) if necessary, sterilized by filtration and used to replace the culture filtrate of L127. The mixed culture was incubated for another 24 h at 27°C; HON production was measured by HPLC.

A. Size of the substance accumulated

Filtration of L138 broth through a membrane passing only molecules less than 10 kDa in size did not alter its ability to stimulate HON production by L127 (Table 10). Therefore, the substance accumulated is relatively small and is unlikely to be an enzyme.

B. Hydrophilicity of the substance accumulated

When the filtrates of L138 broth were extracted with chloroform or ethyl acetate, and the aqueous solution was fed to L127, the ability of the substance to restore HON production was not altered (Table 10), suggesting that the substance is a hydrophilic compound.

C. Stability of the substance accumulated

Heating the culture filtrate to 100°C for 20 min or storing it at -20°C for 24 h did not alter its ability to restore HON production to mutant L127 mycelium (Table 10). Furthermore, adjusting the culture filtrate to pH 1.0 and then incubating at room temperature for 2 h, or at 100°C for 10 min, did not destroy the ability to restore HON production. At

Table 10. Production of HON in converter culture L127 24 h after exchanging its filtrate at 48 h with the 48-h filtrate of L138 cultures treated in various ways.

Treatment	HON production* (mM)
None	1.50
Filtration through a 10 KDa membrane	1.55
Extraction with $CHCl_3$	1.97
Extraction with EtOAc	1.56
Storage at pH 1 / 2h	2.18
Storage at pH 12 / 2 h	2.24
Storage at -20°C / 24 h	1.90
Heated at 100°C / 20 min	1.70
Heated at 100°C / 10 min / pH 1	1.97
Heated at 100°C / 10 min / pH 12	0

* HON production in L127 cultures without exchange of filtrate was 0.28 mM at 48 h and 0.58 mM at 72 h after initial inoculation.

pH 12, this ability was unaffected after 2 h at room temperature but was lost after heating at 100°C for 10 min (Table 10). These results suggested that the substance is relatively stable.

D. Conversion assay to detect small amounts of material

To facilitate purification of the unknown compound accumulated by L138, a bioassay was developed to measure its concentration. This used L127 to convert the intermediate to HON, and then measured HON production. The L138 product was fed to a 10 mm agar plug cut from an L127 plate culture by attaching to the top of the plug a short length of drinking straw able to hold about 200 ul of liquid. In optimization tests, plugs from two to three days surface growth of the converter gave the largest response when fed 120 ul of 48-h L138 culture filtrate and incubated at 27°C for 24 h, before transfer to a bioassay plate. Differences in the dryness of the bioassay plates strongly influenced the diffusion of HON, and thus the size of the inhibition zone. When the agar concentration in the bioassay plate was decreased from 1.5% to 1.2%, the method was reproducible and the size of the inhibition zone increased from 1.5 to 2.0 cm in diameter.

VI. Mutants blocked in the biosynthesis of aspartate family amino acids

Since none of the mutants blocked in HON biosynthesis was auxotrophic, they are presumed to have lesions in the HON-specific part of the biosynthetic pathway, after it branches from primary metabolism. To investigate the relationship between the two pathways, aspartate pathway auxotrophs were isolated, and their HON production was examined.

A. Isolation of auxotrophs

Spores of S. akiyoshiensis treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG;

2mg/ml) for 2 to 3 h gave a survival rate of less than 2%. The spores were plated on MYM agar supplemented with diaminopimelate. When the colonies generated from NTG-treated spores were well sporulated, the agar plates were replicated to MM agar or MM agar supplemented with amino acids to examine their phenotypes. Those colonies requiring an amino acid supplement in MM medium were considered potential auxotrophs.

In the first round of screening, a total of 65,262 colonies from NTG-treated spores yielded 783 (1.20%) auxotrophs. Of these 416 were blocked in pathways other than the biosynthesis of aspartate family amino acids. The remaining 367 were able to grow to various extents on MM medium supplemented with a mixture of threonine, methionine, lysine, isoleucine and diaminopimelate. Further testing of these 367 strains gave five that required only lysine and 99 that required only methionine for growth. Six of them required more than one amino acid for growth. The rest were not further characterized because they were very leaky.

B. Characterization of auxotrophs

The growth requirement, putative mutation site and reversion rate of auxotrophs discussed in this section are shown in Table 11.

1. Lysine-requiring mutants LC175Z and LC461A

The five lysine-requiring mutants were able to grow on lysine but not on a mixture of LL- and meso-diaminopimelate; this suggests that they are blocked in lysA, the gene for diaminopimelate decarboxylase.

Bioassays of cultures grown on MSC agar medium showed that all these mutants were able to produce HON. The production of HON by two of them (LC175Z and LC461A) in MSC medium was measured by HPLC (Table 12). The ability of these mutants to produce

near-wild-type amounts of HON indicates that the pathway to HON biosynthesis branches before the final step to lysine.

2. Methionine or threonine-requiring mutant LC296Y

Mutant LC296Y was able to grow on either methionine or threonine; a plausible explanation is that this is a leaky mutant altered in either a regulatory or structural gene for homoserine dehydrogenase. The mutant cannot make enough homoserine to supply its needs on minimal medium. However, if methionine or threonine is provided in the medium, the mutant needs only to synthesize enough homoserine to make the other amino acid. To determine whether LC296Y was indeed a hsd mutant, the enzyme activities of homoserine dehydrogenase (Table 13) and aspartate kinase (Table 14) for the mutant, revertant (LC296R) and wild-type strain were compared. Mutant LC296Y contained a considerably lower level of homoserine dehydrogenase than the revertant or the wild-type strain, supporting the hypothesis that the mutation was in hsd. Moreover, LC296Y contained a level of aspartate kinase similar to that in the wild-type strain; thus the mutation was not at ask.

The level of HON production (in MSC medium) assayed by HPLC (Table 12) indicated that the hsd mutation did not block the biosynthesis of HON, implying that the branch-point occurs before homoserine dehydrogenase. As shown in Table 12, LC296Y produced more HON than the wild-type S. akiyoshiensis and its revertant, LC296R. Because the increase in HON production could be due to an accumulation of intermediate(s) in the early part of the aspartate pathway (before homoserine dehydrogenase), HON production (Table 15, 16) and growth (Table 17, 18) by the mutant, revertant and wildtype strains were investigated. Cultures were grown in MSN and MSC medium using a washed TSB C culture as the inoculum.

Mutant LC296Y was able to make more HON than the revertant and wild-type strain

Table 11. Growth requirement, putative mutation site and reversion rate of some auxotrophs.

Strain	Growth requirement	Putative mutation	Reversion rate ($X10^{-6}$)
LC175Z	Lys	lysA	5.1
LC461A	Lys	lysA	0.36
LC296Y	Met or Thr	hsd	82
LC224X	Met+Thr	n.d.	n.d.
LC263Z	Lys+Met or Met+Thr	n.d.	2.7
LC153C	Lys+Met+Thr	n.d.	n.d.
LC242B	Lys+Met+Thr	n.d.	n.d.
LC367Y	Lys+Met+Thr	ask	2.7

* n.d., not determined.

Table 12. Concentration of HON (mM) in cultures of auxotrophic mutants and revertants during growth in MSC medium.

	Culture age				
Strain	48 h	72 h	96 h	120 h	144 h
WT	5.25	8.78	9.94	12.9	12.8
LC175Z	0.34	1.28	6.21	9.17	10.8
LC461A	0.54	1.37	6.94	9.58	12.0
LC296Y	0	0	3.24	14.2	16.3
LC296R	0	0.21	3.46	9.25	11.9
LC224X	0	0.17	0.84	0.93	1.04
LC153C	0	0.18	0.67	4.25	8.74
LC263Z	0	0.062	0.35	0.47	0.63
LC263R	0	0	0.19	0.42	0.69
LC242B	0	0	0	0.12	0.36
LC367Y	0	0.12	0.59	1.22	2.01
LC367R	0	0.15	0.67	1.38	2.69

*Spore inoculum was used. The wildtype served as a positive control. The data listed are the averages from three measurements.

Table 13. Comparison of specific homoserine dehydrogenase activity (U/mg protein) in partially purified enzyme preparations (0-65% saturated ammonium sulfate fractions) of auxotrophic mutants and revertants.

	Culture age		
Strain	24 h	48 h	72 h
WT	0.046	0.043	0.041
LC296R	0.045	0.054	0.027
LC296Y	0.016	0.008	0.002
LC224X	0.044	0.055	0.038
LC153C	0.035	0.055	0.023
LC263R	0.054	0.052	0.043
LC263Z	0.044	0.057	0.068
LC242B	0.045	0.051	0.030
LC367R	0.056	0.059	0.043
LC367Y	0.053	0.040	0.039

*Washed mycelium grown in TSB C medium was used to inoculate MME medium containing starch and asparagine as carbon and nitrogen sources, respectively. The culture medium was supplemented to 0.3 mM each with lysine, methionine and threonine. Cultures were harvested at 24, 48 and 72 h; maximum growth (OD_{640}) occurred at 72 h (data not shown). One unit of enzyme activity is defined as the quantity of enzyme required to produce an absorbance change of 1.00 per minute at 340 nm in a reverse direction assay. Protein concentrations was determined using the Bio-Rad Protein Assay Dye Reagent. The enzyme activity shown is the average of three assays. The wild-type strain served as a positive control.

Table 14. Comparison of specific aspartate kinase activity (U/mg protein) in partially purified enzyme preparations (0-65% saturated ammonium sulfate fractions) of auxotrophic mutants and revertants.

	Culture age		
Strain	24 h	48 h	72 h
WT	3.40	3.71	2.68
LC296R	3.53	2.73	1.95
LC296Y	5.27	4.13	1.80
LC224X	6.65	6.74	9.05
LC153C	2.89	3.73	2.34
LC263R	3.27	3.96	2.63
LC263Z	2.98	9.38	4.36
LC242B	1.44	2.85	2.83
LC367R	1.24	2.47	2.59
LC367Y	1.26	2.59	1.94

*Washed mycelium grown in TSB C medium was used to inoculate MME medium containing starch and asparagine as carbon and nitrogen sources, respectively. The culture medium was supplemented to 0.3 mM each with lysine, methionine and threonine. Cultures were harvested at 24, 48 and 72 h; maximum growth (OD_{640}) occurred at 72 h (data not shown). One unit of enzyme activity is defined as the quantity of enzyme required to produce an absorbance increase of 0.001 per minute at 540 nm. Protein concentrations were determined using the Bio-Rad Protein Assay Dye Reagert. The enzyme activity shown is the average of three assays. The wild-type strain served as a positive control.

in both minimal and complex media; in fact, in minimal medium, the revertant LC296R produced very little HON (Table 15). A plausible explanation is that LC296Y carries more than one mutation. The decreased HON production in minimal medium caused by one of the mutations was compensated by the second (hsd) mutation, which elevated HON production. When the hsd mutation was restored in revertants, this compensation was abolished and HON production dramatically decreased. Since there was no significant difference in growth (as cell dry weight yield) between mutant LC296Y and revertant LC296R, it was reasonable to believe that the elevated HON production by LC296Y was not influenced by an accumulation of biomass.

3. Methionine and threonine-requiring mutant LC224X

Mutant LC224X grew most rapidly on MM medium supplemented with methionine and threonine, but growth could be detected, usually after a 24-h delay, on MM medium alone or on MM medium supplemented with any single amino acid in the aspartate family. The mutant phenotype is similar, though not identical to that of LC296Y. Growth and the production of pigments was distinctly better on MM medium supplemented with both methionine and threonine, suggesting that the requirement for these two amino acids was limiting. The results from assaying homoserine dehydrogenase (Table 13) showed that LC224X contained wild-type levels of this enzyme and is thus not an hsd mutant. The large increase in aspartate kinase specific activity (Table 14) suggested that LC224X is either an ask-regulatory mutant or a mutant blocked in an aspartate pathway step that lowers the level of end product(s) and derepresses synthesis of aspartate kinase. As diaminopimelate was not required for its growth, this mutant is unlikely to be an asd mutant. Since this was a very leaky mutant, it was impossible to isolate revertants.

In comparison with the wildtype culture, LC224X produced substantially less HON

in MSC medium (Table 12). Whether this was a result of a second mutation blocked in the HON-specific pathway was not determined.

4. Lysine, methionine and threonine-requiring mutant LC153

Mutant LC153C grew most rapidly on MM medium supplemented with a mixture of lysine, methionine and threonine. Its aspartate kinase activity (Table 14) and homoserine dehydrogenase activity (Table 13) were similar to those in the wild-type and thus did not give any indication that production of these enzymes was impaired. Since LC153C did not require diaminopimelate for growth, it was assumed not to be an asd mutant. The exact location of the mutation has yet to be identified. As LC153C was very leaky, it was not possible to isolate revertants. HON production (Table 12) by this mutant in MSC medium was not severely altered.

5. Lysine, and methionine or methionine and threonine-requiring mutant LC263Z

Mutant LC263Z grew equally well on minimal medium supplemented with combinations of lysine and methionine, or methionine and threonine. No growth was observed on minimal media containing single amino acids. Enzyme assays showed that LC263Z contained more than twice the aspartate kinase activity of the wild-type and revertant (LC263R) at 48 h; thus its ability to produce aspartate kinase was not damaged. Whether this is an ask-regulatory mutant or a mutant blocked in an aspartate pathway step other than ask was not investigated. The similar levels of homoserine dehydrogenase activity produced by LC263R, LC263Z and the wildtype strain suggested the mutation was not in hsd. Since LC263Z did not rely on diaminopimelate for growth, it does not have the phenotype of an asd mutant. In comparison with wild-type cultures, HON production by this mutant was dramatically decreased. The revertant LC263R produced no more HON than the mutant (LC263Z), thus HON production was not restored by the reversion. Whether the lower level

Table 15. Concentration of HON (mM) in wild-type, LC296R and LC296Y cultures grown in MSN medium.*

Culture age (h)	WT	296R	296Y
0	0	0	0
24	0	0	0.08
48	0	0	0.15
72	0	0	0.11
96	0.07	0	0.12
120	0.61	0	0.14
144	1.10	0	1.67
168	1.58	0	1.52
216	0.69	0.53	3.12

*Washed mycelium grown in TSB C medium was used as inoculum. The data listed are the averages of three measurements.

Table 16. Concentration of HON (mM) in wild-type, LC296R and LC296Y cultures grown in MSC medium.*

Culture age (h)	WT	296R	296Y
0	0	0	0
24	3.21	2.07	2.58
48	5.70	5.45	7.50
72	9.10	7.95	11.5
96	10.2	9.52	14.6
120	14.3	11.8	16.3
144	12.9	12.7	15.4

*Washed mycelium grown in TSB C medium was used as inoculum. The data listed are the averages of three measurements.

Table 17. Dry weight (mg/ml culture) of wild-type, LC296R and LC296Y cultures grown in MSN medium.*

Culture age (h)	WT	296R	296Y
0	0	0	0
24	2.02	1.96	1.74
48	3.86	3.51	3.42
72	4.94	4.90	4.52
96	5.12	5.11	5.04
120	4.88	4.91	4.31
144	4.91	5.17	3.64
168	4.49	3.93	3.79
216	4.09	4.15	3.70

*Washed mycelium grown in TSB C medium was used as inoculum. The data listed are the averages of three measurements.

Table 18. Dry weight (mg/ml culture) of wild-type, LC296R and LC296Y cultures grown in MSC medium.*

Culture age (h)	WT	296R	296Y
0	0	0	0
24	3.17	2.53	2.11
48	4.10	3.86	3.86
72	4.37	4.33	4.21
96	4.32	4.35	4.43
120	4.65	5.07	4.47
144	4.83	4.45	4.23

*Washed mycelium grown in TSB C medium was used as inoculum. The data listed are the averages of three measurements.

of HON production was due to an independent mutation that blocked the HON-specific pathway, or whether the restoration of the prototrophic phenotype was due to a suppressor mutation was not examined further.

6. Lysine, methionine and threonine requiring mutant LC242B

A mixture of lysine, methionine and threonine is required to achieve fast growth of mutant LC242B. Enzyme assays (Table 14) indicated this mutant produced slightly less aspartate kinase than the wild-type strain. However, it is unsafe to conclude from this that LC242B is an ask mutant. This might be the result of a difference in growth pattern affecting the synthesis of aspartate kinase. The specific activity of aspartate kinase in LC242B increased more slowly than the wild type. Since LC242B and the wild type produced similar levels of homoserine dehydrogenase (Table 13), and the mutant did not depend on diaminopimelate for growth, LC242B was unlikely to be an asd or hsd mutant. No revertant of this mutant was isolated. HON production (Table 12) in MSC medium was appreciably lower than in the wild type.

7. Lysine, methionine and threonine-requiring mutant LC367Y

Mutant LC367Y required lysine, methionine and threonine to achieve fast growth. On MM medium containing only lysine and methionine, growth was observed one day later than on a medium containing all three amino acids; after another day, growth was observed on MM medium containing only a methionine supplement. No growth was observed on a medium containing lysine or threonine alone, or a combination of these two amino acids. Since under similar conditions, growth of wild type S. akiyoshiensis was inhibited by threonine or a combination of threonine and lysine (data not shown), no conclusion can be drawn from the results with threonine or threonine plus lysine. The homoserine dehydrogenase (Table 13) of LC367R and LC367Y were similar to that of the wildtype strain, and since the mutant did not

require diaminopimelate to grow, the mutation is unlikely to be in hsd or asd. Compared with the wild-type, mutant LC367Y gave relatively low aspartate kinase activity (Table 14, 63% lower at 24 h, and approximately 30% lower at 48 h and 72 h) implicating a mutation in ask. Enzyme assays indicated that the wild-type ability to synthesize aspartate kinase was not restored in LC367R. HON production (Table 12) by LC367R and LC367Y in MSC medium was appreciably lower than in wild-type cultures.

8. Conclusion

The overall results suggested that the biosynthesis of HON is not related to steps after diaminopimelate decarboxylase in the lysine branch, or after homoserine dehydrogenase in the threonine-methionine branch of the aspartate pathway. The results also supported in some ways (and do not contradict) the argument that intermediate(s) in the early part of the aspartate pathway provide precursor(s) for HON. Since chemical mutagenesis often did not give completely blocked auxotrophs, it seemed desirable to isolate a site-specific mutant which might give more decisive information on the branch point leading to the HON pathway.

VII. Molecular cloning of asd from S. akiyoshiensis

A. Shot-gun cloning in E. coli

Since chemical mutagenesis did not yield an auxotroph that could be recognized unabiguously as an asd mutant, cloning of the S. akiyoshiensis asd gene was undertaken to provide information about the biosynthesis and genetics of the aspartate pathway. Access to asd was also expected to facilitate the construction oi an asd-specific mutant. The cloning strategy chosen involved introducing a library of S. akiyoshiensis genomic DNA fragments into an E. coli asd mutant and selection for prototrophic transformants. The bifunctional shuttle plasmid pHJL400 (Larson and Hershbeger, 1986), which contained antibiotic

resistance markers allowing selection in both E. coli and S. akiyoshiensis, was used as the cloning vector (Fig. 11). The strategy is summarized in Fig. 12.

Streptomyces akiyoshiensis genomic DNA was partially digested with Sau3AI and size-fractionated by centrifugation in a sucrose density gradient. The DNA fragments determined by agarose gel electrophoresis to be 6 to 15 kb in size were used to make a genomic library. The vector, pHJL400, was linearized with BamHI and treated with alkaline phosphatase to prevent self ligation. The genomic DNA was then ligated to the phosphatase-treated pHJL400 using an insert:vector ratio of 5:1. By α-complementation testing, 60 - 99% of the ligated plasmid mixture contained inserts. The plasmids were introduced into competent cells of the E. coli asd mutant strain CGSC 6212. Transformants were allowed to grow overnight on L-broth agar containing 100 μg/ml ampicillin and 0.3 mM diaminopimelate, the colonies that grew were replicated on L-broth agar containing only ampicillin. After approximately 39,000 transformants had been screened in this manner, one prototrophic colony was isolated.

A 17.1-kb recombinant plasmid, pJV21, was isolated from the original transformant grown without selection for the Asd^+ phenotype. Therefore, it is expected that the insert represented a true fragment of chromosomal DNA. When purified pJV21 was used to transform the E. coli asd mutants CGSC 5080 and CGSC 6212, all ampicillin-resistant transformants were prototrophs, indicating that the Asd^+ phenotype was associated with the plasmid.

B. Subcloning of the asd-containing segment

To locate the asd gene on the 11.3-kb insert in pJV21, the plasmid was partially digested with SalI and the fragments were subcloned in pHJL400. The subcloning strategy

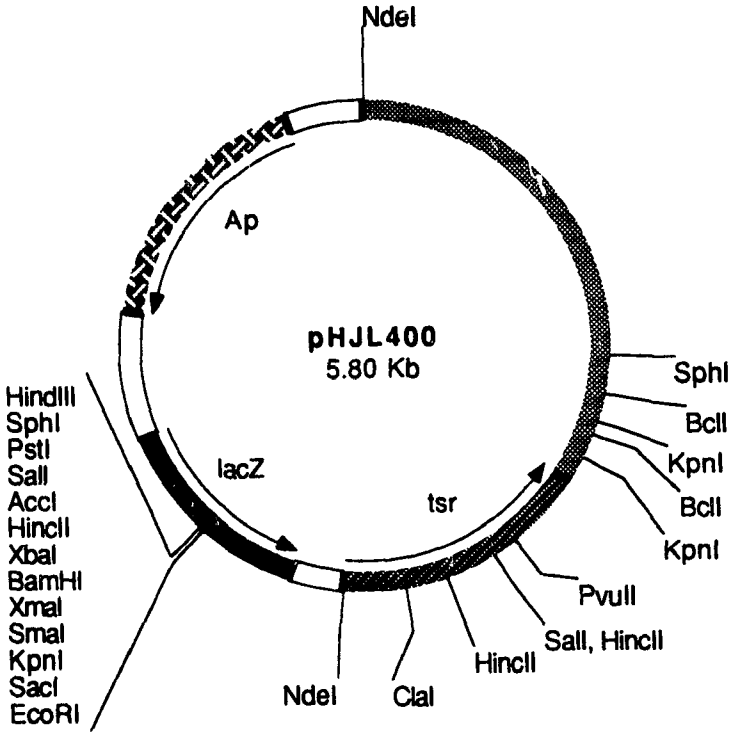


Figure 11. Circular restriction map of pHJL400.

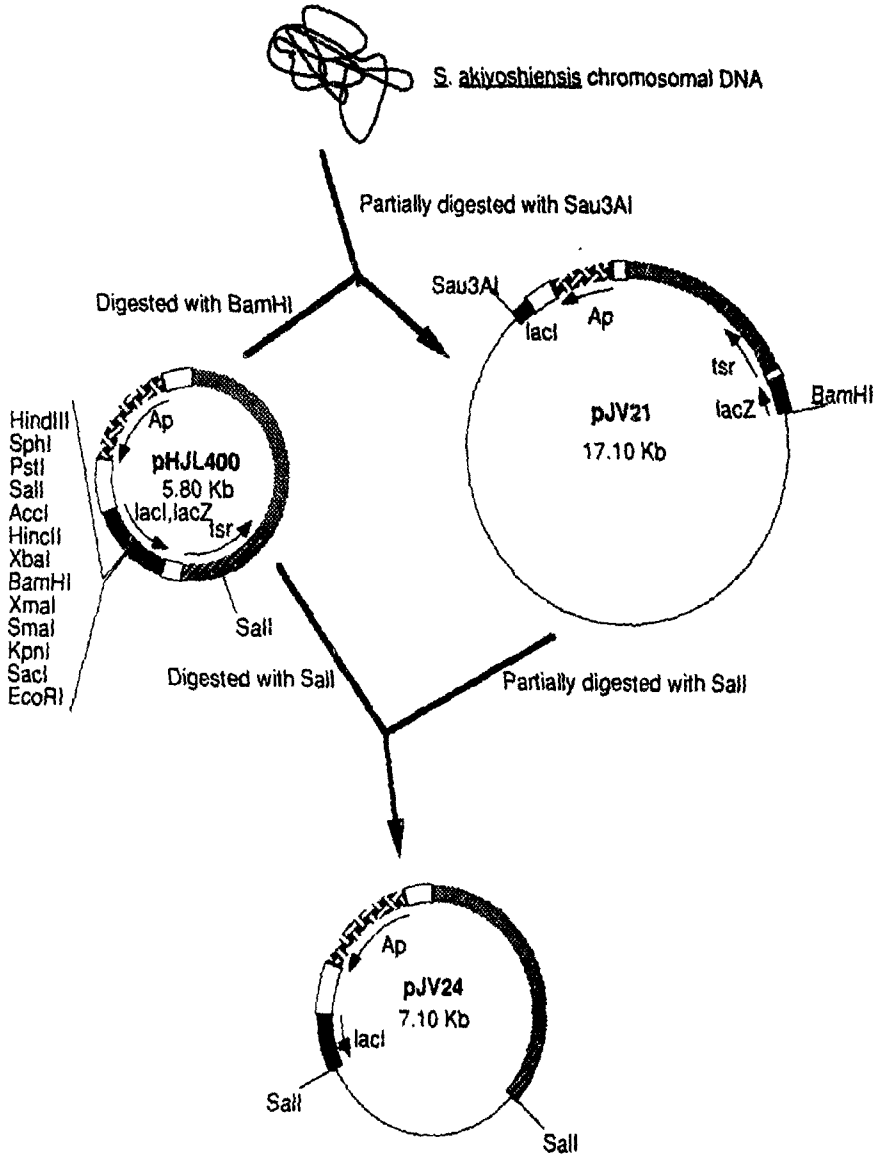


Figure 12. Cloning and subcloning of a S. akiyoshiensis DNA fragment containing asd. For construction of pJV21, the genomic DNA was partially digested with Sau3AI, size-fractionated, and ligated to the BamHI site of pHJL400. For construction of pJV24, the DNA fragments generated by SalI partial digestion of pJV21 were ligated to the SalI sites of pHJL400, created in the vector by removing a SalI-SalI fragment during the construction. The thick line represents vector DNA. The thin line represents S. akiyoshiensis DNA. Most restriction enzyme sites outside the polylinker region are not shown (For reference, see previous Figure).

is shown in Fig. 12.

The partial SalI digest of pJV21 was ligated to the SalI-digested pHJL400, in which a SalI-SalI fragment was removed. The ligation mixture was used to transform competent cells of the E. coli asd mutant CGSC 6212. Transformants were directly selected for the Asd^+ phenotype on L-broth agar supplemented with ampicillin. When Asd^+ colonies were examined for the presence of plasmids, the smallest plasmid found, pJV24, contained an insert of 2.2 kb. The ability of this plasmid to complement the asd mutation was confirmed by using it to transform the E. coli asd mutant CGSC 6212.

Attempts to reduce the size of the fragment in pJV24 while retaining the asd gene were unsuccessful. Complete digestion of pJV24 with SalI generated two SalI-SalI fragments (0.75 kb and 1.4 kb) from the insert. When these fragments were individually ligated to SalI-digested pHJL400, and the ligation mixtures were used to transform the E. coli asd mutant CGSC 6212, no prototroph was isolated. It was tentatively concluded that the 2.2-kb insert in pJV24 was the smallest DNA fragment able to confer the Asd^+ phenotype.

C. pJV21 and pJV24

Plasmid pJV21 and pJV24 were digested with various restriction enzymes singly or in combination. The sizes of the DNA fragments were determined by agarose gel eletrophoresis and comparison with standards prepared by digesting lambda DNA with HindIII or PstI. The restriction fragments obtained indicated that the inserts in both plasmids lacked sites for BglII, ClaI, HindIII, KpnI, NdeI, PstI and XhoI. The sizes of DNA fragments generated by digesting pJV21 and pJV24 with other restriction endonucleases are listed in Table 19 and Table 20, respectively. The insert sizes for pJV21 and pJV24 were confirmed to be 11.3 and 2.2 kb, respectively. Restriction maps of both plasmids are shown in Fig. 13.

Table 19. Fragments generated by digesting pJV21 with restriction enzymes.

Restriction enzyme	Size of fragments (kb)
EcoRI	10.1, 7.0
BamHI	8.8, 7.3, 0.55, 0.45
SstI	8.1, 8.1, 0.95
Hind'II + EcoRI	10.1, 5.8, 1.2
EcoRI + BamHI	8.8, 7.0, 0.55, 0.45, 0.30
EcoRI + SstI	8.1, 7.0, 1.1, 0.95
BamHI + SstI	7.3, 7.1, 0.95, 0.75, 0.55, 0.45

Table 20. Fragments generated by digesting pJV24 with restriction enzymes.

Restriction enzyme	Size of fragments (kb)
SalI	4.8, 1.4, 0.75
BamHI + SstI	5.2, 0.95, 0.80
BamHI + SalI	4.8, 1.4, 0.55, 0.20
SalI + SstI	4.8, 0.75, 0.95, 0.25, 0.20

Figure 13. Restriction map of S. akiyoshiensis DNA inserts in pJV21 and pJV24. Not all of the SalI sites are included in the map of pJV21. The shaded boxes are vector sequences. Horizontal thick lines represent S. akiyosheinsis DNA sequences.

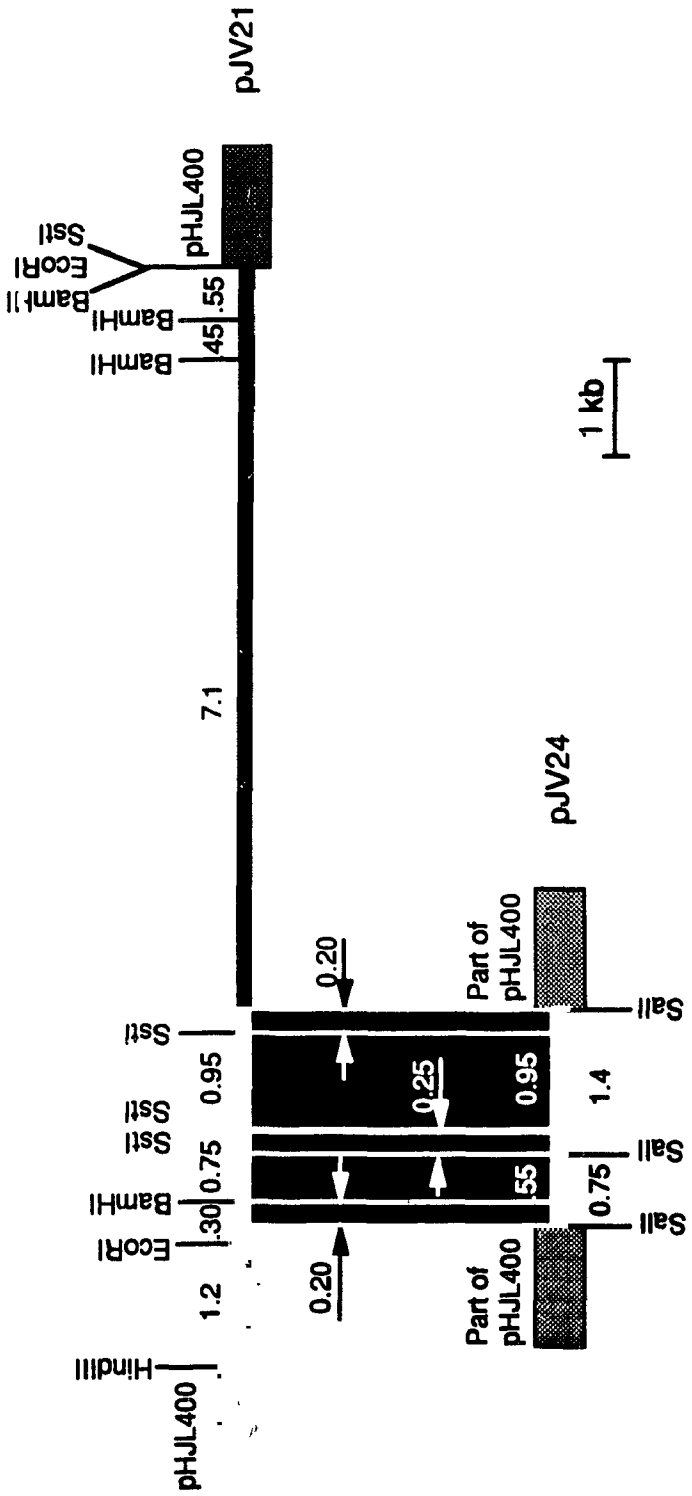

pJV21
pHJL400
SstI
EcoRI
BamHI
55
45
BamHI
BamHI
1 kb
7.1
pJV24
Part of pHJL400
SalI
0.20
SstI
0.95
0.25
0.95
1.4
SstI
SstI
0.75
55
SalI
BamHI
30
0.75
SalI
EcoRI
1.2
0.20
Part of pHJL400
HindIII
pHJL400

Figure 13.

However, not all of the SalI sites detected in pJV21 are identified on the map. The relative positions of two small BamHI fragments (0.55, 0.45 kb) in pJV21 could not be established from a partial digestion with BamHI. They were determined by using the NdeI site, approximately 0.3 kb distant from the BamHI site in the vector polylinker sequence, as a point of reference. The DNA fragments were generated by complete NdeI digestion and partial BamHI digestion of pJV21. The presence of two SstI sites close together in the pJV21 and pJV24 inserts was recognized during subsequent sequencing of the 2.2-kb insert in pJV24.

D. Southern hybridization of genomic DNA with a cloned fragment

To confirm that the asd-containing insert in pJV21 and pJV24 had originated from S. akiyoshiensis, genomic DNA fragments were probed with the 0.75-kb SalI-SalI insert of pJV24. Genomic DNA of S. akiyoshiensis was digested with BamHI or PstI, fractionated by gel electrophoresis and transferred to a nylon membrane. When the membrane was probed with the 0.75-kb SalI fragment and washed at high stringency (0.1X SSC at 65°C), hybridization was observed to a 14.0-kb BamHI fragment and an 11.5-kb PstI fragment (Fig. 14). This indicated that the cloned asd-containing insert was indeed from S. akiyoshiensis.

VIII. Expression of S. akiyoshiensis asd in E. coli

A. Efficiency of expression in E. coli

When pJV21 was used to transform E. coli asd mutants, CGSC 5080 and CGSC 6212, fewer ampicillin-resistant colonies were obtained on ampicillin-LB medium than on ampicillin-LB medium supplemented with diaminopimelate (approximately 30 % for CGSC 5080 and 40% for CGSC 6212 from the same number of transformed cells). Since the efficiency of expression was lower in CGSC 5080, this strain was examined further. Adding IPTG (32

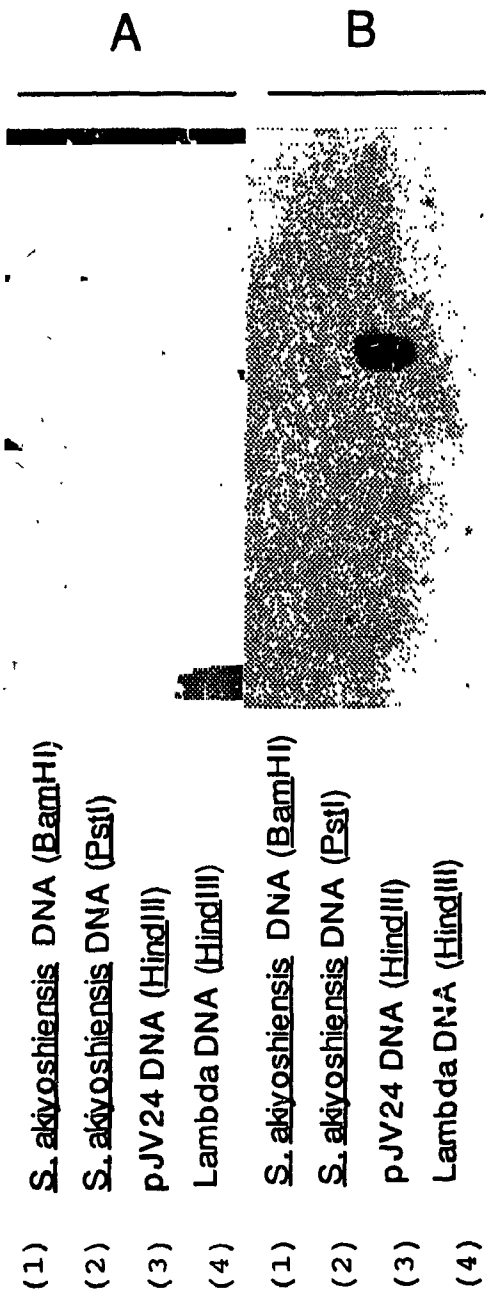


Figure 14. Southern hybridization of S. akiyoshiensis genomic DNA with the asd-containing fragment of pJV24. A: agarose gel electrophoresis of restriction enzyme digests of S. akiyoshiensis genomic DNA and pJV24. B: Southern hybridization of the DNA transferred to a nylon membrane from A. The blot was probed with the Dig-labeled 0.75-kb SalI-SalI fragment from pJV24 and then washed at high stringency (0.1XSSC at 65°C). The samples were: (1) BamHI-digested genomic DNA; (2) PstI-digested genomic DNA; (3) HindIII-digested pJV24 and (4) HindIII-digested Lambda DNA.

μg/ml) to ampicillin-LB medium, increased the number of ampicillin-resistant colonies obtained when CGSC 5080 was transformed with pJV21 or pJV24. However, transformation with pJV24 was more efficient than with pJV21 (Table 21). Furthermore, it took one day longer for the pJV21 transformants to form visible colonies on ampicillin-LB agar than on ampicillin-LB medium supplemented with diaminopimelate.

The results suggested that (1) expression of the S. akiyoshiensis asd gene does not fully complement the mutation in E. coli; (2) IPTG, by inducing the lac promoter and elevating expression of the downstream gene, increased the efficiency of expression; (3) expression of asd was weaker from pJV21 than from pJV24. If the S. akiyoshiensis asd had been expressed from its own promoter, the expression efficiency in the two plasmids should not have been substantially different. Its weaker expression in pJV21 than in pJV24 may be related to the greater distance (1.3 kb) from the lac promoter on the vector to the asd gene in the pJV21 insert. Overall, the results suggested that the lac promoter either increased, or was necessary for, expression of the S. akiyoshiensis asd gene in pJV21 and pJV24.

B. Expression from the lac promoter

To ascertain whether asd from S. akiyoshiensis was indeed expressed from the lac promoter, the pJV21 and pJV24 inserts were excised with HindIII and KpnI and recloned in pTZ18R and pTZ19R. These vectors differ only in having multiple cloning sites oppositely oriented in front of the the lac gene (Fig. 15). After purification, the inserts were ligated to HindIII and KpnI sites generated by digesting pTZ18R and pTZ19R with these enzymes. When the recombinant plasmids (pJV30: pTZ18R ligated with the pJV21 insert; pJV31: pTZ18R ligated with the pJV24 insert; pJV32: pTZ19R ligated with the pJV21 insert; pJV33: pTZ19R ligated with the pJV24 insert) were used to transform the E. coli asd mutant

Table 21. Number of colonies on ampicillin-LB medium alone and supplemented with IPTG (32 μg/ml) or Dap (0.1 mM).*

	Plasmid	
Supplement	PJV21	PJV24
Nil	$1.5X10^4$ (30%)*	$8.0X10^4$ (37%)
IPTG	$2.3X10^4$ (46%)*	$1.9X10^5$ (79%)
Dap	$5.0X10^4$	$2.4X10^5$

*Colonies were visible on day 2. The number in brackets is the percentage of colonies related to the number on the medium with Dap. For transformation of CGSC 5080, 0.1 ug of ccc DNA from pJV21 or pJV24 was used.

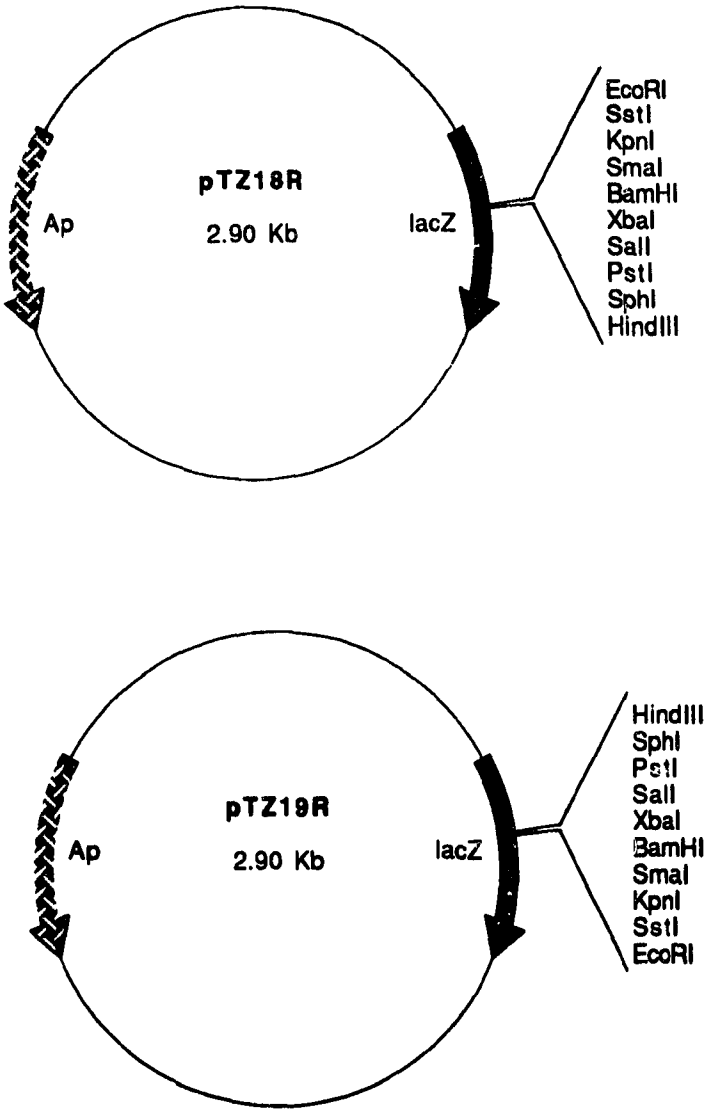


Figure 15. Circular restriction map of pTZ18R and pTZ19R.

CGSC 6212, none of the transformants containing pJV30 and pJV31 grew on ampicillin-LB agar, but transformants containing pJV32 and pJV33 were able to grow.

Since the asd-containing fragments cloned in pJV32 and pJV33 bear the same orientation with respect to the lac promoter as in pJV21 and pJV24 (and thus the asd cloned in pJV30 and pJV31 has the opposite orientation), the results are consistent with expression of the S. akiyoshiensis asd gene in both pJV21 and pJV24 from the lac promoter of the vector.

IX. Attempted complementation of ask in E. coli

Analysis of cloned DNA containing asd genes from Mycobacterium smegmatis (Cirillo et al., 1994), Corynebacterium flavum and Corynebacterium glutamicum (Follettie et al., 1993; Kalinowski, et al., 1990) has shown that asd is clustered with ask or an ask-like sequence. Since these organisms, like S. akiyoshiensis, are high-G+C% Gram-positive bacteria, the pJV21 insert was examined for the presence of an aspartate kinase gene. Both pJV21 and pJV24 were used to transform E. coli CGSC 5074 carrying mutations for all three aspartate kinase genes present in that organism (Theze et al., 1974). The transformants were grown on ampicillin-LB agar and replicated on M9 minimal medium without lysine, methionine and threonine but with other required supplements. None was able to grow, suggesting that either no ask gene was present, or that, if present, it was not expressed.

X. Sequence of the DNA insert in pJV24

A. Recombinant plasmid for sequencing

1. Cloning of the pJV24 insert into pBluescript II SK

The 0.75- and 1.4- kb SalI portions of the insert in pJV24 were subcloned in the SalI

sites of the phagemid vector pBluescript SK(+) (Fig. 16) to generate pJV25 and pJV27, respectively. Since the pBluescript SK (+) recombinant phagemids provided only one of the DNA strands for sequencing, the pJV24 insert was cloned in pBluescript SK (-) in the same orientation to provide the complementary DNA strand. This was done by excising the inserts in pJV25 and pJV27 by double digestion with HindIII and KpnI, using sites that flanked the S. akiyoshiensis DNA, and ligating them to pBluescript SK (-) digested with these enzymes. These constructions generated pJV26 and pJV28, respectively. The 0.75-kb BamHI-SstI DNA fragment in pJV24, which overlapped the two SalI fragments, was also subcloned into the corresponding sites of pBluescript SK (+) to generate pJV29. Table 22 list the sources of inserts cloned into sequencing vectors. Nested sets of deletions were generated from pJV25, pJV26, pJV27 and pJV28.

2. Nested deletions of the inserts

Nested deletions were obtained by the DNase I method (Sambrook et al., 1989). The sequencing plasmids were digested with pancreatic DNase I to create nicks. To generate deletions in the inserts cloned into pBluescript SK(+), the DNase I-treated DNA was digested with XhoI. To generate deletions in the inserts cloned into pBluescript SK (-), the DNase I treated DNA was digested with BamHI. The 5'-protruding DNA ends were filled in with dNTPs using the Klenow fragment of E. coli DNA polymerase I to form blunt ends on plasmids, which were then recircularized. The ligation mixture was used to transform competent E. coli DH5α cells. Plasmids isolated from transformants on ampicillin-LB agar were screened; those of the desired sizes were chosen for DNA sequencing.

3. DNA sequencing

Single strand DNA templates were generated by infecting the strains containing the sequencing plasmids with helper phage VCMS13. In the sequencing reaction, T3 and T7

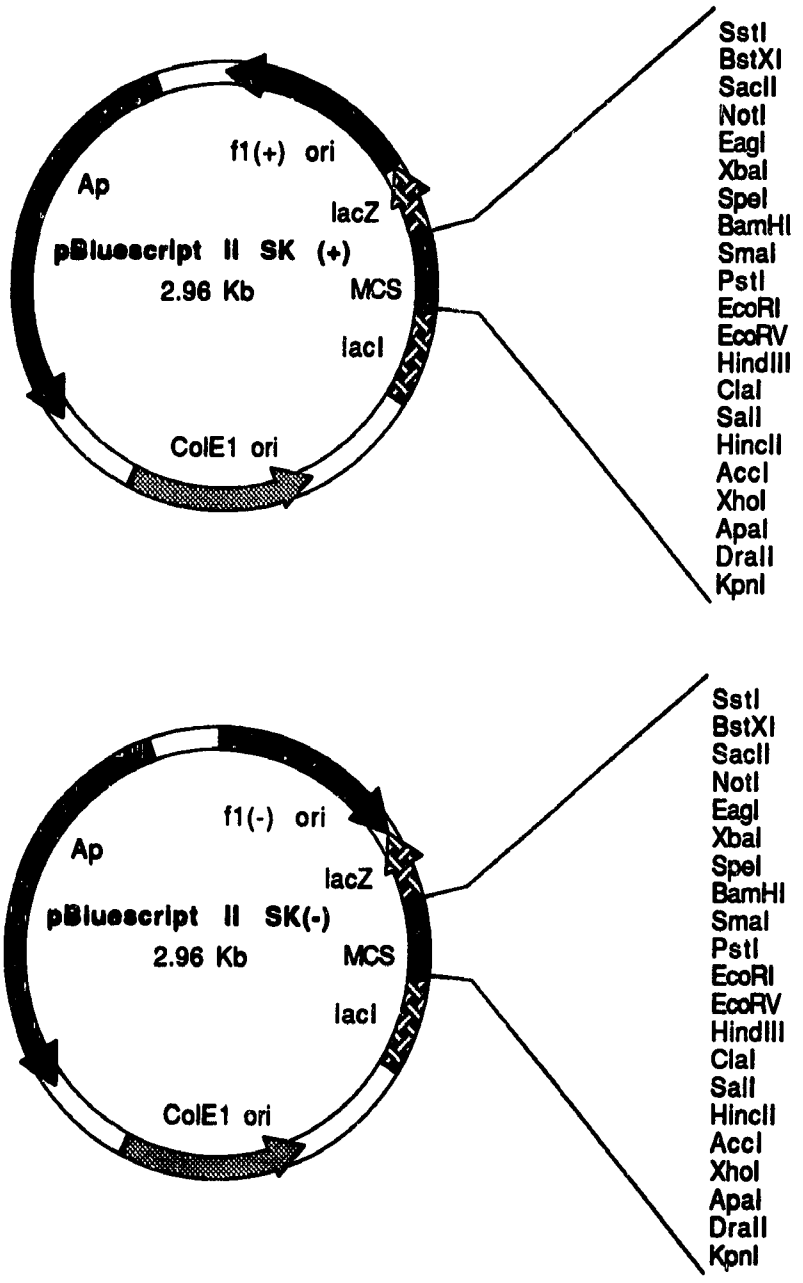


Figure 16. Circular restriction map of pBluescript SK (+) and SK (-).

Table 22. Plasmids used for sequencing.

Plasmid	Vector	Inserts derived from pJV24
pJV25	pBluescript SK(+)	0.75-kb SalI fragment
pJV26	pBluescript SK(-)	0.75-kb SalI fragment
pJV27	pBluescript SK(+)	1.4-kb SalI fragment
pJV28	pBluescript SK(-)	1.4-kb SalI fragment
pJV29	pBluescript SK(-)	0.75-kb BamHI-SstI fragment

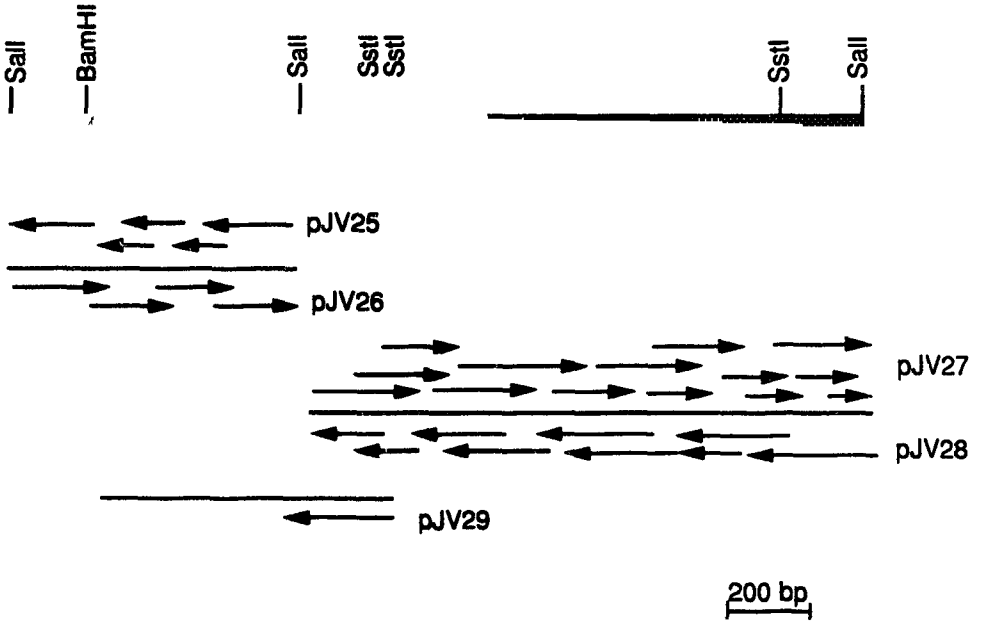


Figure 17 Overall strategy used to sequence the 2.2-kb insert of S. akiyoshiensis DNA in pJV24. The thick line represents the DNA insert. The thin lines represent the inserts in recombinant sequencing phagemids. Arrows represent the length of DNA sequence obtained from individual deletion clones.

primers were used for the DNA polymerase to generate templates from pBluescript SK(-) and SK(+), respectively. Figure 17 shows the strategy for sequencing the pJV24 insert. Figure 18 shows the nucleotide sequence.

B. Open reading frames

Since streptomycete DNA has a high % G+C content, codon usage is biassed in favor of G or C in the third position of each codon (Seno and Baltz, 1989; Wright and Bibb, 1992). The CODONPREFERENCE program of the GCG software was used to identify potential open reading frames (ORFs). The six possible reading frames were analyzed for start and stop codons, GC bias at the third position and the frequency of rare codons based on codon usage encountered in streptomycetes as reported by Wright and Bibb (1992). The results are shown in Fig. 19A and Fig. 19B.

The first region of codon bias (ORF1) was identified in the first reading frame of strand A (sharing the orientation of the lac gene in the vector). There are two possibilities for the translational start codon. One is the ATG at nt 205-207. Upstream of it at nt 191-197, the sequence (AGGTGG) resembles the ribosomal binding site for streptomycetes (Strohl, 1992). The second potential start site is the GTG at nt 160-162. Two possible ribosomal binding sites are present, one at nt 144-148 with the sequence AGGAG and the other at nt 148-151 with sequence GAGG. As the distance between the ribosomal binding sites and the translation start codons is usually 5 to 12 nt in streptomycetes (Strohl, 1992), the latter is more favorable. The ORF terminates with the TAG at nt 1174-1176.

A second region of codon bias (ORF2) was identifed in the first reading frame of strand B (complementary to strand A; therefore, nt 1 in strand B represents nt 2197 in strand A). The only possible start codon in the region where the codon bias begins is located at nt

Figure 18. The nucleotide sequence of the 2.2-kb S. akiyoshiensis DNA in pJV24. The sequence is oriented in the same direction as the lac gene in the cloning vector.

```
    GTCGACACCGCAGGTCCGCAACCATGGAGTGGGACACGCCGTCCCACTCTCCGGGCAGAC
  1 ---------+---------+---------+---------+---------+---------+ 60
    CAGCTGTGGCGTCCAGGCGTTGGTACCTCACCCTGTGCGGCAGGGTGAGAGGCCCGTCTG

    CGGGGAAAGAAGTGGACAGGCGGGCCGTGGTCGGCCGCATGATGGAAAAGCCTCGGCCAA
 61 ---------+---------+---------+---------+---------+---------+ 120
    GCCCCTTTCTTCACCTGTCCGCCCGGCACCAGCCGGCGTACTACCTTTTCGGAGCCGGTT

    GCAACGAGCACGTGAAGAGACGCAGGAGAGGGAGTCACCGTGAGGGTCGGAATCGTCGGA
121 ---------+---------+---------+---------+---------+---------+ 180
    CGTTGCTCGTGCACTTCTCTGCGTCCTCTCCCTCAGTGGCACTCCCAGCCTTAGCAGCCT

    GCCACCGGACAGGTGGGCACGGTCATGCGCAGGATCCTCACGGAGCGGAACTTCCCGGTC
181 ---------+---------+---------+---------+---------+---------+ 240
    CGGTGGCCTGTCCACCCGTGCCAGTACGCGTCCTAGGAGTGCCTCGCCTTGAAGGGCCAG

    ACGGAGCTGCGCCTGTTCGCCTCGGCCCGTTCCGCGGGCACGGAGCTGGACGGCGTGACG
241 ---------+---------+---------+---------+---------+---------+ 300
    TGCCTCGACGCGGACAAGCGGAGCCGGGCAAGGCGCCCGTGCCTCGACCTGCCGCACTGC

    GTGGAGGACGCGGCGACCGCCGACTACACCGGCCTGGACATCGTGCTGTTCTCCGCGGGC
301 ---------+---------+---------+---------+---------+---------+ 360
    CACCTCCTGCGCCGCTGGCGGCTGATGTGGCCGGACCTGTAGCACGACAAGAGGCGCCCG

    GGCGCGACCTCCAAGGCGCTGGCCGAGAAGGTCGCCTCCCAGGGCGCGGTCGTGATCGAC
361 ---------+---------+---------+---------+---------+---------+ 420
    CCGCGCTGGAGGTTCCGCGACCGGCTCTTCCAGCGGAGGGTCCCGCGCCAGCACTAGCTG

    AACTCCTCCGCGTGGCGCAAGCACCCGGAGGTCCCCCTGGTCGTCTCCGAGGTCAACCCG
421 ---------+---------+---------+---------+---------+---------+ 480
    TTGAGGAGGCGCACCGCGTTCGTGGGCCTCCAGGGGGACCAGCAGAGGCTCCAGTTGGGC

    CACGCGATCAAGGACCGCCCCAAGGGCATCATCGCCAACCCGAACTGCACGACGATGGCC
481 ---------+---------+---------+---------+---------+---------+ 540
    GTGCGCTAGTTCCTGGCGGGGTTCCCGTAGTAGCGGTTGGGCTTGACGTGCTGCTACCGG

    GCGATGCCGGTGCTGCGTCCGCTGCACGACGAGGCGGGCCTGGAAGCCCTGGTCGTCGCC
541 ---------+---------+---------+---------+---------+---------+ 600
    CGCTACGGCCACGACGCAGGCGACGTGCTGCTCCGCCCGGACCTTCGGGACCAGCAGCGG

    ACGTACCAGGCGGTCTCCGGTTCCGGCCTCGCGGGCGTCGCCGAGCTGCACGGCCAGACG
601 ---------+---------+---------+---------+---------+---------+ 660
    TGCATGGTCCGCCAGAGGCCAAGGCCGGAGCGCCCGCAGCGGCTCGACGTGCCGGTCTGC

    CAGAAGGTCGTCGCCGACGCCGAGAAGCTCACCCACGACGGTGAGGCGGTGGACTTCCCG
661 ---------+---------+---------+---------+---------+---------+ 720
    GTCTTCCAGCAGCGGCTGCGGCTCTTCGAGTGGGTGCTGCCACTCCGCCACCTGAAGGGC

    GAGCCGGGCGTCTACAAGCGCCCCATCGCCTTCAACGTGCTGCCGCTGGCGGGCAGCATC
721 ---------+---------+---------+---------+---------+---------+ 780
    CTCGGCCCGCAGATGTTCGCGGGGTAGCGGAAGTTGCACGACGGCGACCGCCCGTCGTAG
```

(Figure 18 continued)

```
    GTCGACGACGGCCTGAACGAGACCGACGAGGAGCAGAAGCTCCGCAACGAGTCCCGCAAG
781 ---------+---------+---------+---------+---------+---------+ 840
    CAGCTGCTGCCGGACTTGCTCTGGCTGCTCCTCGTCTTCGAGGCGTTGCTCAGGGCGTTC

    ATCCTGGAGATCCCCGGGCTGAAGGTCTCCGGCACCTGCGTGCGCGTCCCGGTCTTCTCC
841 ---------+---------+---------+---------+---------+---------+ 900
    TAGGACCTCTAGGGGCCCGACTTCCAGAGGCCGTGGACGCACGCGCAGGGCCAGAAGAGG

    GGCCACTCCCTCCAGATCAACGCCCGTTTCGCCCGCCCGATCTCGGCGGACGGCGCGACG
901 ---------+---------+---------+---------+---------+---------+ 960
    CCGGTGAGGGAGGTCTAGTTGCGGGCAAAGCGGGCGGGCTAGAGCCGCCTGCCGCGCTGC

    GAGCTCCTGAAGGACGCCCCCGGCGTCGAGCTCTCCGACATCCCGACCCCCCTCCAGGCC
961 ---------+---------+---------+---------+---------+---------+ 1020
    CTCGAGGACTTCCTGCGGGGGCCGCAGCTCGAGAGGCTGTAGGGCTGGGGGGAGGTCCGG

    GCCGGCAAGGACCCGTCCTACGTCGGCCGCATCCGCAGTGACGAGACGGTGGACAACGGC
1021 ---------+---------+---------+---------+---------+---------+ 1080
    CGGCCGTTCCTGGGCAGGATGCAGCCGGCGTAGGCGTCACTGCTCTGCCACCTGTTGCCG

    CTGGCCCTCTTCGTCTCCAACGACAACCTCCGCAAGGGCGCGGCGCTGAACGCCGTACAG
1081 ---------+---------+---------+---------+---------+---------+ 1140
    GACCGGGAGAAGCAGAGGTTGCTGTTGGAGGCGTTCCCGCGCCGCGACTTGCGGCATGTC

    ATCGCGGAGCTGGTGGCGGCGGAGCTGAAGGGCTAGTCCACGCCCCCTCTCAGCCCCTGC
1141 ---------+---------+---------+---------+---------+---------+ 1200
    TAGCGCCTCGACCACCGCCGCCTCGACTTCCCGATCAGGTGCGGGGGAGAGTCGGGGACG

    GCACCTCGTAGAGAAAGCAGCCGTACTCGACGGCCCGGACGTGGACCAGTGCCACGGCCG
1201 ---------+---------+---------+---------+---------+---------+ 1260
    CGTGGAGCATCTCTTTCGTCGGCATGAGCTGCCGGGCCTGCACCTGGTCACGGTGCCGGC

    GGTCGTCGAATGCTTCTGTGAAGGCCTCGCCGAAACCGGTGGGGTCCTCGACCAGCCGGC
1261 ---------+---------+---------+---------+---------+---------+ 1320
    CCAGCAGCTTACGAAGACACTTCCGGAGCGGCTTTGGCCACCCCAGGAGCTGGTCGGCCG

    CGCCCAGGATGTGCCCCTCGGCCGAGTAGCGGCGGACCGTGCGACGGCTGCCGGTCAACG
1321 ---------+---------+---------+---------+---------+---------+ 1380
    GCGGGTCCTACACGGGGAGCCGGCTCATCGCCGCCTGGCACGCTGCCGACGGCCAGTTGC

    GGAGGGCGTCGGTCTCCGGCCCCTCGCACTCCTCGGCATGGATGAAGACCGGGCCCTGTT
1381 ---------+---------+---------+---------+---------+---------+ 1440
    CCTCCCGCAGCCAGAGGCCGGGGAGCGTGAGGAGCCGTACCTACTTCTGGCCCGGGACAA

    CGTCGCCCCGGGCCCCTACGGCGCTCGTCTCCGACGCCCAGCGGCGGAGGGGTGCGTAGG
1441 ---------+---------+---------+---------+---------+---------+ 1500
    GCAGCGGGGCCCGGGGATGCCGCGAGCAGAGGCTGCGGGTCGCCGCCTCCCCACGCATCC

    AGACCAGGGCCACGCGTTCGCCCGGCTCGCTGTGGCGCAGGCAGCAGCGCAACGGGCTGC
1501 ---------+---------+---------+---------+---------+---------+ 1560
    TCTGGTCCCGGTGCGCAAGCGGGCCGAGCCACCGCGTCCGTCGTCGCGTTGCCCGACG
```

(Figure 18 continued)

```
     CGCCCTCGGTGTCGGCGAAGCCGTCGGTCCGGCGGCCCGCGTCGTCCGTCGTGCGCAGTT
1561 ---------+---------+---------+---------+---------+---------+ 1620
     GCGGGAGCCACAGCCGCTTCGGCAGCCAGGCCGCCGGGCGCAGCAGGCAGCACGCGTCAA

     CCTTCAGGGCGCGCGGGGAGATGGGGCGTGCGGTGTACGCGGTGGTCGTCATGGTCACCA
1621 ---------+---------+---------+---------+---------+---------+ 1680
     GGAAGTCCCGCGCGCCCCTCTACCCCGCACGCCACATGCGCCACCAGCAGTACCAGTGGT

     GACTCACTCGCGCGATCCGCGCTCACCGGCGGAATCCGGACGTCGCGCTCGCCCGCGAAG
1681 ---------+---------+---------+---------+---------+---------+ 1740
     CTGAGTGAGCGCGCTAGGCGCGAGTGGCCGCCTTAGGCCTGCAGCGCGACCGGGCGCTTC

     GGCCCTGCCCGAGGGGTATCCCTAGGACTCGCCCGGGCCGTCGCACGACGCCGATCCTCC
1741 ---------+---------+---------+---------+---------+---------+ 1800
     CCGGGACGGGCTCCCCATAGGGATCCTGAGCGGGCCCGGCAGCGTGCTGCGGCTAGGAGG

     GTGGAAAGATGGCGCAACCCACCCATAACGAGGAGATGACCGCGTGCCTGGCACAAACCT
1801 ---------+---------+---------+---------+---------+---------+ 1860
     CACCTTTCTACCGCGTTGGGTGGGTATTGCTCCTCTACTGGCGCACGGACCGTGTTTGGA

     GACCCGCGAAGAGGCGCAGCAGCGGGCCCAGCTGCTCGCCGTTGAGTCGTACGGGATCGA
1861 ---------+---------+---------+---------+---------+---------+ 1920
     CTGGGCGCTTCTCCGCGTCGTCGCCCGCGTCGACGAGCGGCAACTCAGCATGCCCTAGCT

     GCTCGATCTCTCCGGCGCGCAGGAGGGCGGCACCTACCGGTCCGTGACCACGGTGCGCTT
1921 ---------+---------+---------+---------+---------+---------+ 1980
     CGAGCTAGAGAGGCCGCGCGTCCTCCCGCCGTGGATGGCCAGGCACTGGTGCCACGCGAA

     CGACGTGACGGCCGAGAACGGCGCGGAGTCGTTCATCGACCTGGTGGCGCCGGCCGTGCA
1981 ---------+---------+---------+---------+---------+---------+ 2040
     GCTGCACTGCCGGCTCTTGCCGCGCCTCAGCAAGTAGCTGGACCACCGCGGCCGGCACGT

     CGAGGTGACCCTGAACGGGGACTCCCTCGACCCGGCCGAGGTCTTCGCGGACTCGCGGAT
2041 ---------+---------+---------+---------+---------+---------+ 2100
     GCTCCACTGGGACTTGCCCCTGAGGGAGCTGGGCCGGCTCCAGAAGCGCCTGAGCGCCTA

     CGCCCTGCCCGGTCTGCTCCAGGGCCGCAACATCCTCCGGGTCGTCGCCGACTGCGCGTA
2101 ---------+---------+---------+---------+---------+---------+ 2160
     GCGGGACGGGCCAGACGAGGTCCCGGCGTTGTAGGAGGCCCAGCAGCGGCTGACGCGCAT

     CACCAACACGGGTGAGGGCCTGCACCGGTTCGTCGAC
2161 ---------+---------+---------+------- 2197
     GTGGTTGTGCCCACTCCCGGACGTGGCCAAGCAGCTG
```

Figures 19A and 19B. CODONPREFERENCE analysis of strands A and B, respectively, in the 2.2 kb SalI-SalI fragment for codon bias, third position GC bias, rare codon usage and possible open reading frames. The horizontal axis is the number of nucleotides. The two straight lines in each window represent the average GC content of the entire sequence (upper line) and the random frequency of codon usage (lower line). The two plot lines represent the average GC usage at every third position in a sliding window of 25 triplets (upper plot line) and the codon preference statistic for each codon over a sliding window of 25 triplets. Potential open reading frames are shown as horizontal lines near the bottom of each window. Potential start codons are shown as vertical lines slightly above a horizontal line. Stop codons are shown as vertical lines below a horizontal line. Rare codons, identified from the codon frequency table of Wright and Bibb (1992), are shown as short vetical bars in the bottom of each window.

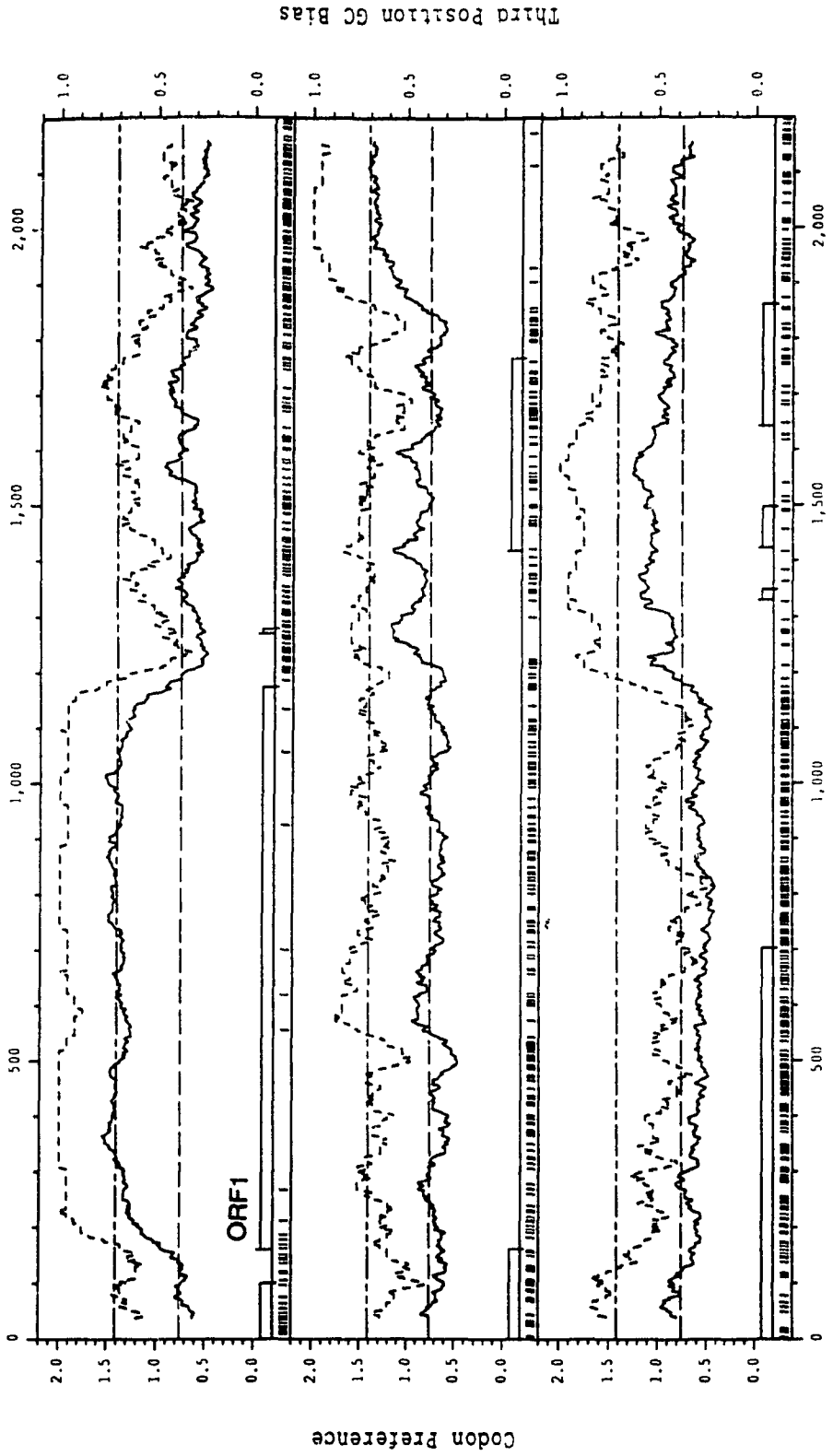

Third Position GC Bias
Codon Preference
ORF1
0
500
1,000
1,500
2,000
0.0
0.5
1.0
1.5
2.0

Figure 19A.

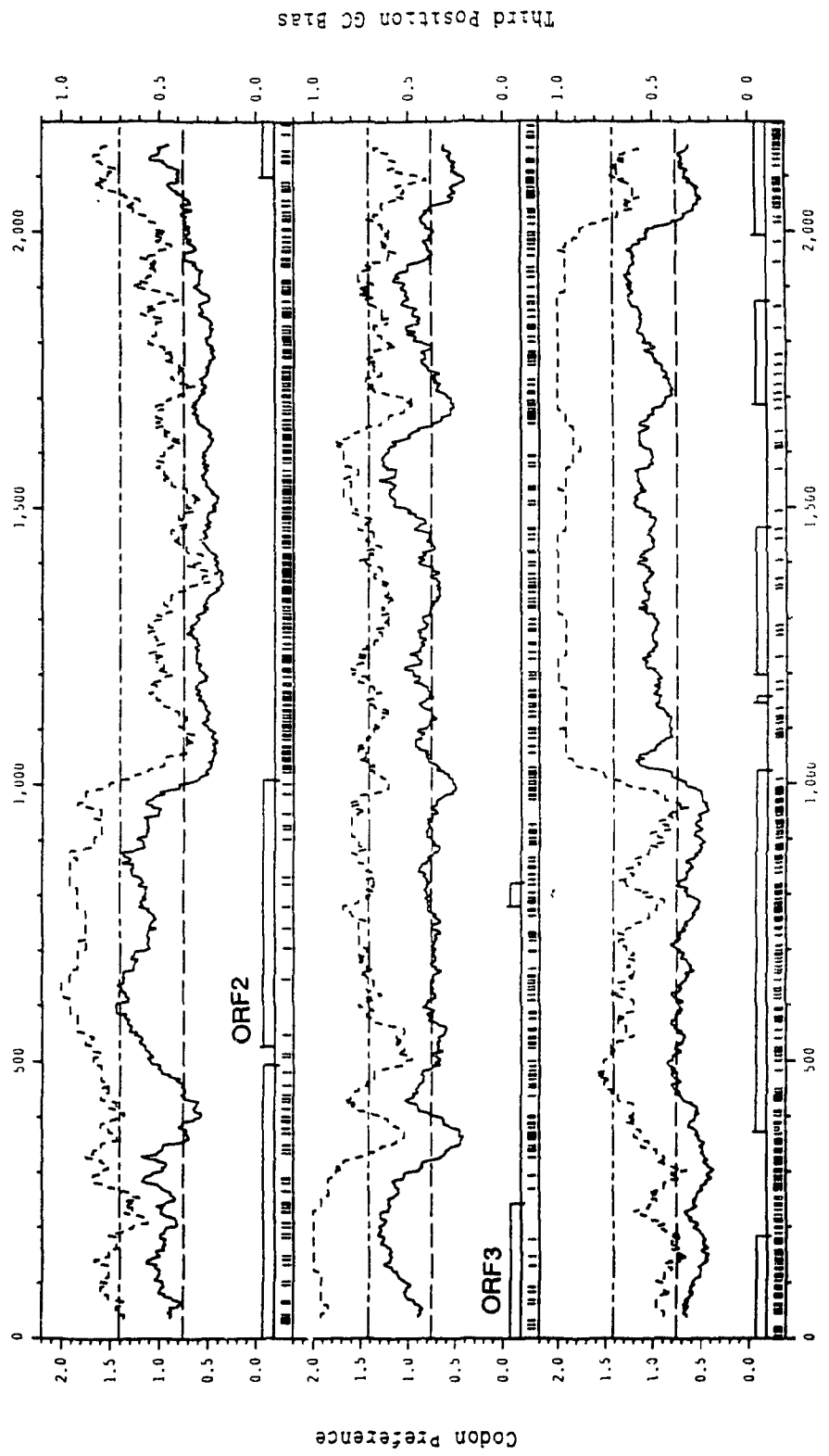


Figure 19B.

526-528, it is preceeded by a possible ribosomal binding site GGTG at nt 518-521. The stop codon TGA is located at nt 1006-1008.

A third region of codon bias (ORF3) was identified in the second reading frame of strand B. There are several possible start codons (GTGs at nt 29 and 35, and ATG at nt 65). Since no plausible ribosomal binding site was present for any of the possible start codons, and the ORF does not contain a region of increased codon bias region where start codon might be located, it is very likely that the ORF begins outside the 2.2-kb SalI-salI fragment; thus ORF3 is incomplete. A stop codon (TAG) is present at nt 242.

1. Analysis of ORF1

Figure 20 shows the nucleotide sequence and deduced amino acid sequence of ORF1. Table 23 shows the codon usage in ORF1, assuming the start codon to be GTG at nt 160-162). Table 24 show the percent G+C for each codon position and the overall average in ORF1. At 70.1%, this is similar to that reported for streptomycetes (Seno and Baltz 1989). 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 E. coli-like -10 and -35 hexamers. An inverted repeat was identified by using the STEMLOOP program of the GCG software. The first sequence begins 45 nt downstream of the TAG stop codon, from nt 1227 to 1244; The second is from nt 1252 to 1269. The RNA secondary structure of the inverted repeat is shown in Figure 21. The binding strength calculated by MFOLD of the GCG program for the corresponding mRNA is -29.2 kcal/mol. This type of structure is typical of a transcriptional terminator.

2. Analysis of ORF2

Figure 22 shows the nucleotide sequence and its deduced amino acid sequence of ORF2. The percent G+C analysis is in Table 24, and the codon usage is listed in Table 25.

Figure 20. Nucleotide and deduced amino acid sequences for ORF1. The numbers above the nucleotide sequence represent nucleotide positions in the sequences. The putative ribosomal binding site (RBS) and some restriction enzyme sites are underlined. An inverted repeat downstream of the stop codon (from 1222 to 1274 nt) is indicated by arrows under the nucleotide sequence.

```
121                                    RBS
GCA ACG AGC ACG TGA AGA GAC GCA GGA GAG GGA GTC ACC GTG AGG GTC GGA ATC GTC GGA
                                                  fMet arg val gly ile val gly
181                                        BamHI
GCC ACC GGA CAG GTG GGC ACG GTC ATG CGC AGG ATC CTC ACG GAG CGG AAC TTC CCG GTC
ala thr gly gln val gly thr val met arg arg ile leu thr glu arg asn phe pro val
241
ACG GAG CTG CGC CTG TTC GCC TCG GCC CGT TCC GCG GGC ACG GAG CTG GAC GGC GTG ACG
thr glu leu arg leu phe ala ser ala arg ser ala gly thr glu leu asp gly val thr
301
GTG GAG GAC GCG GCG ACC GCC GAC TAC ACC GGC CTG GAC ATC GTG CTG TTC TCC GCG GGC
val glu asp ala ala thr ala asp tyr thr gly leu asp ile val leu phe ser ala gly
361
GGC GCG ACC TCC AAG GCG CTG GCC GAG AAG GTC GCC TCC CAG GGC GCG GTC GTG ATC GAC
gly ala thr ser lys ala leu ala glu lys val ala ser gln gly ala val val ile asp
421
AAC TCC TCC GCG TGG CGC AAG CAC CCG GAG GTC CCC CTG GTC GTC TCC GAG GTC AAC CCG
asn ser ser ala trp arg lys his pro glu val pro leu val val ser glu val asn pro
481
CAC GCG ATC AAG GAC CGC CCC AAG GGC ATC ATC GCC AAC CCG AAC TGC ACG ACG ATG GCC
his ala ile lys asp arg pro lys gly ile ile ala asn pro asn cys thr thr met ala
541
GCG ATG CCG GTG CTG CGT CCG CTG CAC GAC GAG GCG GGC CTG GAA GCC CTG GTC GTC GCC
ala met pro val leu arg pro leu his asp glu ala gly leu glu ala leu val val ala
601
ACG TAC CAG GCG GTC TCC GGT TCC GGC CTC GCG GGC GTC GCC GAG CTG CAC GGC CAG ACG
thr tyr gln ala val ser gly ser gly leu ala gly val ala glu leu his gly gln thr
661
CAG AAG GTC GTC GCC GAC GCC GAG AAG CTC ACC CAC GAC GGT GAG GCG GTG GAC TTC CCG
gln lys val val ala asp ala glu lys leu thr his asp gly glu ala val asp phe pro
721
GAG CCG GGC GTC TAC AAG CGC CCC ATC GCC TTC AAC GTG CTG CCG CTG GCG GGC AGC ATC
glu pro gly val tyr lys arg pro ile ala phe asn val leu pro leu ala gly ser ile
781 SalI
GTC GAC GAC GGC CTG AAC GAG ACC GAC GAG GAG CAG AAG CTC CGC AAC GAG TCC CGC AAG
val asp asp gly leu asn glu thr asp glu glu gln lys leu arg asn glu ser arg lys
841
ATC CTG GAG ATC CCC GGG CTG AAG GTC TCC GGC ACC TGC GTG CGC GTC CCG GTC TTC TCC
ile leu glu ile pro gly leu lys val ser gly thr cys val arg val pro val phe ser
901
GGC CAC TCC CTC CAG ATC AAC GCC CGT TTC GCC CGC CCG ATC CCG GCG GAC GGC GCG ACG
gly his ser leu gln ile asn ala arg phe ala arg pro ile ser ala asp gly ala thr
961 SstI                                SstI
GAG CTC CTG AAG GAC GCC CCC GGC GTC GAG CTC TCC GAC ATC CCG ACC CCC CTC CAG GCC
glu leu leu lys asp ala pro gly val glu leu ser asp ile pro thr pro leu gln ala
1021
GCC GGC AAG GAC CCG TCC TAC GTC GGC CGC ATC CGC AGT GAC GAG ACG GTG GAC AAC GGC
ala gly lys asp pro ser tyr val gly arg ile arg ser asp glu thr val asp asn gly
1081
CTG GCC CTC TTC GTC TCC AAC GAC AAC CTC CGC AAG GGC GCG GCG CTG AAC GCC GTA CAG
leu ala leu phe val ser asn asp asn leu arg lys gly ala ala leu asn ala val gln
1141
ATC GCG GAG CTG GTG GCG GCG GAG CTG AAG GGC TAG TCC ACG CCC CCT CTC AGC CCC TGC
ile ala glu leu val ala ala glu leu lys gly AMB
1201
GCA CCT CGT AGA GAA AGC AGC CGT ACT CGA CGG CCC GGA CGT GGA CCA GTG CCA CGG CCG
                                    ----------------------->          <----------
1261
GGT CGT CGA ATG CTT
-----------
```

Figure 20.

Table 23. Codon usage in ORF1.

TTT	phe	F	-	TCT	ser	S	-	TAT	tyr	Y	-	TGT	cys	C	-
TTC	phe	F	8	TCC	ser	S	16	TAC	tyr	Y	4	TGC	cys	C	2
TTA	leu	L	-	TCA	ser	S	-	TAA	OCH	Z	-	TGA	OPA	Z	-
TTG	leu	L	-	TCG	ser	S	2	TAG	AMB	Z	1	TGG	t.o	W	1
CTT	leu	L	-	CCT	pro	P	-	CAT	his	H	-	CGT	arg	R	3
CTC	leu	L	10	CCC	pro	P	6	CAC	his	H	6	CGC	arg	R	12
CTA	leu	L	-	CCA	pro	P	-	CAA	gln	Q	-	CGA	arg	R	-
CTG	leu	L	22	CCG	pro	P	13	CAG	gln	Q	9	CGG	arg	R	1
ATT	ile	I	-	ACT	thr	T	-	AAT	asn	N	-	AGT	ser	S	1
ATC	ile	I	16	ACC	thr	T	8	AAC	asn	N	13	AGC	ser	S	1
ATA	ile	I	-	ACA	thr	T	-	AAA	lys	K	-	AGA	arg	R	-
ATG	met	M	3	ACG	thr	T	11	AAG	lys	K	15	AGG	arg	R	2
GTT	val	V	-	GCT	ala	A	-	GAT	asp	D	-	GGT	gly	G	2
GTC	val	V	24	GCC	ala	A	21	GAC	asp	D	20	GGC	gly	G	24
GTA	val	V	1	GCA	ala	A	-	GAA	glu	E	1	GGA	gly	G	3
GTG	val	V	12	GCG	ala	A	22	GAG	glu	E	22	GGG	gly	G	1

Table 24. Percent G+C of ORF1 and ORF2 compared with average values for Streptomyces genes.

Codon position	ORF1	ORF2	Streptomyces average*
1	69.1	70.8	69.7
2	44.7	57.1	49.9
3	96.5	90.1	90.6
Coding region	70.1	72.6	70.1

*Data obtained from Seno and Baltz (1989)

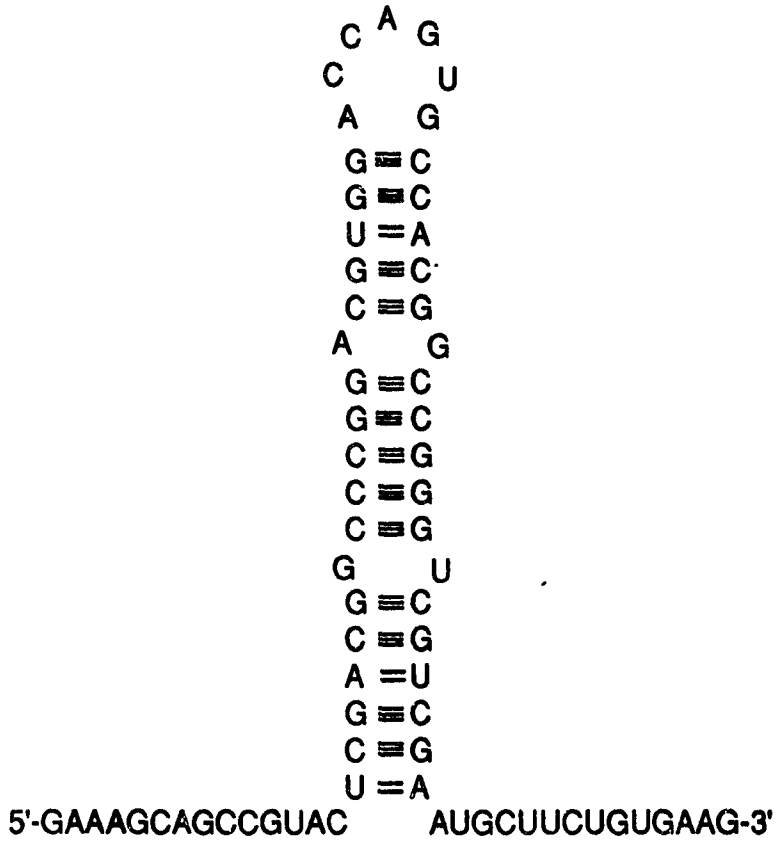


Figure 21. The predicted secondary structure of the transcriptional terminator downstream of ORF1.

```
421
CCC GGG CGA GTC CTA GGG ATA CCC CTC GGG CAG GGC CCT TCG CGG GCG AGC GCG ACG TCC

481                                                    RBS
GGA TTC CGC CGG TGA GCG CGG ATC GCG CGA GTG AGT CTG GTG ACC ATG ACG ACC ACC GCG
                                                         fMet thr thr thr ala
541
TAC ACC GCA CGC CCC ATC TCC CCG CGC GCC CTG AAG GAA CTG CGC ACG ACG GAC GAC GCG
tyr thr ala arg pro ile ser pro arg ala leu lys glu leu arg thr thr asp asp ala
601
GGC CGC CGG ACC GAC GGC TTC GCC GAC ACC GAG GGC GGC AGC CCG TTG CGC TGC TGC CTG
gly arg arg thr asp gly phe ala asp thr glu gly gly ser pro leu arg cys cys leu
661
CGC CAC AGC GAG CCG GGC GAA CGC GTG GCC CTG GTC TCC TAC GCA CCC CTC CGC CGC TGG
arg his ser glu pro gly glu arg val ala leu val ser tyr ala pro leu arg arg trp
721
GCG TCG GAG ACG AGC GCC GTA GGG GCC CGG GGC GAC GAA CAG GGC CCG GTC TTC ATC CAT
ala ser glu thr ser ala val gly ala arg gly asp g u gln gly pro val phe ile his
781
GCC GAG GAG TGC GAG GGG CCG GAG ACC GAC GCC CTC CCG TTG ACC GGC AGC CGT CGC ACG
ala g.u glu cys glu gly pro glu thr asp ala leu pro leu thr gly ser arg arg thr
841
GTC CGC CGC TAC TCG GCC GAG GGG CAC ATC CTG GGC GGC CGG CTG GTC GAG GAC CCC ACC
val arg arg tyr ser ala glu gly his ile leu gly gly arg leu val glu asp pro thr
901
GGT TTC GGC GAG GCC TTC ACA GAA GCA TTC GAC GAC CCG GCC GTG GCA CTG GTC CAC GTC
gly phe gly glu ala phe thr glu ala phe asp asp pro ala val ala leu val his val
961
CGG GCC GTC GAG TAC GGC TGC TTT CTC TAC GAG GTG CGC AGG GGC TGA GAG GGG GCG TGG
arg ala val glu tyr gly cys phe leu tyr glu val arg arg gly OPA   ----------
1021
ACT AGC CCT TCA GCT CCG CCG CCA CCA GCT CCG CGA TCT GTA CGG CGT TCA GCG CCG CGC
------>         <----------------
```

Figure 22 Nucleotide and deduced amino acid sequence of the ORF2 The putative ribosomal binding site (RBS) is underlined An inverted repeat downstream of the stop codon (from nt 1011 to 1026, and from 1033 to 1046) was indicated by arrows under the nucleotide sequences

Table 25. codon usage in ORF2.

TTT	phe	F	1	TCT	ser	S	-	TAT	tyr	Y	-	TGT	cys	C	-
TTC	phe	F	5	TCC	ser	S	2	TAC	tyr	Y	5	TGC	cys	C	4
TTA	leu	L	-	TCA	ser	S	-	TAA	OCH	Z	-	TGA	OPA	Z	1
TTG	leu	L	2	TCG	ser	S	2	TAG	AMB	Z	-	TGG	trp	W	1
CTT	leu	L	-	CCT	pro	P	-	CAT	his	H	1	CGT	arg	R	1
CTC	leu	L	3	CCC	pro	P	3	CAC	his	H	3	CGC	arg	R	13
CTA	leu	L	-	CCA	pro	P	-	CAA	gln	Q	-	CGA	arg	R	-
CTG	leu	L	7	CCG	pro	P	7	CAG	gln	Q	1	CGG	arg	R	4
ATT	ile	I	-	ACT	thr	T	-	AAT	asn	N	-	AGT	ser	S	-
ATC	ile	I	3	ACC	thr	T	8	AAC	asn	N	-	AGC	ser	S	4
ATA	ile	I	-	ACA	thr	T	1	AAA	lys	K	-	AGA	arg	R	-
ATG	met	M	1	ACG	thr	T	5	AAG	lys	K	1	AGG	arg	R	1
GTT	val	V	-	GCT	ala	A	-	GAT	asp	D	-	GGT	gly	G	1
GTC	val	V	7	GCC	ala	A	11	GAC	asp	D	9	GGC	gly	G	13
GTA	val	V	1	GCA	ala	A	4	GAA	glu	E	4	GGA	gly	G	-
GTG	val	V	3	GCG	ala	A	3	GAG	glu	E	12	GGG	gly	G	3

The percent G+C at each position and the overall average in ORF2 are similar to those reported for streptomycetes (Seno and Baltz, 1989). Examination of the region upstream of the translational start codon for sequences similar to promoter recognition sites in streptomycetes (Strohl, 1992) did not identify any E. coli-like -10 and -35 hexamers. By using STEMLOOP, two sequences forming an inverted repeat were identified after the TGA stop codon, from nt 1011 to 1026 and from nt 1033 to 1046 of strand B. The binding strength calculated by MFOLD for the corresponding mRNA is -11.3 kcal/mol.

3. Analysis of ORF3

The deduced amino acid sequence of the incomplete ORF3 is given in Fig. 23. An inverted repeat was identified by using STEMLOOP of the GCG software; it is located immediately after the TGA stop codon, from nt 297 to 306 and from nt 313 to 324 of strand B The binding strength calculated by MFOLD for the corresponding mRNA is -16.5 kcal/mol.

C. The ORF1 product

1. Comparison with databases

When the protein sequences deduced from the open reading frames with start codons at nt 160 and 205 were compared by BLAST with the database in GenBank, they showed a very strong similarity to asd genes from Gram-positive bacteria. The N-terminus deduced from the GTG start codon more closely resembled that of other asd genes; therefore, this N-terminal sequence was used. Those sequences giving a high score in the comparison were compared further by using the BESTFIT program in the GCG software. Table 26 shows the scores in the BLAST comparison of ORF1 from S. akiyoshiensis with the asd genes from C. flavum, C. glutamicum, M. smegmatis and B. subtilis, and gives the percentage of identical

```
 1
TCG ACG AAC CGG TGC AGG CCC TCA CCC GTG TTG GTG TAC GCG CAG TCG GCG ACG ACC CGG
ser thr asn arg cys arg pro ser pro val leu val tyr ala gln ser ala thr thr arg
61
AGG ATG TTG CGG CCC TGG AGC AGA CCG GGC AGG GCG ATC CGC GAG TCC GCG AAG ACC TCG
arg met leu arg pro trp ser arg pro gly arg ala ile arg glu ser ala lys thr ser
121
GCC GGG TCG AGG GAG TCC CCG TTC AGG GTC ACC TCG TGC ACG GCC GGC GCC ACC AGG TCG
ala gly ser arg glu ser pro phe arg val thr ser cys thr ala gly ala thr arg ser
181
ATG AAC GAC TCC GCG CCG TTC TCG GCC GTC ACG TCG AAG CGC ACC GTG GTC ACG GAC CGG
met asn asp ser ala pro phe ser ala val thr ser lys arg thr val val thr asp arg
241
TAG GTG CCG CCC TCC TGC GCG CCG GAG AGA TCG AGC TCG ATC CCG TAC GAC TCA ACG GCG
AMB                                                                      -----
301
AGC AGC TGC GCC CGC TGC TGC GCC TCT TCG CGG GTC AGG TTT GTG CCA GGC ACG CGG TCA
------>          <-------------
```

Figure 23. Deduced amino acid sequence of ORF3. An inverted repeat downstream of the stop codon is indicated by arrows under the nucleotide sequences.

Table 26. Comparison of the amino acid sequence deduced from S. akiyoshiensis ORF1 with those of asd genes from other organisms.

Organism	Scores	% Identity	% Similarity
C. flavum	791	62.5	72.8
C. glutamicum	784	62.2	72.5
M. smegmatis	668	64.0	78.3
B. subtilis	164	46.0	65.4

and similar amino acids. Alignment of the deduced amino acid sequences of Asds from several Gram-positive organisms by the CLUSTAL software (Higgins et al., 1988) is shown in Fig. 24.

2. The Asd protein

The open reading frame with the GTG start codon encodes a 338 amino acid polypeptide with a size of 35,484 daltons. The size is similar to that deduced from the asd of other prokaryotes, such as C. glutamicum (36,275 Da; Kalinowski et al., 1990), B. subtilis (37,861 Da; Chen et al., 1993), S. mutans (38, 897 Da; Cardineau et al., 1987) and E. coli (39,950 Da; Haziza et al., 1982). The amino acid composition of the S. akiyoshiensis polypeptide is given in Table 27. It contained 46.9% hydrophobic, 28.6% hydrophilic, 12.7% acidic and 11.5% basic amino acid residues.

3. The active site of Asd

The deduced amino acid sequence of asd from S. akiyoshiensis contained a sequence similar to the active site of Asd in E. coli (Biellmann et al., 1980; Hazzia et al., 1982) and from other organisms (Fig. 25). The deduced S. akiyoshiensis active site was the same as those from C. flavum, C. glutamicum and M. smegmatis and similar to those from B. subtilis and S. mutans. The deduced active site from S. akiyoshiensis shared least sequence similarity with that from E. coli (Fig. 25).

No alignments above threshold were found when the S. akiyoshiensis Asd sequence was analyzed by the MOTIFS and PROFILESCAN programs of the GCG software for known motifs and function groups.

4. Expression of an incomplete S. akiyoshiensis asd gene in E. coli

As further evidence that ORF1 codes for an asd gene, the BamHI-KpnI fragment of pJV24, including part of the vector sequence, was ligated to pJV21 digested completely with

```
S. akiyoshiensis   M-R---VGIVGATGQVGTVMRRILTERNFPVTELRLFASARSAGTELD--
C. flavum          MTT---IAVVGATGQVGQVMRTLLEERNFPADTVRFFASPRSAGRKIEFR
C. glutamicum      MTT---IAVVGATGQVGQVMRTLLEERNFPADTVRFFASPRSAGRKIEFR
M. smegmatis       MVN---IGVVGATGQGRQVMRNLLEQRNFPATSVRFFASPRSEGKKLTFR
B. subtilis        MGRGLHVAVVGATGAVGQQMLKTLEDRNFEMDTLTLLSSKRSAGTKVTFK
                   *     ...*****     *   * .***    . ...* ** *  .

S. akiyoshiensis   --GVTVEDAATADYTGL---DIVLFSAGGATSKALAEKVASQGAVVIDNS
C. flavum          GTEIEVEDITQATEESLKGIDVALFSAGGTASKQYAPLFAAAGATVVDNS
C. glutamicum      GTEIEVEDITQATEESLKDIDVALFSAGGTASKQYAPLFAAAGATVVDNS
M. smegmatis       GQEIEVENAETADPSGL---DIALFSAGATMSRVQAPRFAEAGVIVVDNS
B. subtilis        GQELTVQE---ASPESFEGVNIALFSAGGSVSQALAPEAVKRGAIVIDNT
                      . *..   *   ..   .. *****.. *.  *      *  *.**.

S. akiyoshiensis   SAWRKHPEVPLVVSEVNP--HAIKDRP--KGIIANPNCTTMAAMPVLRPL
C. flavum          SAWRKDDEVPLIVSEVNPSDKD----SLVKGIIANPNCTTMAAMPVLKPL
C. glutamicum      SAWRKDDEVPLIVSEVNPSDKD----SLVKGIIANPNCTTMAAMPVLKPL
M. smegmatis       SAFRKDPDVPLVVSEVN-FDRDVRGKKLAKGIIANPNCTTMAAMPVLKPL
B. subtilis        SAFRMDENTPLVVPEVNEADLHEH-----NGIIANPNCSTIQMVAALEPI
                   ** * . . **.*.*** .******** .*.  .. * *.

S. akiyoshiensis   HDEAGLEALVVATYQAVSGSGLAGVAELHGQTQKVVADAEKLTHDGEAVD
C. flavum          HDAAGLVKLHVSSYQAVSGSGLAGVETLAKQVAAVGDHNVEFVHDGQAAD
C. glutamicum      HDAAGLVKLHVSSYQAVSGSGLAGVETLAKQVAAVGDHNVEFVHDGQAAD
M. smegmatis       HEEAGLQRLIVSSYQAVSGSGIAGVEELAGQARPVIDGVEQLVHDGSALQ
B. subtilis        RKAYGLNKVIVSTYQAVSGAGNEAVKELYSQTQAILNKEE--IEPEIMPV
                   .   **  . *..******.*  .*  *  *   .        .

S. akiyoshiensis   FPEPGVYKRPIAFNVLPLAGSIVDDGLNETDEEQKLRNESRKILEIPGLK
C. flavum          AGDVGPYVSPIAYNVLPFAGNLVDDGTFETDEEQKLRNESRKILGLPDLK
C. glutamicum      AGDVGPYVSPIAYNVLPFAGNLVDDGTFETDEEQKLRNESRKILGLPDLK
M. smegmatis       YPAPNKYVAPIAFNIVPLAGNYVDDGSGETDEDQKLRNESRKILGIPELL
B. subtilis        KGDKKHY--QIAFNAIPQIDKFQDNGY--TFEEMKMINETKKIMHMPDLQ
                         *   **.* .*  .   *.*   * *. *. **..**. .* *

S. akiyoshiensis   VSGTCVRVPVFSGHSLQINARFARP-ISADGATELLKDAPGVELSDIPT-
C. flavum          VSGTCVRVPVFTGHTLTIHAEFDKA-ITVEQAQEILGAASGVELVDVPT-
C. glutamicum      VSGTCVRVPVFTGHTLTIHAEFDKA-ITVDQAQEILGAASGVKLVDVPT-
M. smegmatis       VSGTCVRVPVFSGHSLSINAEFSQP-ISVERTKELLSAAAGVKLVDVPT-
B. subtilis        VAATCVRLPIQTGHSEAVYIEIDRDDATVEDIKNLLKEAPGVTLQDDPSQ
                   *..****.*. .**.  .   . .   . .   ..*  *.** * * *.

S. akiyoshiensis   -----PLQAAGKDPSYVGRIRSDETVDN--GLALFVSNDNLRKGAALNAV
C. flavum          -----PLAAAGIDESLVGRIRQDSTVDDNRGLVLVVSGDNLRKGAALNTI
C. glutamicum      -----PLAAAGIDESLVGRIRQDSTVDDNRGLVLVVSGDNLRKGAALNTI
M. smegmatis       -----PLAAAGIDDCLVGRIRQDPGVPDGRGLALFVSGDNLRKGAALNTI
B. subtilis        QLYPMPADAVGKNDVFVGRIRKD--LDRANGFHLWVVSDNLLKGAAWNSV
                        *  * * .   ***** *  .    *. * *  *** **** *..

S. akiyoshiensis   QIAELVAAELKG
C. flavum          QIAELLVK----
C. glutamicum      QIAELLVK----
M. smegmatis       QIAELLAADL--
B. subtilis        QIAESLKKLNLV
                   **** .
```

Figure 24. Alignment of the deduced amino acid sequence of asd from S. akiyoshiensis with the sequences of Asds from C. flavum, C. glutamicum, M. smegmatis and B. subtilis. Stars represent identical amino acids. Dots represent similar amino acid.

Table 27. Amino acid composition of Asd from S. akiyoshiensis. n represents the number of residues of the amino acid present in the protein. n(%) represents the percentage of the amino acid present in the protein.

			n	n(%)	Amino acid type
A	ala	alanine	43	12.7	hydrophobic
C	cys	cysteine	2	0.6	hydrophilic
D	asp	aspartic acid	20	5.9	acidic
E	glu	glutamic acid	23	6.8	acidic
F	phe	phenylalanine	8	2.4	hydrophobic
G	gly	glycine	30	8.8	hydrophilic
H	his	histidine	6	1.8	basic
I	ile	isoleucine	16	4.7	hydrophobic
K	lys	lysine	15	4.4	basic
L	leu	leucine	32	9.4	hydrophobic
M	met	methionine	4	1.2	hydrophobic
N	asn	asparagine	13	3.8	hydrophilic
P	pro	proline	19	5.6	hydrophobic
Q	gln	glutamine	9	2.7	hydrophilic
R	arg	arginine	18	5.3	basic
S	ser	serine	20	5.9	hydrophilic
T	thr	threonine	19	5.6	hydrophilic
V	val	valine	36	10.6	hydrophobic
W	trp	tryptophan	1	0.3	hydrophobic
Y	tyr	tyrosine	4	1.2	hydrophilic
Z	---	STOP	1	0.3	

S. akiyoshiensis	GIIANPNCTT
C. flavum	GIIANPNCTT
C. glutamicum	GIIANPNCTT
M. smegmatis	GIIANPNCTT
B. subtilis	GIIANPNCST
S. mutans	GIIACPNCST
E. coli	FVGGNCTVSL

Figure 25. Comparison of the deduced active site of Asd from S. akiyoshiensis with that from other organisms.

these two restriction enzymes. The recombinant plasmid, which lacks the ribosomal binding site and a small segment encoding the N-terminus of the asd protein, did not complement the asd mutation in E. coli.

D. Analysis of the ORF2 product

The deduced amino acid sequence indicated that ORF2 encodes a protein of 160 amino acids, with a size of 17,430 Da. When the deduced protein sequence was compared with the GenBank database by BLAST, it did not show strong sequence similarities to any known proteins. The sequence of amino acids 85 to 115 showed 48% identity and 51% of similarity to the sequence of amino acids 344 to 374 in phosphoglycerate kinase of the protozoon, Crithidia fasciculata (GenBank accession number P25055); the sequence of amino acids 14 to 58 showed 37% identity and 51% similarity to the sequence of amino acids 150 to 194 in Barley (Hordeum vulgare) ferrochelatase (GenBank accession number D26105). Since the homologies were observed only in portions of the sequence, and the scores in BLAST comparison were only 62 and 60 for the phosphoglycerate kinase and the ferrochelatase, respectively, the low degree of similarity to known proteins in the database was too low for the function of the ORF2 protein to be predicted from sequence information

E. Analysis of the ORF3 product

When the deduced amino acid sequence of the incomplete ORF was compared with the database in GenBank by BLAST, it did not show strong similarity to any known proteins. The closest amino acid sequence (score of 63) was ORF8 protein of retroviral pseudoprotease (GenBank accession number: H34768), followed by human AF-9 gene product (score of 60; GenBank accession number: L13744). Therefore, the function of the ORF3 protein could not

be deduced from sequence analysis.

XI. Efforts to construct an asd-specific mutant by gene disruption

A. Transformation of S. akiyoshiensis

1. Culture conditions yielding mycelium suitable for protoplast formation

To create an S. akiyoshiensis asd mutant by gene disruption or replacement, it is necessary to have a DNA transformation system. In a preliminary study (data not shown), the medium commonly used to grow streptomycetes for protoplasting (Hopwood et al., 1985) gave sparse and very clumpy growth of S. akiyoshiensis; this resulted in a low yield of protoplasts. Therefore, culture conditions that provided dispersed growth and large amounts of mycelium were explored. The growth morphology of S. akiyoshiensis in three media containing the same amount of Trypticase Soy Broth with either glycerol (TSB A), glucose (TSB B) or sucrose, yeast extract and magnesium chloride (TSB C) was tested. After 48-h incubation of cultures inoculated with spores, the mycelium grown in TSB C gave the least clumpy growth

The initial pH of these media was 7.0. Since Glazebrook et al. (1992) found that the most dispersed growth of S. akiyoshiensis was obtained at pH 5.5 in a chemically defined medium, the growth morphology in TSB C medium at initial pH values of 5.7, 6.1, 6.4, 6.7 and 7.2 was compared. Growth was dispersed at pH 5.7 and 6.1, and remained so when the medium was supplemented with 0.5% glycine, a component facilitating breakage of cell walls during protoplasting (Hopwood et al., 1985). The TSB C medium developed in this study was subsequently used to prepare seed cultures as well as cultures for plasmid isolation or protoplast formation.

2. Formation and transformation of protoplasts

Except for a few modifications (indicated in the Materials and Methods section), the conditions used for preparing protoplasts, for transformation and for regeneration of transformed protoplasts were as described by Hopwood et al. (1985).

To establish optimum conditions, pHJL400 or pIJ702 isolated from Streptomyces lividans (gifts from F. Arhin) were used to transform the protoplasts of S. akiyoshiensis. Two thiostrepton-resistant colonies were obtained from the transformation with pHJL400 and 10 from the transformation with pIJ702. To increase the transformation efficiency, strains containing pHJL400 or pIJ702 were cured of plasmids by plating them on MYM without selection. When pIJ702 isolated from S. akiyoshiensis was used to transform protoplasts made from a pIJ702-cured strain, the transformation efficiency increased to $4.3X10^5$ transformants per μg ccc DNA.

B. Construction of a recombinant plasmid for disrupting asd

When the DNA sequence of the S. akiyoshiensis asd gene became known, a plasmid with a DNA insert containing an internal region of the coding sequence of the gene could be constructed. As the vector, the segregationally unstable pHJL400 was used. This plasmid is lost from the host very rapidly when plasmid-based selection is not applied, allowing integrants to be recognized when selection is reinstated. The internal fragment of the asd gene cloned in pHJL400 was the 0.75-kb BamHI-SstI fragment. The fragment was ligated to pHJL400 linearized with the same enzymes. When the recombinant plasmid was used to transform the E. coli asd mutant CGSC 6212, it did not complement the mutation. Disruption of the asd gene in S. akiyoshiensis using this plasmid is in progress (J. He, personal communication).

DISCUSSION

Secondary metabolites are biosynthesized from primary pathway intermediates and products, among them those of the aspartate pathway. White et al. (1988) studied the biosynthesis of HON and its relationship to the aspartate pathway by using isotopically labelled precursors, and concluded that HON is biosynthesized from aspartate and acetate (White et al., 1988; 1990). To understand the biosynthesis of HON in more detail, it is necessary to know more about the aspartate pathway in streptomycetes. Unfortunately little information about this pathway is yet available, and a more direct approach to the clarification of HON biosynthesis seemed worth investigating. An attempt was therefore made to develop an in vitro system for HON production.

I. In vitro synthesis of HON

The assumption made initially was that all the necessary enzymes would be present in suitably prepared cell-free extracts, and that HON would be synthesized if appropriate substrates and cofactors were provided under compatible conditions. However, various factors can affect the outcome. The cofactors included in the reaction mixture, although appropriate for the hypothesized pathway, might not be the correct ones. Instability and loss of activity for any single enzyme would prevent synthesis of the final products. Evidence obtained in the study suggested that HON does not accumulate inside the mycelium. This suggests that it is actively exported, so the final step in its biosynthesis may occur in the cell membrane rather than in the cytoplasm. Since HON is an antimetabolite of homoserine dehydrogenase, its presence inside the cell would likely inhibit the biosynthesis of methionine, threonine and isoleucine, and ultimately protein synthesis. A membrane protein jointly serving as a biosynthetic enzyme and a transport protein would provide a resistance mechanism.

Membrane proteins were not included in the cell-free extracts tested in the present study, and therefore HON production in vitro may have failed because of a need for functional membrane proteins. Because of the many potential lacunae in the in vitro system, the reason for the negative results obtained were difficult to diagnose.

II. Aspartate kinase

Aspartate kinase is the first enzyme of the aspartate pathway; it catalyzes the conversion of aspartate to aspartyl phosphate. It is, therefore, a crucial enzyme in the cell-free biosynthesis of HON from aspartate; assays for its activity showed that the conditions used to obtain cell-free extracts gave little or no aspartate kinase activity. Since aspartyl phosphate is not commercially available to test as a substrate for in vitro HON synthesis, examining conditions for optimum production of aspartate kinase was a potential means to ensure that aspartyl phosphate was available for use as a substrate in the in vitro system. Whereas the enzyme activities in extracts from SC medium and SN medium were negligible, the conditions that had been used by Mendelovitz and Aharonowitz (1982) to obtain aspartate kinase in Streptomyces clavuligerus were more promising. These were improved further when it was shown that, the activity of aspartate kinase in S. akiyoshiensis was influenced by the nitrogen and carbon sources in the growth medium. A combination of asparagine and starch supported the highest specific activity.

Aspartate kinase activity in cell-free extracts was affected by the solutes present. When these were removed and the protein fraction precipitated with ammonium sulfate at 65% saturation was assayed, the total enzyme activity increased appeciably. That the increase was due to release from feedback inhibition by end products of aspartate pathway was supported by the effect of lysine and threonine on aspartate kinase activity in the protein fraction

precipitated with 65% saturated ammonium sulfate. With lysine and threonine at 5 mM, the enzyme activity decreased to approximately 10%. This can be attributed to concerted feedback regulation by lysine and threonine. In this respect, aspartate kinase in S. akiyoshiensis is similar to that in S. clavuligerus (Mendelovitz and Aharonowitz, 1982). Concerted feedback inhibition for aspartate kinase is common among microorganisms (Cohen et al., 1969). Proteolysis also affected aspartate kinase activity; adding PMSF to the buffer used to prepare cell-free extracts, and to ammonium sulfate precipitated proteins after they had been redissolved, was beneficial.

Aspartate kinase activity was not found in mycelium grown in MSC medium or SN medium. Since the latter had been optimized to obtain a high level of HON biosynthesis (Glazebrook et al., 1992), such results suggest that (1) aspartate kinase may not be involved in the biosynthesis of HON; or (2) aspartate kinase activity is low during HON production, even though, the enzyme is required for HON biosynthesis or (3) aspartate kinase activity cannot be accurately measured in vitro due to instability. The presence of more than one enzyme is also possible, the minor activity being responsible for HON production. In bacilli, one aspartate kinase isozyme that is not by itself sufficient for growth is present throughout growth and sporulation (Zhang, et al., 1990).

III. Isolation and characterization of mutants blocked in HON biosynthesis

Because the initial efforts to obtain in vitro biosynthesis of HON gave disappointing results, an alternative approach was initiated in parallel with the enzyme studies. One of the common approaches to study the biosynthesis of antibiotics is to use blocked mutants. Such an approach has been used to study the biosynthesis of bialaphos (Imai, et al., 1984; 1985), tylosin (Baltz and Seno, 1988), chloramphenicol (Doull et al., 1985) and various other

antibiotics.

A. Bioassay of HON produced by single colonies

The purpose of a bioassay for HON was to use it in the isolation of nonproducing mutants. Whether such a bioassay is feasible depends on HON being the only antibiotic in the culture broth inhibiting growth of the test organism, in this case S. cerevisiae. Paper chromatography of samples from fractionated and unfractionated culture supernatants showed that the only region showing antibiotic activity was associated with the presence of HON. Therefore, it was reasonable to believe that the bioassay could safely be used in the isolation of HON-nonproducing mutants.

Because a large number of colonies must be screened, isolating blocked mutants depends on having an easy method of detecting them. As a strategy to develop a bioassay for HON, the agar-plug method was explored. This is not the easiest way to screen large numbers of colonies. Overlaying a sensitive strain on agar plated with antibiotic-producing colonies is more convenient. However, three practical reasons had to be considered. (1) Difficulties would be introduced with overlays of S. cere[illegible]producing colonies needed to be purified. (2) The medium used [illegible] medium for HON production. A thin layer of agar co[illegible] visiae did not support adequate growth for this orga[illegible]le it difficult to detect inhibition zone around colonies. [illegible] zones (1-1 5 cm in diameter), only 10 to 15 colonies could [illegible] dish without the risk of misinterpreting the results. Technically, it would [illegible] control the number of colonies with the desired accuracy.

B. Characterization of the mutants

Chemical mutagenesis was used to generate HON-nonproducing mutants. Since the production of pigments in these blocked mutants appears similar to that in the wild type, they are unlikely to be blocked in the steps regulating secondary metabolism in general. The fact that all blocked mutants were prototrophs indicated that they were blocked in the HON-specific pathway. This does not necessarily imply that the biosynthesis of HON involves reactions different to those used in primary metabolism. The biosynthesis of HON may use "duplicate" secondary metabolic enzymes. An example of this is in P. aeruginosa where there are two sets of anthranilate synthetase genes. One is involved in the biosynthesis of tryptophan and the other is involved in the biosynthesis of a secondary metabolite, pyocyanine (Essar et al., 1990).

The cross-feeding studies distinguished four groups of blocked mutants. One group showed no cosynthesis behaviour and may be blocked in one or more regulatory steps. The remaining three groups were capable of cross-feeding to restore HON production. Thus the biosynthetic pathway must contain at least three HON-specific steps. These are probably involved in pathway reactions that make intermediate(s) or the final product, but two other possibilities cannot be excluded: (1) a mutant may be blocked in the biosynthesis of a cofactor required specifically for the formation of HON. A precedent for this is the discovery during characterization of S. lincolnesis mutants that a riboflavin derivative, 7,8-didemethyl-8-hydroxy-5-deazariboflavin, accumulated by one mutant, restored lincomycin production in another mutant (Coats et al., 1989). (2) HON production may require the stimulation of a bacterial pheromone. In the biosynthesis of streptomycin in S. griseus, production of the antibiotic by some nonproducing mutants was restored by feeding culture filtrates of wild-type or other nonproducing mutants containing A-factor (Khokhlov et al., 1967). However, when

the supernatant of wild-type cultures was fed to mutants L167 and L127, each putatively blocked in early HON-specific steps, the production of HON was not elevated. The apparent absence of any stimulatory substance in the supernatants of wild-type cultures suggests that mutant L167 and L127 are not blocked in the production of an intraspecific regulatory agent such as a pheromone.

The conclusion from treating mutant cultures with sodium borohydride was that no keto-amino acids were present among the substances accumulated in L127 and L138. This implies that the substances accumulated are different from the postulated intermediates in the biosynthetic pathway. The hypothetical biosynthetic pathway assumed at the begining of this study must, therefore, be viewed with caution.

The substance accumulated in L138 and stimulating the production of HON in L127 is hydrophilic and relatively stable. The latter property will facilitate purification of the compound and structure determination. Preliminary chromatography on cation and anion exchange resins (data not shown) suggested that the substance lacks a free amino group. This situation occurs in the biosynthesis of bialaphos in S. hygroscopicus; the amino group is protected with a N-acetyl group which provides resistance for the cell and is cleaved only in the final step of the biosynthesis (Murakami et al., 1986).

No functional groups have yet been identified in the substance accumulated by mutant L138. Successful isolation of the compound relies heavily on a conversion assay, based on that developed in the present study, in which the production of HON by the converter (L127) is measured. Isolation of the L138 product is in progress (K. C. Smith, private communication).

IV. Isolation and characterization of auxotrophic mutants

The goal in isolating auxotrophs of the aspartate pathway was to obtain HON$^-$ mutants blocked in the early part of the pathway, in particular those mutants blocked at aspartate kinase and aspartate semialdehyde dehydrogenase. Theoretically, these mutants should exhibit growth requirements for at least diaminopimelate, methionine and threonine. Although some mutants showed growth requirements for more than one amino acid, their phenotypes were not clear cut. This may be a characteristic of such mutants in streptomycetes. In a thorough search for auxotrophs in S. coelicolor A3(2) (Hopwood and Sermonti, 1962), no ask and asd mutant was identified. Interestingly, no diaminopimelate-requiring mutant was identified in this study. Since the medium used in testing for a diaminopimelate requirement was MYM, and tests with the E. coli asd mutant CGSC 5080 showed no growth on MYM agar, but good growth when diaminopimelate was added, asd mutants of S. akiyoshiensis requiring a comparable level of diaminopimelate to E. coli asd mutants, should have been detected. However, if the amount of diaminopimelate required to support an asd mutant of S. akiyoshiensis is much lower than for E. coli, such a mutation might have been overlooked. This possibililty is supported by evidence that E. coli ask mutant CGSC 5074, which has a growth requirement for lysine, methionine and threonine due to mutations in its three aspartate kinase genes (Theze et al., 1974), did not require a diaminopimelate supplement to grow. It is also possible that no S. akiyoshiensis dap mutant was isolated because this species lacks a diaminopimelate transport system. Chen et al. (1993) have suggested this to account for their failure to obtain asd-specific mutants in bacilli. No diaminopimelate-requiring mutant has been found in other Gram-positive bacteria, except for a leaky diaminopimelate-requiring mutant in S. coelicolor A3(2) (Hopwood and Kieser, 1990).

The large number of methionine-requiring mutants may reflect multiple needs for

methionine in essential pathways due to its role as a precursor of methyl donors in vivo.

A. The putative ask mutant LC367Y

LC367Y contained less aspartate kinase than the wild-type strain. This could be due to a regulatory mutation, an incomplete mutation in a structural gene or a mutation inactivating one of several ask genes. Since there has been no report on the isolation of ask mutants in streptomycetes, and a thoroughly search for auxotrophs in S. coelicolor A3(2) did not identify any ask mutants, it is likely that more than one aspartate kinase is present in S. akiyoshiensis. In a preliminary experiment (data not shown), the cell extracts of wild-type S. akiyoshiensis were fractionated by precipitation with 0-45% and 45%-65% saturated ammonium sulfate. When lysine, which activated the aspartate kinase, was introduced into assay mixtures with the two partially purified fractions, substantial differences were seen between the two fractions. This may well be due to the presence of more than one aspartate kinase. Since HON production by LC367Y was markedly decreased from the wild-type level, the results suggest that ask is involved in HON biosynthesis.

B. The putative hsd mutant LC296Y

Enzyme assays for aspartate kinase and homoserine dehydrogenase suggested that LC296Y is an hsd mutant. The growth requirements of this strain may have been caused by a regulatory mutation, an incomplete mutation in a structural gene, or a mutation blocking one of two sets of hsd such as are present in E. coli. The fact that the mutant produced more HON than the wild-type strain indicated the mutation affects the biosynthesis of HON. Two possible scenarios were considered: (1) because of the mutation, feedback regulation by end-products (methionine and threonine) of the pathway was impaired. Therefore, more aspartyl

phosphate and perhaps aspartate semialdehyde were available, and were shunted to the HON-specific pathway. This increased the production of HON. (2) the hsd mutation caused a change in primary or secondary metabolism by decreasing the methionine concentration in the cellular pool, thus affecting the supply of methyl donors. Of the many biochemical processes that could be altered, some would indirectly affect HON production. When the results with hsd mutant LC296Y are considered along with those obtained with ask mutant LC367Y, the first scenario seems more likely.

C. The putative lysA mutants LC175X and LC461A

Diaminopimelate could not restore the growth of these strains suggesting that the mutation was in the gene for diaminopimelate decarboxylase. However, the possibility that diaminopimelate is not taken up by S. akiyoshiensis means that this is not a definitive test for such a mutant. In any case, since both mutants produced near wild-type amounts of HON, the biosynthesis of HON was not affected by the step after diaminopimelate decarboxylase.

D. Other auxotrophic mutants requiring methionine, threonine or lysine

Although the growth requirements for mutant LC153C, LC224X, LC242B and LC263Z suggested that these mutants might be blocked at steps in the early part of the aspartate pathway, the enzyme assays for aspartate kinase and homoserine dehydrogenase indicated that none of them was blocked in ask or hsd. Since they did not need diaminopimelate to grow, they are not likely to be asd mutants, but it is difficult to determine their mutations from growth requirements. These mutants were not further characterized.

E. Summary

The simplest interpretation of the results is that the biosynthesis of HON is not related to the steps after homoserine dehydrogenase and diaminopimelate decarboxylase, and that aspartate kinase is involved in HON biosynthesis. However, it is possible that the decreased HON production in a putative ask mutant is due to a second mutation; multiple mutations are not uncommon during treatment with NTG. Therefore, it would be advantageous to obtain a site-specific mutant blocked at a step in the early part of the aspartate pathway. Since S. akiyoshiensis may have more than one set of aspartate kinase genes, asd should be a more favorable gene for constructing a site-specific mutant that could be used in determining the branch-point to HON. This approach required that the S. akiyoshiensis asd gene should first be cloned.

V. Cloning and expression of the S. akiyoshiensis asd gene in E. coli

Since no asd mutants have been identified in streptomycetes and other Gram-positive organisms, the approach to cloning asd was to complement an E. coli mutant. Such an approach was used in the cloning of asd genes from S. mutans (Jagusztyn-Krynicka et al., 1982). The asd genes from other Gram-positive organisms, such as C. flavum (Follettie et al., 1993), C. glutamicum (Kalinowski et al., 1990) and B. subtilis (Chen et al., 1993), have also been expressed in E. coli asd mutants. Because the aspartate pathway in bacteria is highly conserved, it was anticipated that the S. akiyoshiensis asd protein would function in E. coli, if expressed. Most streptomycete genes do not possess the -10 and -35 hexameric sequences recognized by E. coli RNA polymerase containing sigma-70 (Strohl 1992); therefore, their promoters are not recognized. Although some streptomycete promoters, such as the promoters for S. coelicolor hisBd (Limauro et al., 1990), S. lividans argG (Meade,

1985) and S. lividans pabAB (Arhin and Vining, 1993), are recognized in E. coli, expression of most streptomycete genes requires an E. coli vector promoter upstream of the genes. In some cases, a spontaneous deletion in the cloned segment upstream the gene is needed, probably to remove terminator or requlatory sequences from the region between the gene and the vector promoter. For example, during expression of S. acrimycini chloramphenicol acetyltransferase in E. coli (Gil et al., 1985), a 0.7-kb sequence upstream of the gene was deleted. Sequence analysis suggested that DNA fusion had occurred via homologous recombination between two short repeat sequences spaced 0.7 kb apart (Murray et al., 1989). With these consideration in mind, it seemed reasonable to expect that the S. akiyoshiensis asd gene could be cloned by complementation in E. coli.

To maximize the possibility of expression, random fragments of genomic DNA more than 6-kb in size were ligated into a site in the vector downstream of the lac promoter. This provided an opportunity for expression of the S. akiyoshiensis asd gene in case its own promoter was not recognized by the E. coli transcription system. The recombinant plasmid pJV21 eventually isolated by complementation of the asd mutation in E. coli did, in fact, express the cloned S. akiyoshiensis gene from the lac promoter of the vector. The main evidence for this is that when the inserts in pJV21 and pJV24 were cloned downstream of the lac promoter in opposite orientations, asd was expressed only when the orientation to the lac promoter matched that of the recombinant plasmid initially isolated. Additional support came from the fact that asd in pJV24 was expressed in E. coli more efficiently than in pJV21, (the gene in pJV24 is 1.5-kb closer to the lac promoter than that in pJV21). DNA sequence data for the cloned asd was also consistent with failure of the S. akiyoshiensis asd promoter to be recognized in E. coli.

Since subcloning by complete digestion of the pJV21 insert with SalI did not yield any

recombinant plasmids complementing the asd mutation in E. coli, partial SalI digestion of the insert was explored as a strategy for removing DNA segments to localize the asd gene. This yielded pJV24 containing a 2.2-kb insert with a SalI site in the middle. Attempts to further narrow down the asd gene by a partial SalI and a complete SstI digestion were not successful. The reason for this became apparent when the DNA sequence was determined; two adjacent SstI sites and a SalI site are located inside the asd gene.

VI. Sequence analysis of the 2.2-kb SalI-SalI fragment containing asd

A. Open reading frames and codon usage

The nucleotide sequence of the 2.2-kb SalI-SalI fragment showed that it consisted of 2197 bp. Because of their high G+C content, streptomycete genes are biased towards codons containing these bases, particularly in the third position. Analysis of the nucleotide sequence with the CODONPREFERENCE program and a codon usage table calculated by Wright and Bibb (1992) identified two complete open reading frames (ORF1 and ORF2) and an incomplete open reading frame (ORF3). ORF1 and ORF2 have 69.1% and 70.8% G+C, respectively, at the first codon postion; 44.7% and 57.1%, respectively, at the third codon position; and 96.5% and 90.1%, respectively, at the third codon position. This is comparable to the average frequency in streptomycetes of 69.7% at the first codon position, 49.9% at the second codon position, and 90.6% at the third position (Seno and Baltz, 1989). The overall G+C contents of 70.1% and 72.6% in ORF1 and ORF2, respectively, are similar to the average (70.1%) for streptomycetes.

B. Transcriptional and translational control

1. Promoter sequence

Some streptomycete promoters are similar to those recognized by E. coli sigma-70 (Strohl, 1992). The space between their -10 and -35 consensus sequences varies from 16 - 18 bp, as in E. coli. However, most streptomycetes do not have promoters containing the -10 and -35 consensus characteristics of E. coli. ORF1 and ORF2 belong in the latter category and would not be expected to be recognized by E. coli sigma-70 like RNA polymerase.

2. Transcriptional terminator

Inverted repeat sequence downstream of the translational stop codon can form stem-loop mRNA secondary structures and serve as transcriptional teminators in E. coli. Similar structures have been found in streptomycetes, and were present in ORF1, ORF2 and ORF3.

3. Ribosomal binding sites

In most streptomycetes, translational starts are preceded by a ribosomal binding site similar to that identified in E. coli by Shine and Dalgarno (1974). This sequence is complementary to a sequence near the 3'-end of 16S rRNA and aligns the ribosome with mRNA to initiate translation. The 3'-end of S. lividans 16S rRNA contains a sequence GAUCACCUCCUUUCU-3' (Bibb and Cohen, 1982). In ORF1 the putative ATG start codon is preceded by a possible ribosomal binding site AGGTGG; the putative GTG codon is preceded by the possible ribosomal binding site GAGG. The distance between the suggested ribosomal binding sites and start codons are in the average range (5-12 bp) for streptomycetes (Strohl, 1992). A possible ribosomal binding site, GGTG, is located 8 bp before the ATG start codon of ORF2.

C. Comparative analysis of the amino acid sequence deduced from ORFs

1. ORF1

The deduced amino acid sequence for ORF1 showed strong similarity to aspartate

semialdehyde dehydrogenase genes in the GenBank database. Noteworthy among these were the asd genes from C. flavum (GenBank accession number L16848), C. glutamicum (Kalinowski et al., 1990), M. smegmatis (Cirillo et al., 1994) and B. subtilis (Chen et al., 1993). In particular, asd from S. akiyoshiensis shares more than 62% identical and 72% similar amino acids with asd from corynebacteria and mycobacteria. Since they are all high % G+C Gram-positive bacteria, this presumably reflects their close evolutionary relationship.

Based on amino acid sequence comparison, GTG at nt 160-162 of strand A is most likely to be the transcriptional start codon. It gives a deduced protein more in line with the size of asd proteins from other Gram-positive organisms. In addition, a computer alignment of the protein showed that the amino acid sequence lying between nt 160-162 and the alternative start codon (ATG at nt 205-207 of strand A) was very similar to the corresponding region of asd genes from other Gram-positive organisms.

The deduced amino acid sequence contained a region very similar to the active site suggested for aspartate semialdehyde dehydrogenase in E. coli (Biellmann et al., 1980; Hazzia et al., 1982) and S. mutans (Cardineau and Curtiss, 1987). The conclusion that ORF1 encodes an asd gene was supported by failure of an incomplete ORF1, in which the putative ribosomal binding site and a portion of deduced N-terminal sequence were deleted, to complement the asd mutation in E. coli.

2. ORF2 and ORF3

Since a BLAST database search in GenBank did not show strong similarities between the deduced amino acid sequences for both ORF2 and ORF3 and any other protein sequence, it was not possible to deduce the function of their products. This, and the absence of other ORFs in the neighbourhood of asd had implications for the organization of aspartate pathway genes.

D. Gene organization for aspartate kinase and aspartate semialdehyde dehydrogenase

Comparisons of five Streptomyces genomes has shown extensive structural conservation of primary metabolism genes (Stuttard, 1988). Therefore, the information obtained in S. akiyoshiensis should be useful in streptomycetes generally. To date, no clear-cut mutant blocked in core reactions of the aspartate pathway, namely aspartate kinase and aspartate semialdehyde dehydrogenase, has been reported, so that the loci for these genes in streptomycetes have not been mapped. DNA sequence data for the S. akiyoshiensis asd gene and its adjacent region should give an indication of whether these genes are clustered.

Because there are strong sequence similarities among the asd genes from corynebacteria, mycobacteria and bacilli, and the asd in these organisms are all clustered with ask or ask-like sequences, it was anticipated that S. akiyoshiensis ask and asd genes would be clustered. In two high %G+C Gram-positive organisms, corynebacteria (Folleittie et al., 1993; Kalinowski, et al, 1991) and mycobacteria (Cirillo et al., 1994), asd is located immediately downstream of akα and akß, which encode the α- and ß-subunits of aspartate kinases. Follettie et al. (1993) investigated expression of the C. flavum asd-ask operon and proposed that either these three genes are expressed from the promoter upstream of akα, or the promoter upstream of akß is responsible for expression of the two downstream genes. In bacilli, asd is followed by dapG (Chen et al., 1993), one of the three aspartate kinase genes responsible for diaminopimelate biosynthesis. These two genes are organized in an operon with other genes involved in diaminopimelate biosynthesis.

The failure of pJV21 and pJV24 to complement an E. coli ask mutant did not necessarily mean that no ask gene was present in these plasmids. However, the DNA sequence downstream of asd in S. akiyoshiensis does not show any ask-like ORF. In fact, there is no ORFs downstream of, and transcribed in the same orientation as asd in the 2.2-kb

insert. The complementary sequence (on strand B) contains a complete ORF (ORF2) in this region and an incomplete ORF (ORF3), but their deduced amino acid sequences (as noted above) do not match those of ask genes. Sequence analysis of the region upstream of asd in pJV21 has not identified any ask-like ORF, but there is an ORF that encodes a putative sigma factor (J. He, personal communication). Since the original transformant containing pJV21 was not exposed to selection for Asd^+, the insert in this plasmid would not have encountered conditions favoring a spontaneous deletion to allow expression of the streptomycete asd gene in E. coli. Therefore, the sequence data indicating that ask genes are absent from the region upstrea n of asd in S. akiyoshiensis are valid evidence that asd is not clustered with an ask gene. This is similar to the situation in E. coli where none of the ask genes is clustered with asd

VII. Efforts to construct an asd-specific mutant

A. Transformation of S. akiyoshiensis

Before a site-specific S. akiyoshiensis mutant can be constructed, a DNA transformation system is needed. Suitable procedures were developed during the present studies.

Dispersal of mycelium is an important prerequisite for preparing protoplasts and for plasmid isolation. Well dispersed growth provides access to the mycelial surface during lysozyme treatment. Since a medium containing Trypticase Soy Broth (TSB) gave relatively dispersed growth of S. akiyoshiensis in an early experiment, its suitability as a major component in media was tested. The results suggested that TSB C medium gave relatively dispersed growth. This was improved by using results from a morphological study of S. akiyoshiensis (Glazebrook et al., 1992) showing that pH 5.5 gave more dispersed growth than

a neutral pH. In TSB C medium S. akiyoshiensis gave optimally dispersed growth at pH 5.7-6.1. This medium also gave superior results in preparing seed cultures or cultures for plasmid and chromosomal DNA isolation.

Development of a transformation system for S. akiyoshiensis facilitated the genetic study, and the procedure is now being used in gene disruption/replacement experiments to create an asd-specific mutant. Since a relatively high transformation efficiency was achieved, this organism can be used as a cloning host for genes from other streptomycetes.

B. Gene disruption and replacement

Homologous integration is the basis for molecular analysis of the function of a gene. It is also used routinely to construct site-specific mutants. If an asd mutant could be constructed, it would provide a definite answer to where HON biosynthesis branches from the aspartate pathway. Moreover, an asd mutant is potentially important to the biotechnology industry. A plasmid carrying an asd gene would be stably maintained in an asd mutant without selection of markers on the plasmid. This could replace the use of antibiotics to select and maintain strains producing certain biotechnology products. The latter practices are not permitted by the U. S. Food and Drug Administration. A balanced lethal system of this type has been used to express foreign antigen genes in a Salmonella typhimurium vaccine strain (Nakayama et al., 1988). As a very efficent transformation system has been established for S. akiyoshiensis, and Streptomyces have been used to express medically important recombinant proteins, an Asd^+ expression-cloning vector stably maintained in an S. akiyoshiensis asd mutant should be potentially useful.

A variety of plasmid vectors can be used to transform streptomycetes and are stably maintained. However, recombinant plasmids carrying homologous DNA are not stable and

are often integrated into the chromosome by a Campbell type recombination (Campbell, 1962). A single cross-over between an internal fragment of a plamid-borne gene and the chromosomal gene would give two incomplete segments of the gene in the chromosome; thus the gene is disrupted. Gene replacement uses recombinant plasmids carrying an inactivated gene. A selectable marker, usually for antibiotic resistance gene, is inserted into the gene to create a mutation. If double cross-overs occur between the recombinant plasmid carrying an inactivated gene and the chromosome, the wild-type gene can be replaced with the inactivated one. The frequency of a single or double cross-over recombination is proportinal to the size of the homologous DNA sequence. However, 300 bp of homologous DNA is sufficient to cause integration of a recombinant plasmid into the Saccharopolysora erythaea chromosome (Weber et al., 1990).

To obtain a disruption or replacement mutant, the autonomous plasmid must be lost after the integration event so that the integrant strain can be identified. Various vectors can be used to achieve this. Nonreplicative vectors are usually E. coli plasmids lacking a streptomycete replicon. Such vectors have been successfully used in the construction of an S. ambofaciens mutant defective in spiramycin biosynthesis (Richardson et al., 1990). Conditional vectors, such as the temperature-sensitive pGM plasmids, which are stably maintained in the host below 34°C and are lost at 39°C, have been used to construct a mutant (pat) blocked in the resistance gene for bialaphos in S. viridochromogenes (Muth et al., 1989). The segregatively unstable vector pHJL400, lacking the par sequence involved in partition, and thus lost at a very high frequency when antibiotic marker is not selected, has been used to generate insert-directed integrations in S. griseofuscus (Larson and Hershberger, 1990) and in S. venezuelae (Paradkar, 1991).

Single stranded vectors have been shown effective in avoiding restriction barriers and

thereby increasing the integration rate (Hilleman et al., 1991). A single-stranded non-replicative vector has been used successfully to construct mutants defective in the biosynthesis of the polyketide antibiotic griseusin in S. griseus (Yu et al., 1994). Since the asd gene and its sequence are now available, it should be possible to construct an asd mutant by one of the above procedures.

SUMMARY AND CONCLUSIONS

Synthesis of HON in an in vitro system containing cell extracts, putative substrates and cofactors could not be demonstrated. Some likely reasons for this were investigated, and an alternative approach through the isolation of mutants was explored. To make possible the isolation of mutants blocked in HON biosynthesis, bioassays based on antifungal activity were developed. By screening NTG-treated S. akiyoshiensis spores for mutants that lacked such activity, nonp[illegible]ag mutants were isolated. Cross-feeding of mixed mutant cultures identified four distinct groups. One of them failed to show any cosynthesis, and was classified as a regulatory group. For the remaining three groups the sequence of the mutations in HON biosynthesis was determined by analysis of feeder converter interactions. This distinguished at least three biosynthetic steps; since none of the blocked mutants was auxotrophic, these must be HON-specific reactions.

To identify where the HON-specifc pathway branches from the primary pathway, auxotrophic mutants blocked in the biosynthesis of aspartate family amino acids were isolated by screening NTG-treated S. akiyoshiensis spores. Putative mutants in the genes for diaminopimelate decarboxylase (lysA), homoserine dehydrogenase (hsd) and aspartate kinase (ask) were identified from their growth requirements and enzyme activities. Since HON production by putative lysA mutants was similar to that of the wild-type strain, the HON-specific pathway was presumed not related to the steps after diaminopimelate decarboxylase. The putative homoserine dehydrogenase (hsd) mutant produced more HON than a revertant and the wild-type strain. This suggested that the HON-specific pathway does not require the steps after homoserine dehydrogenase, but may involve the steps that precede this enzyme. This argument was supported by the substantial decrease in HON production by the putative aspartate kinase (ask) mutant compared with the wild-type strain.

To confirm that the early part of the aspartate pathway is involved in HON biosynthesis, and to develop the procedures necessary for site-specific mutagenesis, a partial Sau3AI digest of chromosomal DNA was shot-gun cloned in the BamHI site of pHJL400. Transformants expressing asd were selected by complementation of an E. coli asd mutant. This yielded plasmid (pJV21) carrying asd on a 10.3-kb insert. The asd on pJV21 was then localized to a 2.2-kb fragment by SalI partial digestion. The complete nucleotide sequence of the 2.2-kb SalI-SalI fragment was analyzed by the CODONPREFERENCE program to detect potential ORFs. Two complete (ORF1 and ORF2) and one incomplete (ORF3) ORFs were identifed.

In a BLAST comparison with the database in GenBank, ORF1 showed strong amino acid sequence identity with the asd genes from Gram-positive organisms. As further evidence that ORF1 encodes an asd gene, a small portion of the putative N-terminal sequence and the ribosomal binding site were removed. The truncated recombinant plasmid failed to complement the asd mutation in E. coli. Since neither ORF2 nor ORF3 showed strong amino acid sequence similarity to known proteins in a BLAST comparison, their functions could not be deduced. Analysis of the regions upstream and downstream of asd in S. akiyoshiensis did not detect any ask-like sequence (J. He, personal communication) and therefore, the organization of the genes for aspartate kinase and aspartate semialdehyde dehydrogenase in S. akiyoshiensis is apparently different from that in two high-% G+C Gram-positive organisms, namely corynebacteria (Follettie, et al., 1993) and mycobacteria (Cirillo et al., 1994), where asd is preceded by an ask gene. It also differs from that in the low-% G+C Gram-positive bacilli, where asd is followed by an ask gene (Chen et al., 1993).

To facilitate molecular biology studies in S. akiyoshiensis, the culture conditions for preparing protoplasts were optimized and an efficient DNA transformation system was

developed.

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