CHARACTERIZATION AND REGULATION OF THE *speA* GENE IN *ESCHERICHIA COLI*

by

Robert C. Moore, Jr.

Dissertation submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements of the degree

DOCTOR OF PHILOSOPHY

in

Veterinary Medical Sciences

APPROVED:

S.M. Boyle, Chairman

D. Dean

J. Johnson

G. Lacy

T. Larson

N. Sriranganathan

August, 1990
Blacksburg, Virginia

ABSTRACT

In *Escherichia coli*, the *speA* gene encodes biosynthetic arginine decarboxylase (ADC), the first enyme in a putrescine biosynthetic pathway. ADC converts arginine to agmatine, which is hydrolyzed by agmatine ureohydrolase, encoded by the *speB* gene, to putrescine and urea. ADC is negatively regulated by mechanisms requiring either cAMP and cAMP receptor protein (CRP) or putrescine. A 3,236 base pair (bp) *BalI-AccI* restriction fragment derived from plasmid pKA5, which contains a 7.5 kilobase (kb) *E. coli* genomic fragment in pBR322, was subcloned into pGEM-3Z to produce plasmids pRM15 and pRM59. Both pRM15 and pRM59 overexpress ADC and the DNA sequence of the *BalI-AccI* fragment in each plasmid was determined. A 2,119 bp restriction fragment containing 730 bp 5' to *speA*, the *speA* promoter, and 1,389 bp (463 amino acids) of the 5'-end of *speA* was used to construct transcriptional (pRM161 and pRM162) and translational (pRM65) *speA-lacZ* fusion plasmids. The presence of the predicted 160,000 and 157,000 dalton ADC::β-galactosidase hybrid proteins produced by pRM65 was confirmed by Western blots. Single copies of transcriptional or translational *speA-lacZ* fusions were transferred to the chromosomes of *E. coli* strains CB806, KC14-1, and MC4100 using lambda phage. The presence of a second copy of the *speA* promoter in the *E. coli* chromosome did not affect cAMP or putrescine-mediated regulation of ADC or the growth rate of any of the strains.

E. coli strains carrying single copies of *speA-lacZ* fusions were grown in the absence or presence of either cAMP or putresine, or both. The β-galactosidase activities of each strain was measured and compared to the ADC activity. Addition of 5 to 10 mM cAMP to the strains inhibited ADC activity, but did not effect β-galactosidase activity, indicating post-

translational control of ADC. The sensitivity of each strain to 10 mM cAMP and the degree of the inhibition (4 to 40%) was strain dependent. Addition of 10 mM putrescine repressed *speA-lacZ* fusions (40 to 47%) and inhibited ADC activity in all strains. The inhibition of ADC activity in *E. coli* strains supplemented with both 5 mM cAMP and 10 mM putrescine was additive. As the expression of the *speA-lacZ* fusions were repressed by 10 mM putrescine, but not by 5 mM cAMP, cAMP and putrescine regulate ADC by independent mechanisms.

This dissertation is dedicated to my parents, Bob and Ann,

with love and graditude.

ACKNOWLEDGEMENTS

In every person's life there are people who "make a difference". I want to thank two people in particular who have helped me develop as a person and a scientist. I began my scientific training in the laboratory of Dr. John Dunn, who taught me how to handle failure, appreciate success, and enjoy science. Dr. Stephen Boyle, my graduate advisor, allowed me to learn from my mistakes, expected the most of me, and showed me how to succeed in spite of the obstacles.

I would like to thank my advisory committee, Dr. Dennis Dean, Dr. John Johnson, Dr. George Lacy, and Dr. Nammalwar Sriringanathan for their helpful suggestions and guidance during the project. Dr. Tim Larson provided assistance and graciously acted as a substitute on my defense committee. I have also had the pleasure of working with a great group of fellow graduate students, Maria Szumanski, Leigh Busse, Lynn McGonagle, John McQuiston whom I would like to thank for their encouragement and support during times of extreme stress. Thanks also to Lisa Barroso, John Kromhout and Erin Latimer for all their assistance. I would like to express my graditude to Sirkka for her patience and understanding, for it would not have been the same without her. Finally, I want to extend my deepest appreciation to Durin for his company and friendship during the best and the worst of times.

Parts of my dissertation were supported by a Cunningham Fellowship and a Graduate Research Development Award from Virginia Polytechnic Institute and State University. Additional support was provided by a Grant-in aid from Sigma Xi.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADC = biosynthetic arginine decarboxylase

ADI = biodegradative arginine decarboxylase

ARG = arginase

AUH = agmatine ureohydrolase (agmatinase)

CTAB = hexadecyltrimethyl ammonium bromide

cAMP = cyclic adenosine-3',5'-monophosphate

CRP = cAMP receptor protein

Da = daltons

DapDC = diaminopimelic acid decarboxylase

HDC = histidine decarboxylase

IPTG = isopropylthio-β-D-galactopyranoside

LDC = lysine decarboxylase

MAT = methionine adenosyltransferase

MOPS = 3-(N-morpholino)-propanesulfonic acid

ODC = ornithine decarboxylase

ONPG = o-nitrophenyl-β-D-galactopyranoside

ORF = open reading frame

ppGpp = guanosine tetraphosphate

pppGpp = guanosine pentaphosphate

SAM = S-adenosylmethionine

UV = ultraviolet

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

POLYAMINES IN *E. coli*

Description of polyamines

Polyamines are low molecular weight (<500 Da), aliphatic cations present in all cells. The most common polyamines are putrescine (a diamine) and its derivative, spermidine (a triamine). Prokaryotes usually contain levels of putrescine several fold higher than spermidine. Eukaryotes contain putrescine, but generally have higher levels of spermidine and spermine. Spermine (a tetra-amine) is present in all eukaryotic cells, but is absent in most prokaryotes. At physiological pHs, these basic compounds have a high affinity for all negatively charged components of the cell. Polyamines affect many biological processes in *Escherichia coli*, including DNA replication (Geiger and Morris, 1980; Seyfried and Morris, 1979), RNA transcription (Abraham, 1968), protein synthesis (Algranati and Goldemberg, 1977; Atkins et al., 1975), and biogenesis of ribosomes (Echandi and Algranati, 1975a; Igarashi et al., 1981). Intracellular levels of polyamines and polyamine derivatives influence the growth rate of cells. As the growth rate of cells increase, the intracellular concentration of polyamines and polyamine biosynthetic enzymes also increases (Boyle et al., 1977). *E. coli* polyamine auxotrophs have reduced rates of growth and nucleic acid synthesis compared to auxotrophs supplemented with polyamines (Dion and Cohen, 1972b). Acetylated and oxidized derivatives of polyamines when added exogenously, inhibit many functions in bacteria and mammalian cells. For example, oxidized spermine inhibits nucleic acid and protein synthesis in both *E. coli* and tissue culture. Polyamine derivatives also inactivate DNA and RNA viruses (Bachrach, 1973; Cohen, 1971).

The mechanisms regulating intracellular polyamine levels and the expression of polyamine biosynthetic enzymes are influenced by a variety of effectors. This complex control of expression is not surprising considering the myriad of processes that polyamines appear to influence. Although the existence of polyamines has been known for over 300 years, specific physiological roles have yet to be clearly defined. Research on polyamines has been reviewed for bacteria (Tabor and Tabor, 1985), plants (Smith, 1985), fungi (Stevens and Winther, 1979), and animals (Pegg, 1986). This introduction will focus on the synthesis and regulation of polyamines, their biosynthetic enzymes, and cAMP-mediated gene regulation in bacteria.

Discovery and growth requirement

Antoni van Leeuwenhoek first described polyamines in 1678 in a report to the Royal Society of England on the study of human semen. He wrote "When this matter had stood a little while, some three-sided bodies were seen in it, terminating at either end in a point; some of the length of the smallest grain of sand, and some were a little bigger" (Leeuwenhoek, 1678). We now know that Leeuwenhoek was describing the inorganic phosphate of the polyamine, spermine. It was not until 1888, after this polyamine had been found in other animal tissue, that A. Ladenburg and J. Abel chose the name spermine due to its high concentrations in semen (Ladenburg and Abel, 1888).

Polyamines were reported to be required for growth in 1948 by Herbst and Snell (Herbst and Snell, 1948). They isolated a factor, putrescine, from orange juice that was essential for normal growth of the bacterium *Haemophilus parainfluenzae* (Herbst and Snell, 1948). The putrescine requirement of *H. parainfluenzae* was confirmed in 1981 by March and Boyle (March and Boyle, 1981), who showed that the bacterium lacked putrescine biosynthetic enzymes and was unable to synthesize putrescine. Polyamine mutants of

Aspergillus nidulans (Sneath, 1955), *Neurospora crassa* (Deters et al., 1974), and *Saccharomyces* (Cohn et al., 1980; Whitney and Morris, 1978) were later isolated that also require putrescine for optimum growth. The use of naturally occurring and induced polyamine mutants remains a valuable tool in understanding the biosynthesis and regulation of polyamines and their role in cell metabolism.

Polyamine transport

Little is known about how polyamines are transported across the cell envelope. Putrescine and spermidine are actively transported into the cell against a gradient (Tabor and Tabor, 1966, 1985). Höltje (1978) found that polyamines entered the cell by the same transport system used by streptomycin and is dependent on a proton motive force (Kashiwagi et al., 1986). The import of putrescine in *E. coli* is strongly inhibited by monovalent cations. The divalent magnesium ion inhibited the uptake of spermidine and spermine, but not putrescine. Although the intracellular concentrations of free polyamines remains undetermined in *E. coli*, the levels are probably very low due to the high affinity of polyamines for many of the negatively charged components of the cell. Boyle et al. (1977) calculated that the concentration of putrescine and spermidine in *E. coli* would be 42 mM if they were unbound.

Excretion of putrescine is also an energy dependent process. There is an inverse relationship between intracellular putrescine levels in *E. coli* and the osmolality of the culture medium (Munro et al., 1972). *E. coli* excrete putrescine when grown in a high osmolality culture, resulting in decreased intracellular putrescine levels (Munro et al., 1972; Munro and Sauerbier, 1973). The replacement of K^+ ions with Na^+ in a K^+ transport mutant leads to a five- to eight-fold increase in putrescine production (Rubenstein et al., 1972). *E. coli* treated with α-methylornithine, an inhibitor of ornithine decarboxylase,

resulted in a 60% decrease in intracellular putrescine and spermidine, yet the Na^+ and K^+ concentrations were unaffected (Gunther and Peter, 1979). Therefore, polyamines do not appear to play a significant role in osmoregulation.

Polyamine regulation of growth rate

A number of findings suggests that the stimulatory effect of putrescine and spermidine on the growth of polyamine-starved bacteria is due to the control of ornithine synthesis by polyamines. Cunningham-Rundles and Maas (1975) used an *E. coli* polyamine auxotroph to show that arginine can partially replace the polyamine requirement for growth. The ability of arginine to increase the growth rate can be explained by either of two mechanisms. Arginine could reduce intracellular ornithine levels by feedback inhibition and repression of the arginine biosynthetic pathway or arginine could induce putrescine biosynthesis. It is now clear that arginine effects the growth rate of *E. coli* by controlling intracellular ornithine levels. *E. coli* polyamine auxotrophs grown in the absence of polyamines contained high levels of ornithine (Goldemberg and Algranati, 1977). When putrescine or spermidine was added to culture medium, almost all of the ornithine disappeared. Later studies by Cataldi and Algranati (1989) showed that the growth rate of several *E. coli* polyamine mutants grown in minimal medium decreased when intracellular concentrations of ornithine were high. In the absence of polyamines, the cellular ornithine concentration increased to levels that were toxic. Upon addition of either putrescine, spermidine, arginine, citrulline, or arginosuccinic acid, the growth rate increased. The addition of arginine did not alter intracellular polyamine levels, but the addition of arginine, putrescine, or spermidine caused a 40-fold reduction in intracellular ornithine levels. These results indicate that arginine and polyamines can regulate the growth rate of *E. coli* by controlling ornithine biosynthesis and thus reducing the toxic effects of ornithine accumulation.

Polyamine starvation

Polyamine starvation has a dramatic affect on protein synthesis in bacteria. There was a reduction in the elongation rates of β-galactosidase enzyme in polyamine-starved cells. The reduction was proportional to the growth rate (two-fold), with no change in the rate of protein turnover (Jorstad and Morris, 1974; Morris, 1973; Morris and Hansen, 1973). Polyamines can stimulate protein synthesis *in vitro* and enhance the fidelity of translation (Algranati and Goldemberg, 1977; McMurry and Algranati, 1986). The translation rate of an *E. coli* polyamine auxotroph increased several fold when supplemented with polyamines compared to polyamine starved cells (Echandi and Algranati, 1975a, 1975b). In eukaryotic systems, spermidine or spermine produces a greater stimulation of translation than does putrescine (Atkinset al., 1975).

Polyamines and ribosomes

Ribosomes were the first macromolecular structure found to contain polyamines (Algranti and Goldemberg, 1977). The role of polyamines in translation was analyzed by reconstituting ribosomes using 30S or 50S ribosomal subunits purified from either a polyamine-starved or -supplemented polyamine auxotroph (Echandi and Algranati, 1975a). In *E. coli*, the 30S subunit is responsible for formation of an initiation complex with mRNA and intiation factors. The 50S subunit aligns the aminoacylated tRNA anticodons with the mRNA codons. The reduced translation rate of polyamine starved cells was partially explained by the discovery that the 30S ribosomal subunit had a decreased affinity for the 50S subunit. Therefore, these cells formed fewer functional ribosomes (Echandi and Algranati, 1975b). In 1981, Igarashi et al. measured levels of 30S subunit ribosomal proteins in ribosomes obtained from an *E. coli* polyamine auxotroph starved for polyamines (Igarashi et al., 1981). They found that ribosomes from polyamine depleted *E. coli* contained less

ribosomal protein S1 compared to the ribosomes from polyamine supplemented bacteria. This result was confirmed using an auxotroph blocked in a different step in polyamine biosynthesis. Igarashi concluded that the depletion of S1 was responsible for the reduced rate of translation in polyamine starved cells.

Streptomycin, a cationic antibiotic, inhibits translation by binding to the 30S subunit of the ribosome. The antibiotic enters *E. coli* via an inducible polyamine transport system (Höltje, 1978). The interaction of streptomycin and ribosomes appears to require polyamines. Streptomycin added to a culture of a *E. coli* polyamine auxotroph reduced the level of protein synthesis only when the cells were supplemented with polyamines. Streptomycin inhibited the rate of translation less in polyamine starved cells. In contrast, the absence of polyamines conferred streptomycin resistance. In *E. coli*, a *strA* (also known as *rpsL*) point mutation alters the structure or conformation of the S12 ribosomal protein in the 50S ribosomal subunit and produces a streptomycin resistant phenotype. Introduction of the *strA* mutation into an *E. coli* polyamine auxotroph produced a strain with an absolute requirement for polyamines (Tabor et al., 1981). This suggests that the addition of polyamines *in vivo* and *in vitro* improves the fidelity of translation by binding to the 50S ribosomal subunit to stabilize the polysome (Tabor and Tabor, 1985).

Stringent response

Amino acid starvation of *E. coli* results in the cessation of protein and RNA synthesis, and the accumulation of guanosine tetra- (ppGpp) and penta-phosphate (pppGpp). This stringent response causes a 10- to 20-fold reduction in the synthesis of ribosomal RNA and transfer RNA. As a result, there is an approximately three-fold reduction in total mRNA synthesis in the cell. A mutation in the *E. coli relA* locus produces a relaxed response to amino acid starvation. *E. coli relA* cells fail to produce ppGpp when starved for amino acids,

but remained blocked in protein synthesis. Consequently, *relA* cells do not coordinately inhibit the initiation of transcription of ribosomal RNA operons or reduce the transcriptional efficiency of the cell. The control of RNA synthesis, cellular RNA levels, and the stability of the RNA are unaltered in polyamine mutants starved for amino acids (Morris and Hansen, 1973b, 1973c; Srinivasan, 1973). These findings lead to the conclusion that putrescine and spermidine are not absolutely required for normal growth.

Polyamines and nucleic acids

By their basic nature, polyamines form strong non-covalent interactions with the acidic phosphate groups of nucleic acids (Bachrach, 1973; Cohen, 1971; Tabor and Tabor, 1976). Polyamines aid in the condensation of bacteriophage DNA into particles by reducing the electrostatic repulsion between phosphate groups in the backbone of the nucleic acid helix. For example, phage T4 DNA is packaged with enough putrescine and spermidine to neutralize approximately 50% of its negative charge (Ames and Dubin, 1960). Polyamines also affect the rate of DNA synthesis. A conditional putrescine auxotroph grown in the presence of exogenous arginine exhibited a rapid increase in RNA and DNA synthesis (Young and Srinivasan, 1972). When T4 phage infect an *E. coli* polyamine auxotroph, there is a reduction in the rate of phage DNA synthesis and maturation (Dion and Cohen, 1972a). These effects are reversed by the addition of polyamines 15 min prior to infection. Polyamine limited growth of *E. coli* reduced the rate of DNA replication fork movement in proportion to the growth rate (Geiger and Morris, 1978, 1980). Under normal growth conditions, the growth rate affects the initiation, but not the movement of the replication fork.

Polyamines and DNA supercoiling

Polyamines may affect the degree of supercoiling of the bacterial chromosome.

Spermidine stimulates *Micrococcus luteus* DNA gyrase *in vitro*, increasing the degree of negative supercoiling of the circular DNA chromosome. (Liu and Wang, 1978). Conversely, spermidine inhibits topoisomerase I, which relaxes supercoiled DNA (Lipetz et al., 1980). Therefore, spermidine has the potential to increase the degree of negative supercoiling of DNA. The degree of supercoiling can influence the expression of bacterial genes both *in vivo* and *in vitro* (Horwitz and Loeb, 1988). For example, DNA gyrase expression is induced in bacteria as the degree of supercoiling decreases (Menzel and Gellert, 1987).

AMINO ACID DECARBOXYLASES

Description

In 1940, during an investigation of the mechanism of amine production, Gale discovered six amino acid decarboxylases, including those specific for arginine, ornithine, and lysine (Gale, 1940). The expression of each of these enzymes required an acidic culture pH (pH<6) and the presence of its substrate. Later studies of putrescine biosynthesis in *E. coli* revealed the existence of two sets of arginine and ornithine decarboxylases (Morris and Pardee, 1965, 1966). The biosynthetic decarboxylases are responsible for the synthesis of putrescine in cells grown in neutral minimal medium. The biodegradative decarboxylases degrade arginine and ornithine in cells grown in an acidic enriched medium. Biosynthetic amino acid decarboxylases are found in all strains of *E. coli*, but the biodegradative forms are often absent. For example, biodegradative ornithine decarboxylase is found in only one of ten strains of *E. coli* (Applebaum et al., 1977). Biodegradative amino acid decarboxylases are thought to have evolved by divergence from the biosynthetic forms (Applebaum et al., 1977) and serve as an adaptive mechanism to regulate intracellular pH in cells grown in an acidic culture (Gale, 1946).

Biodegradative amino acid decarboxylases

In 1946, Gale proposed that amino acid decarboxylases might be important in maintaining the intracellular bicarbonate concentration under acidic growth conditions (Gale, 1946). The physiological role of biodegradative decarboxylases was investigated using an *E. coli* strain with defective biodegradative arginine (ADI) and ornithine decarboxylases. These enzymes were found to be required for growth in an acidic medium. In 1972, Recsei and Snell (1972) isolated and characterized a mutant of *Lactobacillus* that lacked histidine decarboxylase but retained ornithine decarboxylase. This mutant was unable to grow at a culture pH of 4.3 even when supplemented with histidine. When ornithine was added to the culture, both the wild type and the mutant grew at pH 4.3. These results indicated that the major defect in the histidine decarboxylase mutant was its inability to regulate the culture medium pH; decarboxylation of ornithine restored that ability. Based on these results, Recsei and Snell proposed that biodegradative amino acid decarboxylases function to neutralize the acid byproducts of carbohydrate fermentation by consuming protons.

E. coli produces ADI when grown anaerobically in an enriched acidic medium containing arginine. Under these growth conditions the enzyme represents approximately 3% of the total protein in a crude extract. ADI has been purified to homogeneity, crystallized, and has a pH optimum of 5.2 (Blethen et al., 1968; Boeker and Snell, 1968; Boeker et al., 1969, 1971). Arginine is the preferred substrate, but cadaverine and ornithine can be used at a low rate. The enzyme requires pyridoxal phosphate for activity, with 10 pyridoxal phosphate binding sites per molecule. The pyridoxal phosphate binds to the lysine next to a histidine residue, like several other amino acid decarboxylases (Boeker et al., 1969). The ADI gene maps to a region between 89 and 2 minutes on the *E. coli* chromosome (Maas et al., 1970). Recently, Mud*lac* fusions were constructed to the *E. coli* K-12 ADI (Auger et al., 1989).

These fusions have been mapped by P1 transduction to 93.4 minutes, near the *melAB* operon.

Biodegradative ornithine decarboxylase has been purified to homogeneity from *E. coli* strain UW44 cultured in a rich medium containing excess ornithine (Applebaum et al., 1975, 1977; Morris and Boeker, 1983). The enzyme has a pH optimum of 6.9, much higher than most biodegradative decarboxylases. In these growth conditions ornithine decarboxylase represents approximately 7% of the total protein in a crude extract. At pH 7.0 ornithine decarboxylase exists as a dimer, is specific for ornithine and requires pyridoxal phosphate for activity.

Biosynthetic amino acid decarboxylases

A second form of ornithine decarboxylase, unlike the form described by Gale, was discovered by Morris and Pardee (Morris and Pardee, 1965, 1966) during a study of polyamine biosynthesis. Biodegradative ornithine decarboxylase was not induced when *E. coli* is grown in neutral minimal medium, yet the bacteria still decarboxylated ornithine. This biosynthetic form of ornithine decarboxylase (ODC) is found in all strains of *E. coli.* The enzyme has been purified to homogeneity and represents approximately 0.023% of the total protein. The activity is specific for ornithine, having less than 0.1% decarboxylase activity toward arginine or lysine. Native ODC is a dimer, with identical subunits of 80,000 Da. The enzyme has a pH optimum of 8.1 (compared to 6.9 for the biodegradative form), and requires pyridoxal phosphate. ODC does not react with antibodies produced to the biodegradative form. The regulation of ODC is very complex. The enzyme is inhibited by both putrescine and spermidine (Applebaum et al., 1977; Höltta et al., 1974), repressed by cAMP (Wright and Boyle, 1982), and activated by guanosine triphosphate (GTP) and other nucleotides (Applebaum et al., 1977; Höllta et al., 1974). The *E. coli speC* gene, encoding

ODC has been cloned (Boyle et al., 1984) and sequenced (Barrosa et al., 1990). Deletion mutants lacking ODC activity have been obtained (Hafner et al., 1979a). The *speC* gene maps to 63.4 min on the *E. coli* chromosome (Boyle et al., 1984; Cunningham-Rundles and Maas, 1975; Hafner et al., 1979a). ODC activity assays have localized the gene to pLC20-5 in the Clarke-Carbon library (Clarke and Carbon, 1976). Southern hybridization using a *speC* probe has localized the gene to lambda clones 3D11 and 1G7 from the Kohara library (Satishchandran et al., 1990). These lambda clones map to 64.0 min. on the *E. coli* K-12 chromosome (Bachmann, 1987, Satishchandran et al., 1990).

POLYAMINE BIOSYNTHESIS

Purescine biosynthetic pathways

If putrescine was only produced by the decarboxylation of ornithine, arginine levels would regulate cellular putrescine levels. In 1961, Maas (Maas, 1961) reported that arginine repressed the formation of most of the enzymes of the arginine biosynthetic pathway. In *E. coli*, arginine controls the formation of ornithine (and arginine) through feedback inhibition of N-acetylglutamate synthetase. Therefore, an additional pathway would be necessary for the formation of putrescine when exogenous arginine is available, as in the intestine, or the bacterium would be starved for putrescine. An alternative solution to ornithine limitation in the presence of arginine would be to hydrolyze arginine to ornithine. The ornithine obtained from arginine could then be used by ODC to produce putrescine. Many eukaryotes and some bacteria possess an arginase which hydrolyzes arginine to ornithine. *E. coli* lacks arginase and is therefore incapable of converting arginine to ornithine.

A second pathway (Pathway II) leading to the production of putrescine was discovered

that uses biosynthetic arginine decarboxylase (ADC) to convert arginine to agmatine (Morris and Pardee, 1966). The agmatine is hydrolyzed to putrescine and urea by a second enzyme, agmatine ureohydrolase (AUH). In unsupplemented medium, 75 to 90% of the putrescine in *E. coli* is synthesized by the ornithine pathway (Morris et al., 1970). When an *E. coli* culture was supplemented with arginine, the ornithine pool decreased to undetectable levels, and no putrescine was produced from ornithine (Morris and Koffron, 1969). Removal of the exogenous arginine resulted in a four- to fifty-fold reduction in the rate of conversion of arginine to putrescine, depending on the strain (Morris and Koffron, 1969; Morriset al., 1970). There was little effect on the rate of protein synthesis when exogenous arginine was removed due to sufficient endogenous arginine synthesis. As the specific activities of ADC and AUH did not change following arginine supplementation (Morris et al., 1970), Morris proposed that the intracellular concentration of ornithine controlled which of the two pathways was used to produce putrescine. Consequently, arginine acts as a signal for derepression of Pathway II (putrescine via arginine) and repression of Pathway I (putrescine via ornithine). The absence of arginase in *E. coli* may have required the evolution of these two parallel pathways for putrescine synthesis in the presence of exogenous arginine.

Biosynthetic arginine decarboxylase

ADC was isolated from *E. coli* strain UW44 grown aerobically in a minimal medium at neutral pH and represented approximately 0.07% of the total protein from these cells. The enzyme has been purified to homogeneity and crystallized (Wu and Morris, 1973). Arginine, but not ornithine or lysine, acts as a substrate. Native ADC is a tetramer with a molecular mass of 280,000 daltons (Da). ADC differs from biodegradative arginine decarboxylase in several characteristics. The biosynthetic form has a pH optimum of 8.4, compared to 5.2

for the biodegradative form. The enzyme has an absolute requirement for Mg^{++} and binds two pyridoxal phosphate molecules.

When Wu and Morris (Wu and Morris, 1973) initially purified biosynthetic arginine decarboxylase, their preparation resolved into two major and three minor bands on a native gel. On denaturing gels, the two major bands migrated with molecular masses corresponding to 74- and 70,000 Da. After separating the major bands on a native gel, it was determined that both forms decarboxylated arginine. Radiolabeling of minicell preparations containing a plasmid bearing the *speA* gene, encoding ADC, confirmed the existence of these two monomeric forms (Boyle et al., 1984). Immunoprecipitation of ADC isolated from pulse-labeled *E. coli* also showed two major bands of 74- and 70,000 Da, with minor bands of 30- to 40,000 Da (Buch and Boyle, 1985a). The minor bands disappeared when the label was chased, suggesting that they were probably nascent chains of arginine decarboxylase. The same pulse-chase experiments showed that the 74,000 Da precursor polypeptides are processed to a 70,000 Da mature form (Buch and Boyle, 1985a). Selective disruption of the cell envelope revealed that the 70,000 Da, but not the 74,000 Da species, is localized within the inner periplasmic space. The translocation of the ADC monomer into the periplasm was confirmed using an *E. coli* strain that can be blocked in protein transport (Buch and Boyle, 1985a). When transport was blocked, the 74,000 Da, but not the 70,000 Da species, accumulated in the cytoplasm. These results explained the previous finding by Tabor (1969b) that *E. coli* produced more putrescine from exogenous arginine than from endogenous arginine. It has been estimated that approximately 80% of the exogenous arginine is converted into protein and 20% to polyamines. An *E. coli* mutant blocked in arginine biosynthesis between acetylornithine and ornithine formation was grown in a chemostat with limiting amounts of labelled citrulline and arginine. Approximately 40 to

60% of the putrescine produced by these bacteria was derived from exogenous arginine. It is now clear that the periplasmic location of ADC allows the enzyme to channel exogenous arginine preferentially into putrescine. The agmatine produced by ADC during the import of arginine is converted to putrescine in the cytoplasm by AUH.

The *speA*, *speB*, and *metK* genes

An 8.0 kb *Eco*RI fragment containing the *speA*, *speB*, and *metK* genes was identified on plasmid pLC2-5 from the Clarke-Carbon *E. coli* genomic library (Hafner et al., 1979b). This fragment was cloned into the *Eco*RI site of pBR322 to create plasmid pKA5 (Tabor et al., 1983; Boyle et al., 1984). *E. coli* transformed with plasmid pKA5 overexpresses ADC (*speA*), AUH (*speB*), and methionine adenosyltransferase (MAT) (*metK*). *E. coli* deletion mutants that are defective in biosynthetic arginine decarboxylase have been isolated (Hafner et al., 1979a; Tabor et al., 1983b). The *speA* gene maps to 62.8 min on the *E. coli* map (Hafner et al., 1979a) or 63.5 min on the *E. coli* K-12 map (Satishchandran et al., 1990). Southern hybridization using a *speA* probe localized the gene to lambda clones 1H10 and 23G4S of the Kohara library (Satishchandran et al., 1990). The *speA* gene is duplicated in *E. coli* strain UW44 (Satishchandran, personal communications). A genomic fragment including the *metK*, *speA*, and at least part of the *speB* gene is duplicated as an inverted repeat directly adjacent to the original genes in the 63 min region. The function of this second set of genes is currently under investigation.

Agmatine ureohydrolase

AUH, the second enzyme in Pathway II, converts the agmatine produced by ADC into putrescine and urea (Morris and Pardee, 1966). Hydrolysis of agmatine by AUH is the only known pathway for urea production in *E. coli* (Morris and Koffron, 1967). Except for a few ureopathogenic strains, *E. coli* lacks urease and urea accumulates in the medium as

putrescine is synthesized from Pathway II. Thus, urea produced by *E. coli* is usually an indication of the putrescine production through Pathway II. Mutants in *speB* were isolated by screening for strains defective in urea production (Morris and Jorstad, 1970) or for strains requiring putrescine when grown in the presence of exogenous arginine (Hirschfield et al., 1970; Maas et al., 1970; Hafner et al., 1979a; Morris and Jorstad, 1970; Tabor et al., 1983). The gene encoding AUH, *speB*, has been cloned (Tabor et al., 1983; Boyle et al., 1984) and sequenced (Szumanski and Boyle, 1990). AUH was purified to homogeneity from overproducing a strain transformed with a plasmid encoding *speB* (Satishchandran and Boyle, 1984). There does not appear to a biodegradative form of AUH (Satishchandran and Boyle, 1984). Agmatine induces the expression of AUH (Satishchandran and Boyle, 1984). Unlike arginine and ornithine decarboxylases, AUH expression is not inhibited by putrescine or spermidine. However, AUH expression is repressed by cAMP and cAMP receptor protein (CRP) (Satishchandran and Boyle, 1984). Both monocistronic and polycistronic mRNA encoded by *speB* have been identified (Szumanski and Boyle, 1990). There appears to be a rho-independent terminator located between the *speA* and *speB* genes. How transcription of the *speB* genes occurs if this structure terminates transcription, or if it plays a role in regulating *speB* is unknown. The *speB* gene maps to 62.9 min on the Kohara *E. coli* map and 63.5 min on the *E. coli* K-12 chromosome (Moore and Boyle, 1990; Satishchandran et al., 1990). Southern hybridization using a *speB* probe localized the gene to lambda clones 1H10 and 23G4S of the Kohara library (Satishchandran et al., 1990).

Spermidine synthesis

In *E. coli*, putrescine can be converted into the tri-amine, spermidine. MAT, encoded by the *metK* gene, adenylates methionine to produce S-adenosylmethionine (SAM). The *metK* gene was isolated from the Clarke-Carbon plasmid library and cloned on a plasmid,

pKA5 (Tabor et al., 1983; Boyle et al., 1984). It has not been possible to obtain deletion mutants in *metK* as these mutations are lethal. The *metK* gene maps to 63.7 min on the *E. coli* K-12 chromosome (Satishchandran et al., 1990). Southern hybridization with a *metK* probe located the gene on lambda clones 1H10 and 23G4S from the Kohara library (Satishchandran et al., 1990). A second copy of the *metK* gene has been identified in the 64 min region of the *E. coli* chromosome (Satishchandran, personal communications). The conversion of putrescine to spermidine requires S-adenosylmethionine decarboxylase (*speD*), spermidine synthase (*speE*) and S-adenosylmethionine (SAM), an intermediate in many metabolic pathways. Purified *E. coli* SAM decarboxylase is composed of six identical subunits, with a molecular mass of 108,000 Da (Markham et al., 1982, 1983; Wickner et al., 1970). In contrast to most other decarboxylases, SAM decarboxylase requires pyruvate, not pyridoxal phosphate, as a cofactor. Spermidine synthase catalyses the conversion of putrescine and decarboxylated SAM to spermidine and thiomethyladenosine (Tabor et al., 1958; Hannonen et al., 1972; Raina and Hannonen, 1971). Purified spermidine synthase contains two subunits, each with a molecular mass of 73,000 Da. Cadaverine and spermidine can act as substrates, but at slower rates than with putrescine (Bowman et al., 1973).

The *speD* and *speE* genes form an operon, located at 2.9 minutes on the *E. coli* chromosome (Xie et al., 1989). The *speD* and *speE* genes have been cloned and sequenced. Both point and deletion mutants of *speD* have been isolated (Hafner et al., 1979a; Tabor and Tabor, 1976; Tabor et al., 1978, 1983). Tight *speD* mutants grow at 75% of the wild type rate and contain twice as much putrescine as wild type *E. coli* (Tabor et al., 1978). These mutants grew at twice the rate compared to strains depleted of putrescine. This result implies that putrescine can substitute, albeit less efficiently, for the growth promoting effects of spermidine.

Spermine synthesis

A second aminopropyltransferase has been purified from animal tissue. This enzyme catalyzes the transfer of a second aminopropyl group, also derived from SAM, to spermidine to produce the tetra-amine, spermine (Hannonen et al., 1972; Raina and Hannonen, 1971). Spermine is not usually found in *E. coli*., although small amounts were detected in *speA*, *speB*, *speC* mutants grown in the presence of spermidine (Hafner et al., 1979b).

REGULATION OF POLYAMINE LEVELS

Pathways and effectors

Intracellular polyamine levels in *E. coli* are regulated by a number of interactive pathways and enzyme effectors (see Figs. 1 and 2). More detailed information on the *E. coli* polyamine biosynthetic pathways and their regulation can be found in reviews on arginine biosynthesis and metabolism (Glansdorff, 1987; Cunin et al., 1986), polyamines in microorganisms (Tabor and Tabor, 1985), and amino acid decarboxylases (Morris and Fillingame, 1974). Both ornithine and arginine are part of the arginine biosynthetic pathway (Fig. 2). These substrates can be used by either of two parallel pathways leading to the formation of putrescine. The arginine and pyrimidine biosynthetic pathways compete for carbomylphosphate, an intermediate in both pathways (Fig. 2). Arginine feedback inhibits arginine biosynthesis, limiting ornithine pools. Methionine is converted to S-adenosylmethionine (SAM), an intermediate in a number of biosynthetic pathways, including spermidine synthesis. The polyamine biosynthetic enzymes are also regulated by several effectors. Arginine and ornithine decarboxylases are both competitively inhibited by putrescine and spermidine (Morris et al., 1970). There is a reduction in the amount of $^{14}CO_2$ released from labelled ornithine or arginine when *E. coli* grown in a chemostat are

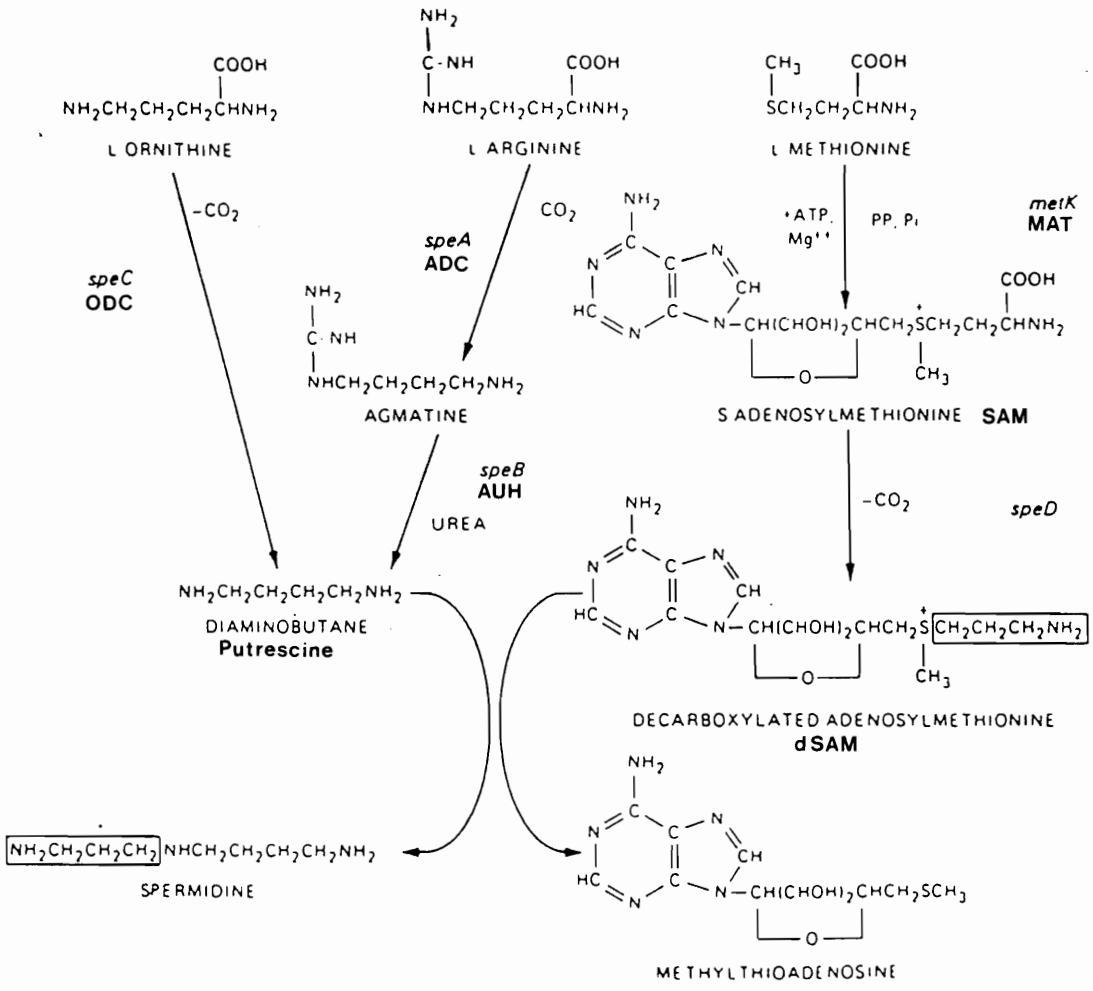


Figure 1. Putrescine (diaminobutane) and spermidine biosynthetic pathways in *E. coli*. The bacterium lacks arginase and cannot convert arginine to ornithine. ODC is ornithine decarboxylase; ADC is arginine decarboxylase; AUH is agmatine ureohydrolase; MAT is methionine adenosyltransferase; SAM is S-adenosylmethionine. Taken from Tabor and Tabor, 1985.

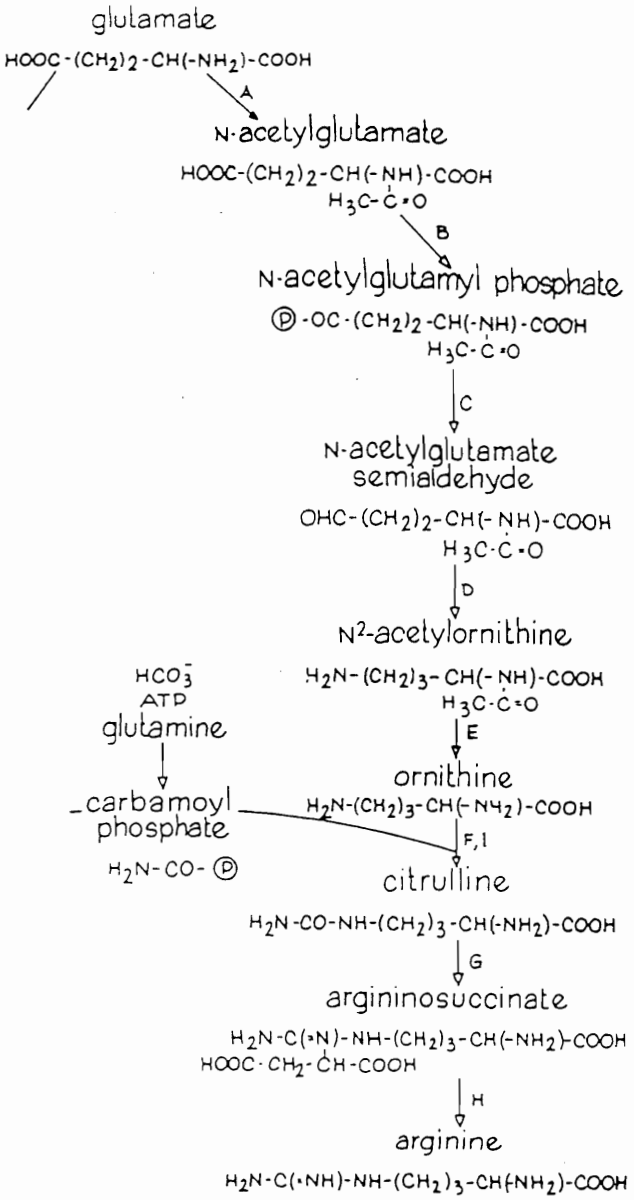


Figure 2. The arginine biosynthetic pathway in *E. coli*. A is N-acetylglutamate synthetase; B is N-acetylglutamate 5-phosphotransferase; C is N-acetylglutamate 5-phosphate reductase; D is N-acetylornithine aminotransferase; E is N-acetylornithinase; F is ornithine carbamoyltransferase; G is argininosuccinate synthetase; H is argininosuccinase; I is carbamoylphosphate synthetase. Taken from Cunin et al., 1986.

supplemented with putrescine or spermidine (Tabor and Tabor, 1969b). Although the actual amounts of ADC and ODC were not determined in these experiments, the activities of both enzymes decreased *in vivo* and *in vitro*, indicating both repression and inhibition. While these observations clearly show that putrescine and spermidine cause a reduction in the activities of ADC and ODC, it is not clear how this regulation occurs. The activity of the enzymes could be controlled at the level of transcription, translation, or post-translation. Polyamines are known in influence the rate and fidelity of both transcription and translation. Thus, these polyamines may negatively regulate ADC by reducing either the rate of mRNA synthesis or translation of the mRNA into protein. There are reports that putrescine induces the synthesis of ODC and ADC "antizymes" that noncompetitively inhibit the enzymes activities (Fong et al., 1976; Kyriakidis et al., 1978). The term antizymes refers to regulatory proteins that are specifically induced by an endproduct of the enzyme (Heller et al., 1976). ODC antizymes have been reported in cultured cancer and normal mammalian cells (Fong et al., 1976; Heller et al., 1976), rat liver, (Fujita et al., 1984), germinating barley (Kyriakidis, 1983), and *E. coli* (Kyriakidis et al., 1978; Heller et al., 1983). Three ODC antizyme proteins were reported in *E. coli*, one with an acidic pI of 3.5 and two with basic a pI of 9.5 (Heller et al., 1983). The basic proteins, designated antizyme 1 and antizyme 2, also inhibit ADC activity, but have no effect on the activity of any biodegradative decarboxylases. Antizyme 1 and 2 contain amino acid sequences that are identical to *E. coli* ribosomal proteins S20/L26 and L34, respectively (Panagiotidis and Canellakis, 1984). Kashiwagi and Igarashi (1987) have shown that other ribosomal proteins inhibit ODC activity competitively. Ribosome-bound antizymes did not inhibit ODC and ribosomes eliminated antizyme inhibition of the enzyme. It remains unclear whether these basic antizymes function as inhibitors of ODC activity *in vivo*.

Regulation of the *spe* genes by cAMP and cAMP receptor protein

The expression of the putrescine biosynthetic enzymes is inhibited by a mechanism requiring cyclic adenosine-3',5'-monophosphate (cAMP) and cAMP receptor protein (CRP) (Wright and Boyle, 1982). *E. coli* strain KC14 contains wild type adenylate cyclase (*cya)* and cAMP receptor protein (*crp)* genes and produces increased ADC, AUH, and ODC activities when grown in the presence of glucose. *E. coli* KC13 has a deletion in the *cya* gene and therefore is unable to synthesize cAMP. In the absence of cAMP, *E. coli* KC13 showed a derepression of the putrescine biosynthetic genes. Addition of cAMP to the culture medium restored repression to wild type levels. *E. coli* KC42 has a second deletion in the *crp* gene encoding CRP. Addition of cAMP to *E. coli* strain KC42 (*cya*, *crp*) failed to restore repression to wild type levels, indicating that CRP is required for cAMP-mediated repression of the putrescine biosynthetic enzymes. Because the intracellular concentration of cAMP is inversely related to the growth rate of *E. coli*, cAMP levels act to coordinate the production of polyamines with the rate of growth.

It should be noted that ODC and ADC present in some strains of *E. coli* do not respond to repression by glucose (Shaibe et al., 1985a). Halpern's group showed that the ADC and ODC synthesis of the *cya* derivative of *E. coli* strain CS101B, unlike strain KC13 (*cya*) or strain CA8303 (*cya*), was not affected by cAMP. The synthesis of β-galactosidase in the *cya* or *crp* derivatives of CS101B was repressed by glucose and induced by the addition of cAMP. After confirming that cAMP repressed ODC and ADC in strain CA8303, Halpern concluded that the discepancy in results of the two laboratories can be explained by differences in the strains used in each study. An additional difference in strains was in their growth rate on glucose and succinate; strain CA8303 had a much slower growth rate than strain CS101B or its derivatives.

cAMP LEVELS AND GENE REGULATION

cAMP control of gene expression

In bacteria, cAMP acts as a secondary messenger to signal changes in the glucose concentration. The greater the limitation of glucose as a carbon source, the greater the intracellular cAMP concentration (Alpher and Ames, 1977; Buettner et al., 1973; Epstein et al., 1975; Pastan and Adhya, 1976). cAMP-CRP controls a number of biological phenomena, including transcriptional initiation (Ullmann et al., 1979), cell division (Utsumi et al., 1981), cell morphology (Aono et al., 1978; Harwood and Peterkofsky, 1975), and the life cycle of phages (Hong et al. et al., 1971; Grodzicker, 1972). At least 30 operons are controlled by cAMP in *E. coli* (Pastan and Adhya, 1976). Of these approximately 20 genes are positively regulated by cAMP-CRP (Rickenberg, 1974; Pastan and Adhya, 1976). A model for the cAMP-mediated stimulation of genes was proposed in 1970 by Pastan and Perlman (Pastan and Perlman, 1970) for the *E. coli* lactose operon in which the addition of lactose or a lactose derivative induced the operon. This "catabolite repression" could be overcome by the addition of cAMP to the growth medium (Pastan and Perlman, 1970). A drop in glucose levels results in the derepression of many catabolic operons, depending on what sugars are available in the medium.

Regulation of cAMP levels

In *E. coli*, the level of cAMP is determined by its rate of synthesis by adenylate cyclase, the rate of degradation by phosphodiesterase (*cpd*), and the rate of excretion by an energy dependent mechanism. Degradation of cAMP by cAMP phosphodiesterase does not play a major role in determining cAMP levels, since cAMP levels in *E. coli* cAMP phosphodiesterase mutants vary inversely with the carbon source (Buettner et al., 1973). The rate of excretion of cAMP from *E. coli* grown on different carbon sources does not

vary, suggesting that excretion does not regulate cAMP levels (Epstein et al., 1975).

One mechanism regulating cellular cAMP levels involves a sugar-dependent phosphoenolpyruvate:sugar phosphotransferase system (PTS) inhibition of adenylate cyclase activity (Harwood and Peterkofsky, 1975; Dills et al., 1980). Bacteria grown with glycerol synthesize more cAMP than bacteria growing on glucose, but produce less adenylate cyclase (Janacek et al., 1979). It should be noted that partially purified adenylate cyclase is extremely labile and exhibits very low activities compared to those found *in vivo* (Majerfeld et al., 1981). Therefore, the best procedure for determining adenylate cyclase activity uses toluenized cells (Harwood and Peterkofsky, 1975). The inhibition of cAMP synthesis is partially responsible for catabolite repression (Pastan and Adhya, 1976; Epstein et al., 1975). The ability of the cell to synthesize cAMP upon removal of a carbon source permits the bacterium to adapt to conditions of catabolite repression very rapidly.

Regulation of adenylate cyclase activity by cAMP-CRP

The activity of adenylate cyclase is severely inhibited by the cAMP-CRP complex. CRP-deficient *E. coli* synthesize more adenylate cyclase than does the parent strain, but the partial repression of adenylate cyclase does not explain the difference in the amount of cAMP produced by wild type and *crp* derivatives (Majerfeld et al., 1981). The *in vivo* rates of synthesis of cAMP synthesis are several hundred-fold higher in *E. coli crp* mutants compared to the wild type without a parallel increase in adenylate cyclase activities (Joseph et al., 1982). An *E. coli* strain carrying the *crp* gene on a plasmid that overproduces CRP severely inhibited adenylate cyclase activity, although the *in vivo* rate of cAMP synthesis is similar to the parental strain. These results indicate that CRP controls the activity rather than the synthesis of adenylate cyclase.

The *cya* gene from *Salmonella typhimurium* has been cloned and overexpressed in *E. coli*

on a multicopy plasmid (Wang et al., 1981). Adenylate cyclase was overproduced 20-fold, but the cAMP level increased only 60%. The rate of excretion of cAMP in the overproducing strain was only 75% higher than the wild type and phosphodiesterase activity was unaltered. These results suggest that the activity of *S. typhimurium* adenylate cyclase is inhibited when *cya* is overexpressed.

Bankaitis & Bassford (1982) investigated the regulation of *cya in vivo* using *cya-lac* transcriptional and translational fusions. cAMP levels produced extremely weak effects on the *cya-lac* fusions and were not considered physiologically significant. They proposed that cAMP-CRP complex is involved, directly or indirectly, as a negative element in the post-translational regulation of adenylate cyclase.

Botsford & Drexler (1978) proposed a model for regulation of *cya* at the transcriptional level. There is evidence indicating that at least part of the regulation of *cya* by cAMP-CRP is at the level of transcription (Jovanovich, 1985; Aiba, 1985; Mori and Aiba, 1985; Kawamukai et al., 1985). Aiba et al. showed that a *crp E. coli* produced about five-fold more *cya* mRNA than do wild type cells (Aiba, 1985). DNA footprinting experiments revealed that cAMP-CRP protects a region between +11 and -20 bp in the 5'-end of the *cya* gene containing a CRP consensus sequence. The difference in the transcription rate of *cya in vivo* and *in vitro* are insufficient to explain the increased cAMP synthesis in *crp* mutants. This indicates that the control of cAMP synthesis also involves regulation of adenylate cyclase activity.

The autogenous regulation of adenylate cyclase and CRP by the cAMP-CRP complex ensures that the proper concentration of cAMP and CRP will be maintained under a variety of growth conditions. In the presence of glucose, the expression of *cya* and *crp* are derepressed due to low levels of cAMP. Although cells accumulate adenylate cyclase and

CRP, the cAMP level remains low because both CRP and glucose independently inhibit the activity of adenylate cyclase. When glucose is depleted, the inhibition of adenylate cyclase is removed and the cells immediately begin to synthesize cAMP. An increase in intracellular cAMP levels results in a rapid increase in the levels of cAMP-CRP complex in the cells. cAMP-CRP binds to specific DNA sequences to effect gene and operon expression, including the repression of *crp*. As cAMP-CRP levels increase, the rate of transcription of *cya* and *crp* decrease and adenylate cyclase activity is inhibited. This ensures that the appropriate concentrations of cAMP and CRP are maintained during growth in fluctuating environmental conditions.

Direct regulation of transcription by cAMP-CRP

The cAMP-CRP complex regulates many genes in *E. coli* by binding to specific sequences within or near promoter sequences. The mechanism by which cAMP-CRP regulates gene expression is usually direct, by affecting the initiation of transcription (de Crombrugghe et al., 1984). Most CRP binding sites are 22-bp long and begin with the highly conserved sequence, 5'-TGTGA-3' and end with a second, symmetrically related motif. This two-fold symmetry appears to be important because a completely symmetrical site has a higher affinity for CRP than the best naturally occuring site (*lac*) (Jansen et al., 1987). The *crp* gene has been cloned and sequenced (Aiba et al., 1982; Cossart and Gicquel-Sanzey, 1982). In the absence of cAMP, CRP is a dimer of identical subunits (M_r 22,500), that shows weak, nonspecific binding to DNA. It is estimated that between 1,000 to 3,000 dimers of CRP are present in the cell (Zubay, 1980; Guiso and Blazy, 1980). Each subunit of CRP contains two domains which are covalently connected by a hinge region (Aiba and Krakow, 1981; Eilen et al., 1978; McKay et al., 1982). The larger, amino-terminal domain is responsible for the subunit contacts that form the dimer and contains a pocket

formed by a series of antiparallel β-sheets in which cAMP binds. cAMP acts as a ligand to induce a conformational change in CRP. One molecule of cAMP binds per CRP subunit, resulting in a conformation with a higher affinity for specific sites on DNA (Takahashi et al., 1979, 1983; Saxe and Revzin, 1979). The smaller, carboxy-terminal domain of CRP is responsible for binding to DNA.

Indirect regulation of transcription by cAMP-CRP

The cAMP-CRP complex also acts to regulate genes indirectly. When intracellular cAMP levels are high, the concentration of CRP levels off. cAMP-CRP strongly inhibited transcription of the *crp* gene *in vitro* (Aiba, 1983) and *in vivo* (Cossart and Gicquel-Sanzey, 1985). This negative autogenous regulation of *crp* expression is a consequence of activation of a divergent promoter located within the *crp* promoter region that produces an antisense (divergent) RNA (Okamoto and Freundlich, 1986). This antisense RNA binds to the 5'-end of *crp* mRNA and inhibits *crp* transcription. Thus, CRP levels are autoregulated, preventing an increase in the non-specific binding of cAMP-CRP to DNA.

OBJECTIVES

While many bacterial genes and operons are induced by cAMP, others are repressed. Examples of negatively regulated genes include *speA*, *speB*, *speC* (Wright and Boyle, 1982), *crp* (Aiba, 1983), *cya* (Majerfeld et al., 1981), glutamate synthase (Prusiner et al., 1972), *rrnB* (Glaser et al., 1980), spot 42 RNA (Sahagan and Dahlberg, 1979), and the P2 promoter of the galactose operon (Musso et al., 1977). These genes show a less than 10-fold repression by cAMP under the conditions studied. Mallick and Herrlich (1979) proposed that negative control by cAMP occurred at concentrations below those required for positive control. They suggested that two conformational states exist for CRP. One state saturates CRP with

cAMP at low cAMP concentrations and negatively regulate genes. A second state saturates CRP with cAMP at high cAMP levels and positively regulates genes. The mechanism by which cAMP and CRP negatively regulate genes other than the *crp* gene remains unknown. In *E. coli* , the activity of ADC is derepressed 1.5 to three-fold in either *cya* or *crp* mutants. The addition of cAMP to cultures of wild type or *cya* strains causes 35 to 60% repression, but does not cause repression in *crp* strains. Thus, the focus of this dissertation is to determine if cAMP and putrescine regulate *E. coli* ADC at the transcriptional, translational, or post-translational level.

CHAPTER I

Moore, R.C. and S.M. Boyle. 1990. Nucleotide sequence and analysis of the *speA* gene encoding biosynthetic arginine decarboxylase in *Escherichia coli*. J. Bacteriol. 172:4631-4640.

Satishchandran, C., G.D. Markham, R.C. Moore, and S.M. Boyle. 1990. Locations of the *speA*, *speB*, *speC*, and *metK* genes on the physical map of *Escherichia coli*. J. Bacteriol. 172:4748.

INTRODUCTION

Putrescine (1,4-diaminobutane), a diamine required for optimal growth, is produced in *E. coli* and many plants by either of two pathways related to arginine biosynthesis (Glansdorff, 1987; Tabor and Tabor, 1985). In *E. coli*, biosynthetic arginine decarboxylase (ADC), encoded by the *speA* gene, acts upon arginine to produce agmatine, which is converted into putrescine by agmatine ureohydrolase (AUH). A second pathway uses ornithine decarboxylase (ODC) to produce putrescine directly from ornithine. While ODC is common to all cells, ADC appears to be unique to bacteria and plants. These two parallel pathways remain distinct in *E. coli* as the species lacks arginase and is therefore unable to convert arginine to ornithine. In the absence of exogenous arginine, putrescine is produced primarily from the ODC pathway, but when arginine is added to the growth medium, ornithine levels decline due to inhibition of arginine biosynthesis (Tabor and Tabor, 1969a). The ADC pathway thereby ensures that putrescine is produced in the presence of exogenous arginine (Morris and Koffron, 1970), e.g., in the intestinal tract.

ADC can be found in two distinct forms: biodegradative and biosynthetic. Biodegradative arginine decarboxylase (ADI) is induced in some strains of *E. coli* when they

are grown in an acidic enriched medium containing arginine (Gale, 1946; Morris and Boeker, 1983). Biodegradative amino acid decarboxylases appear to play a role in regulating pH by consuming protons and thus neutralizing the acidic products of carbohydrate fermentation (Gale, 1946). Biosynthetic ADC is produced when *E. coli* is grown in minimal media at neutral pH; approximately 0.07% of the protein found in *E. coli* UW44 crude extracts is biosynthetic ADC (Wu and Morris, 1973). Native biosynthetic ADC is a tetramer of 280,000 Da which requires both Mg^{2+} and pyridoxal phosphate as cofactors (Morris and Fillingame, 1974; Wu and Morris, 1973). Monomers of ADC are synthesized as 74,000 Da precursor polypeptides which are posttranslationally processed to a 70,000 Da mature form which is localized within the inner periplasmic space (Buch and Boyle, 1985a). The periplasmic location of ADC explains the finding that more putrescine is produced from exogenous arginine than from endogenous arginine, since arginine can be preferentially channeled into putrescine as it is being transported into the cell (Tabor and Tabor, 1969b). The *speA* gene and ADC are subject to metabolic control. Putrescine and spermidine repress the *speA* gene and feedback inhibit ADC (Tabor and Tabor, 1969a). Cyclic AMP (cAMP) also negatively regulates *speA* gene expression either directly or indirectly via binding to cAMP receptor protein (CRP)(Wright and Boyle, 1982). The experiments described in this dissertation present the nucleotide sequence of the *speA* gene and compare the properties of the deduced ADC amino acid sequence to those of purified ADC (Wu and Morris, 1973) and other decarboxylases.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

The bacterial strains and plasmids used in this study are described in Table 1. *E. coli*

Table 1. *E. coli* and plasmids used in Chapter I.

Strain	Genotype	Source
E. coli		
DH5α	F⁻Φ80 *lacZM15 (lacZYA-argF)U169 recA1 endA1 hsdR17* ($r_k^- m_k^+$) *supE44 thi-1* lambda-*gyrA96 relA1*	BRL
CB806	F⁻ Δ*lacZ* $lacY^+$ *galK rpsL thi recA56 phoA8*	Schneider
Plasmids		
pKA5	pBR322 with a 8.0-kilobase (kb) *Eco*RI fragment	Boyle
pGEM-3Z	High-copy number sequencing plasmid	Promega
pRM15	3.2 kb *Bal*I-*Acc*I fragment in pGEM-3Z	This study
pRM59	3.2 kb *Acc*I-*Bal*I in reverse orientation in pGEM-3Z	This study
pMC1403	High-copy number *lacZ* translational fusion vector	Casadaban
pRM65	2.2 kb *Bam*HI fragment in pMC1403	This study

strains were grown in either Luria broth (LB)(Maniatis et al., 1982) (1% tryptone, 0.5% yeast extract, and 1% NaCl), Terrific broth (1.4% tryptone, 2.7% yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4)(Tarrof and Hobbs, 1987), or MOPS medium (Niedhardt et al., 1974) supplemented with 1 ug/ml thiamine, 0.2% glucose and 50 μg/ml of each naturally occurring L-amino acid except arginine. Ampicillin was used at 100 ug/ml.

Enzymes and reagents.

Restriction endonucleases, Klenow fragment of DNA polymerase I, T_4 DNA ligase, exonucleases III and VII, and halogenated indolyl-ß-D-galactoside (Bluo-Gal) were purchased from Bethesda Research Laboratories, Inc. (BRL)(Gaithersburg, MD). Sequenase was purchased from U. S. Biochemical Corp. (Cleveland, OH). Avian myeloblastosis reverse transcriptase was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). [α-^{35}S]dATP and 1-^{14}C-L-arginine were from Dupont, NEN Research Products (Boston, MA). ß-galactosidase affinity column and antisera were purchased from Sigma Chemical Co. (St. Louis, MO). SeaPlaque low-melt agarose was from FMC BioProducts (Rockland, ME).

Plasmid screening.

Plasmids were isolated and purified using standard methods (Maniatis et al., 1982). The size of supercoiled plasmid DNA was determined using a phenol-chloroform/quick screen method. Bacterial colonies were picked from a plate, resuspended in 40 ul 1X STE (20 mm TRIS, pH 7.5, 100 mM NaCl, 10 mM EDTA), lysed by the addition of one half volume of phenol-chloroform (1:1) and vortexed. RNaseA (50 ug/ml, final concentration) was mixed with supernatants, incubated at 37^{o}C for 15 minutes and electrophoresed on a 0.8% agarose gel to separate chromosomal and plasmid DNA. Plasmid size was estimated by comparing the mobility of supercoiled forms of deletion plasmids to the vector (pGEM-3Z) and undeleted plasmid (pRM15).

Subcloning of *speA* gene.

The *speA* gene is located on a 8.0 kb *Eco*RI fragment on plasmid pKA5 (Boyle et al., 1984). Plasmid pKA5 was cleaved with *Bal*I and *Acc*I to produce a 3236 base-pair (bp) fragment (Fig. 3). Restriction fragments were blunt-ended by treatment with the Klenow fragment of DNA polymerase and separated by electrophoresis on a 0.7% low-melting SeaPlaque agarose gel (Struhl, 1986). The agarose containing the 3236 bp *Bal*I-*Acc*I fragment was excised, melted at 60°C, and the 3236 bp fragment ligated into the *Sma*I site of pGEM-3Z. Ligation products were transformed (Hanahan, 1983) into *E. coli* DH5α. Clones containing plasmids bearing the *speA* gene were selected by their ability to overexpress ADC (see Enzyme Assays).

To obtain deletions in the opposite orientation, the 3282 bp insert from pRM15 was removed by digestion with restriction endonucleases *Hin*dIII and *Eco*RI. Restriction products were blunt-ended by treatment with the Klenow fragment of DNA polymerase and separated by electrophoresis on a 0.7% SeaPlaque agarose gel. The blunt-ended 3282 bp *Hin*dIII-*Eco*RI fragment was excised from the gel, ligated into the *Sma*I site of a new pGEM-3Z and transformed into *E. coli* DH5α. Clones were assayed for their ability to overproduce ADC. Clone pRM59 expressed levels of ADC comparable to that produced by pRM15 and was selected for further study.

Exonuclease deletions of *speA*.

Overlapping deletions of plasmid pRM15 were generated using exonuclease III (Henikoff 1984). Plasmid pRM15 was cleaved with *Sal*I and *Sph*I, digested with exonucleases III and VII, followed by treatment with the Klenow fragment of DNA polymerase to create blunt ends. Deleted DNA fragments were separated by electrophoresis in 0.7% SeaPlaque agarose; the DNA bands were excised, recircularized with

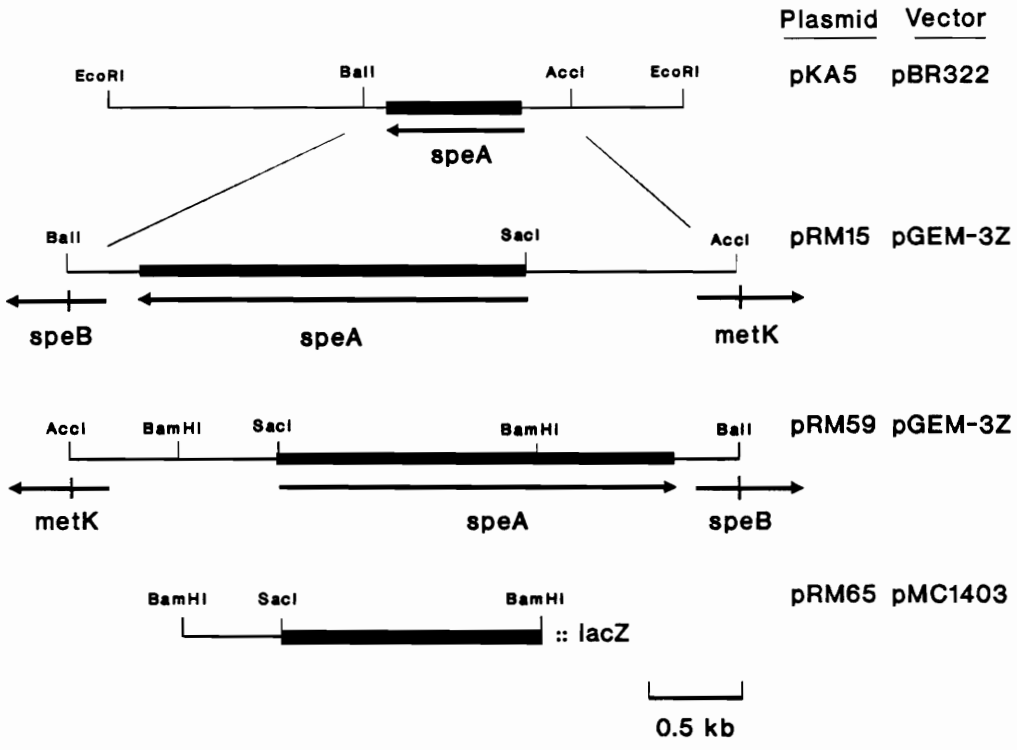


Figure 3. Physical map of plasmids. Thick lines indicate *speA* (arginine decarboxylase) gene; arrows, direction of transcription; arrow with bar, truncated *speB* (agmatine ureohydrolase) and *metK* (methionine adenosyltransferase) genes.

T_4 DNA ligase and transformed into *E. coli* DH5α. Deletion plasmids were sized using the phenol-chloroform quick screen method, then clones were screened for ADC enzyme activity to determine the approximate location of the *speA* gene within pRM15. Deletions of pRM59 were constructed as previously described except that the deletions were not separated on a SeaPlaque gel following treatment with Klenow fragment. Plasmids were sized using the phenol-chloroform quick screen method and clones were analyzed with an ADC enzyme assay (Fig. 4). All plasmids were restriction mapped to confirm the extent of deletions.

Dideoxy sequencing.

Overlapping deletions were sequenced by the dideoxy-chain termination method of Sanger (Sanger et al., 1977) using SP6 and T7 sequencing primers (Promega Corp.). The DNA sequence was determined using either avian myeloblastosis reverse transcriptase (Houts, et al., 1979) or Sequenase following the protocols supplied except sequencing primers were hybridized following an alkaline denaturation. Plasmid DNA for sequencing reactions using avian myeloblastosis reverse transcriptase was purified by banding on CsCl gradients. Plasmid templates sequenced using Sequenase was isolated using a modified alkaline lysis method (Ish-Horowicz and Burke, 1981) from overnight 3 ml cultures. Both strands of overlapping DNA fragments were sequenced.

Construction of a *speA-lacZ* fusion.

A translational *speA-lacZ* fusion was constructed by digesting pRM15 with *Bam*HI. The restriction fragments were separated by electrophoresis on a 0.7% SeaPlaque agarose gel. A 2,119 bp *Bam*HI fragment was excised, ligated into the *Bam*HI site of pMC1403 and transformed into *E. coli* DH5α. *E. coli* strains were screened for ß-galactosidase production on LB plates containing 100 ug/ml ampicillin spread with 100 ulof a 20 mg/ml solution of Bluo-Gal. Plasmids containing *speA-lacZ* fusions were isolated and mapped with *Pst*I and

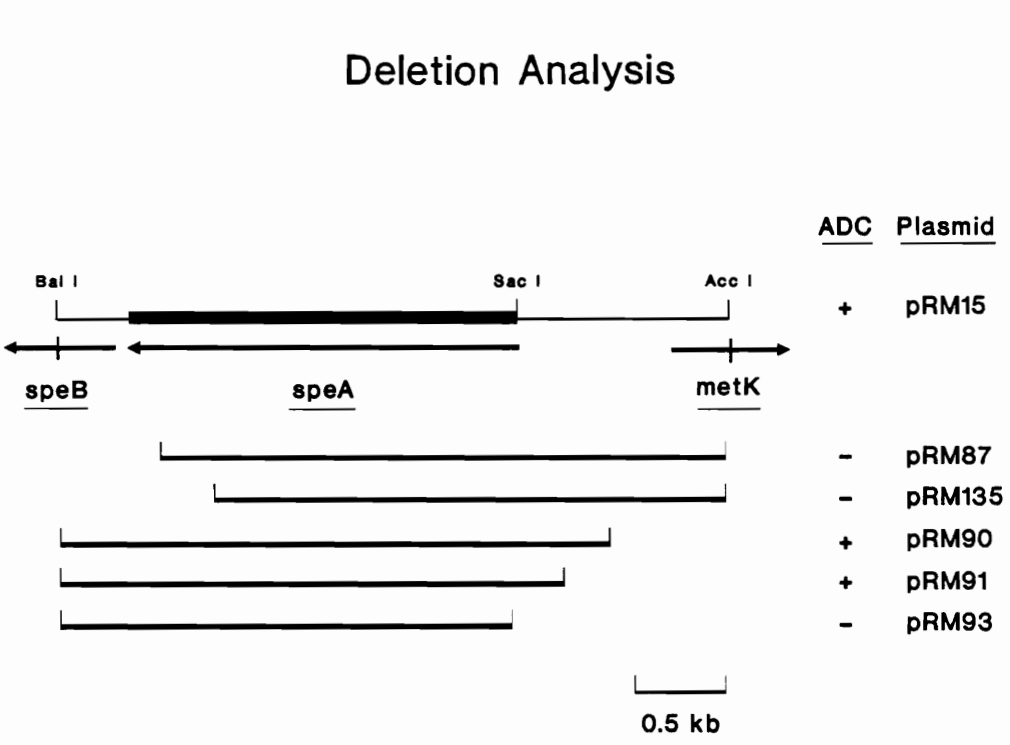


Figure 4. Location of *speA* by deletion analysis. Region contained in deletion plasmids are indicated by thick lines. The *speB* and *metK* open reading frames are truncated in pRM15. Arginine decarboxylase activity is relative to *E. coli* DH5α containing pRM15 and designation of plasmids is listed on the right.

*Sac*I to determine the orientation of the 2,119 bp insert within pMC1403. The orientation of the *Bam*HI insert was confirmed by sequencing the *lacZ* junction. Plasmid pRM65 was chosen for further study.

Western Blots.

E. coli CB806 transformed with pRM65 was grown in a shaking waterbath at 37°C overnight in 5 ml of LB medium. Cells were recovered by centrifugation and lysed by sonication. ADC::ß-galactosidase fusion proteins were separated using a Protosorb *lacZ* immunoaffinity column. Approximately 50 ug of protein was loaded into each well of a 3% stacking gel with a 5% polyacrylamide gel. Samples were loaded a second time in the reverse order to create a mirror image for an immunoblot. Following electrophoresis the gel was cut in two; one half was stained with Coomassie blue and the second half was blotted to a nitrocellulose membrane. The membrane was probed with ß-galactosidase antisera to identify ß-galactosidase and ADC::ß-galactosidase hybrid proteins. The size of the fusion proteins was estimated by comparison with the mobility of molecular weight standards.

Protein determinations.

Protein concentrations were determined using the Bradford method (Bradford, 1976)(Bio-Rad Laboratories, Richmond, CA).

Enzyme assays.

E. coli strains were grown in a shaking waterbath at 37°C to a density of 70 Klett units in either LB or MOPS medium supplemented with thiamine (1 ug/ml), glucose (0.2%) and all amino acids (50 ug/ml) except arginine. ß-galactosidase activity was assayed by the method of Miller (1972). The lactose operon of *E. coli* strains was induced by addition of isopropyl-ß-D-thiogalactopyranoside (5 mM, final concentration). ADC activity was assayed

by quantitation of $^{14}CO_2$ released as previously described (Wright and Boyle, 1982).

Computer Analysis.

Analysis of the DNA sequence was carried out using the sequence analysis software from ICR (Fox Chase Cancer Institute) and the University of Wisconsin Genetic Computer Group sequence analysis software package installed on a VAX8800. The nucleic acid and predicted amino acid sequences of ADC were used to search for similar proteins in the GenEMBL and NBRF databases using the Wordsearch program.

RESULTS

Subcloning of *speA* gene.

A plasmid, pLC2-5, was identified from Clarke-Carbon *E. coli* genomic library that overexpresses ADC (Clarke and Carbon, 1976). An 8,000 bp *Eco*RI fragment from pLC2-5 was ligated into the *Eco*RI site of pBR322 yielding the hybrid plasmid pKA5 (Tabor et al., 1983). This 8.0 kb *Eco*RI fragment carries the *speA*, *speB* and *metK* genes which overexpress ADC, AUH and methionine adenosyltransferase (MAT) respectively (Boyle et al., 1984). Deletion of a *Bal*I fragment from pKA5 was shown to effect AUH, but not ADC or MAT activity. A computer search of the *metK* gene sequence (Markham et al., 1984) revealed an *Acc*I site approximately 3.2 kb downstream from the *Bal*I site in pKA5, within the *metK* ORF (Fig. 3). A 3236 *Bal*I-*Acc*I fragment from pKA5 was subcloned into pGEM-3Z and when clones containing recombinant plasmids were assayed for ADC activity, eight out of fifteen clones overproduced ADC. A single clone, pRM15, was chosen for further use. Crude extracts of *E. coli* DH5α cells containing pRM15 produced a 4.3-fold increase in ADC specific activity compared to *E. coli* DH5α and 1.5 times more activity than *E. coli* bearing pKA5. Crude extracts of *E. coli* DH5α transformed with plasmid pRM59 produced

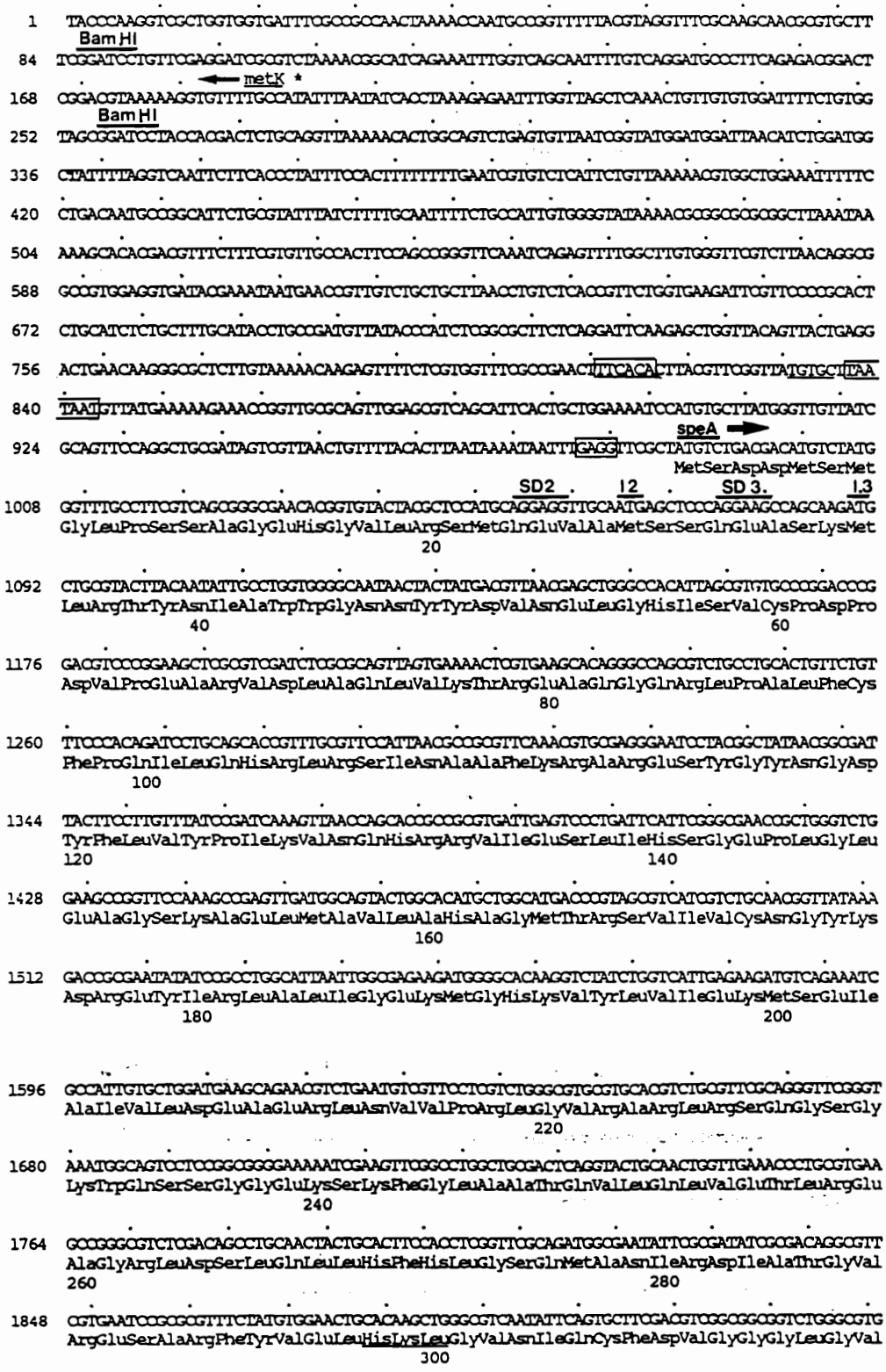
a 6.2-fold increase in ADC specific activity compared to *E. coli* DH5α and 2.8 times more than *E. coli* bearing pKA5. The apparent increase in the level of ADC expression of *E. coli* containing either pRM15 or pRM59 compared with cells containing pKA5 may be due to either the loss of a regulatory element present on pKA5 or may simply be a reflection of the difference in the plasmid copy number. A slight increase in ADC activity of cells bearing pRM59 is probably due to a transcriptional fusion between the *speA* gene and the *lacZ* promoter in the vector, pGEM-3Z.

Sequence analysis.

The sequence of the 3,236 base pair *Bal*I-*Acc*I fragment carried by pRM15 and pRM59 was determined by the dideoxy-chain terminating method of Sanger (1977) for both strands from overlapping DNA fragments (Fig. 5). A large ORF (ORF1) contains 1,974 nucleotides, begins with an AUG initiation codon (Met) at nucleotide 987 and ends with a GAG codon (Glu) at nucleotide 2,960. ORF1 is capable of encoding a 73,980 Da polypeptide consisting of 658 amino acids with a predicted isoelectric point of 4.70.

Sequences resembling *E. coli* consensus for -35 (TTCACA) and -10 (AATAAT) promoter regions are located at nucleotides 811 to 816 and 839 to 844 respectively. A possible CRP binding consensus sequence (TGTGC) was found at nucleotides 831 to 835, a ribosome binding site, GAGG, was present at nucleotides 977 to 980, and a potential transcription terminator was located at nucleotides 3,030 to 3,067. A probable pyridoxal phosphate binding site, H-K-L, is located in ADC from residues 298 to 300.

Partial ORFs corresponding to the *speB* and *metK* genes were also identified (Fig. 3). The *speA* and *speB* (ORF2) ORFs are found on the same DNA strand. ORF2 begins 140 nucleotides downstream from ORF1, continues for 135 nucleotides (45 amino acids) and terminates at a *Bal*I site within the agmatine ureohydrolase (*speB*) ORF. A 37 bpstem-loop


BamHI
metK *
BamHI
speA
MetSerAspAspMetSerMet
SD2
I2
SD 3.
I3
GlyLeuProSerSerAlaGlyGluHisGlyValLeuArgSerMetGlnGluValAlaMetSerSerGlnGluAlaSerLysMet
LeuArgThrTyrAsnIleAlaTrpTrpGlyAsnAsnTyrTyrAspValAsnGluLeuGlyHisIleSerValCysProAspPro
AspValProGluAlaArgValAspLeuAlaGlnLeuValLysThrArgGluAlaGlnGlyGlnArgLeuProAlaLeuPheCys
PheProGlnIleLeuGlnHisArgLeuArgSerIleAsnAlaAlaPheLysArgAlaArgGluSerTyrGlyTyrAsnGlyAsp
TyrPheLeuValTyrProIleLysValAsnGlnHisArgArgValIleGluSerLeuIleHisSerGlyGluProLeuGlyLeu
GluAlaGlySerLysAlaGluLeuMetAlaValLeuAlaHisAlaGlyMetThrArgSerValIleValCysAsnGlyTyrLys
AspArgGluTyrIleArgLeuAlaLeuIleGlyGluLysMetGlyHisLysValTyrLeuValIleGluLysMetSerGluIle
AlaIleValLeuAspGluAlaGluArgLeuAsnValValProArgLeuGlyValArgAlaArgLeuArgSerGlnGlySerGly
LysTrpGlnSerSerGlyGlyGluLysSerPheGlyLeuAlaAlaThrGlnValLeuGlnLeuValGluThrLeuArgGlu
AlaGlyArgLeuAspSerLeuGlnLeuLeuHisPheHisLeuGlySerGlnMetAlaAsnIleArgAspIleAlaThrGlyVal
ArgGluSerAlaArgPheTyrValGluLeuHisLysLeuGlyValAsnIleGlnCysPheAspValGlyGlyGlyLeuGlyVal

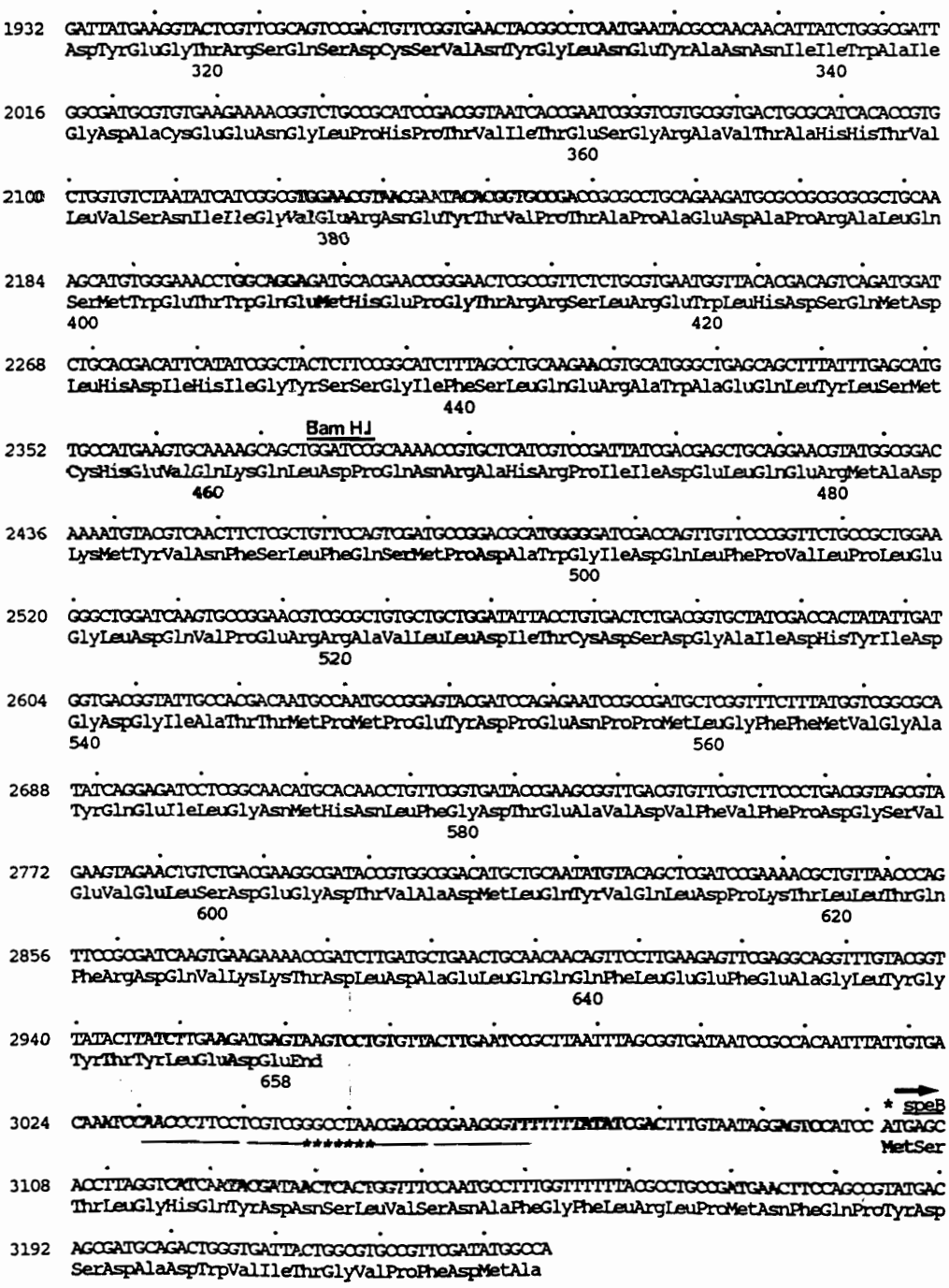


Figure 5. Nucleotide and deduced amino acid sequence of *speA*. Promoter and ribosome binding consensus sequences (boxed), CRP binding site and pyridoxal phosphate binding consensus (underlined), translation initiation sites of *speB* and *metK* (*), and rho-independent transcriptional terminator (————***————) are indicated. Alternate Shine-Dalgarno (SD2, SD3), translation initiation (I2, I3), and *Bam*HI sites are indicated.

structure, located at nucleotide 3,031 to 3,067, is present in the 140 base pair intergenic region separating the *speA* and *speB* genes. The existence of this stem-loop structure was confirmed by S1 analysis (Szumanski and Boyle, 1990). The *metK* open reading frame (ORF3), divergent from *speA*, begins 794 nucleotides upstream from ORF1, continues for 192 nucleotides (64 amino acids) and terminates at an *Acc*I site within the methionine adenosyltransferase (*metK*) ORF (Markham et al., 1984).

Identification of the *speA* gene and promoter.

The location of the *speA* gene was determined by assessing the ability of clones bearing deletions in pRM15 or pRM59 to overexpress arginine decarboxylase (Fig. 4). ADC expression was maintained when 564 nucleotides (424 bases upstream of ORF1) of the 5' end of the *Bal*I-*Acc*I fragment were removed. This treatment results in the loss of the *metK* ORF and a large portion of the intergenic region between the *speA* and *metK* genes. The ability to express ADC is lost when 355 nucleotides of the 3' end of the *Bal*I-*Acc*I fragment are removed. In this case the *speB* ORF and 80 bp of ORF1, which encodes ADC, has been removed.

The *speA* promoter and 1389 nucleotides (463 amino acids) of ORF1 were ligated to the *lacZ* gene in the vector pMC1403 to produce a *speA-lacZ* translational fusion, pRM65. *E. coli* CB806 bearing pRM65 produced a 42-fold increase in ß-galactosidase activity relative to *E. coli* CB806 containing pMC1403 (1,250 Units in pRM65 and 30 Units in pMC1403). Western blots probed with ß-galactosidase antisera identified three proteins in *E. coli* DH5α containing pRM65: 160,000 and 156,000 Da ADC::ß-galactosidase fusion proteins and a 116,353 Da ß-galactosidase monomer (Fowler and Zabin, 1978)(Fig. 6). The 160,000 Da protein results from the fusion of 44,000 Da of ADC to the 116,353 Da of ß-galactosidase. The presence of an additional 156,000 Da fusion protein containing 41,000 Da of ADC is

consistent with the processing of ADC; a 74,000 Da ADC precursor is processed to the 70,000 Da form (Buch and Boyle, 1985a). The size of these two fusion proteins further suggests that translation initiates at or near the beginning of ORF1. The difference of 4,000 Da in molecular weight of these two fusion proteins also suggests that the 160,000 Da form contains a signal peptide that is removed to produce the 156,000 Da form (Buch and Boyle, 1985a).

The predicted molecular mass of an ADC monomer, 73,980 Da, is identical to the 74,000 Da estimated from denaturing gels (Buch and Boyle, 1985a; Wu and Morris, 1973). The deduced amino acid composition of ORF1 is also in good agreement with the amino acid analysis of ADC performed by Wu and Morris (1973)(Table 2). The results of deletion analysis, immunoblots of ADC::β-galactosidase fusion proteins, amino acid composition and molecular weight of a protein predicted by ORF1 all indicate that ORF1 encodes arginine decarboxylase.

Sequence Comparisons of ADC and amino acid decarboxylases.

The results of a computer search of the GenEMBL and NBRF databases revealed that ADC contains small regions of identity with five other decarboxylases and two arginases. A six amino acid region, D-V-G-G-G-L, is conserved in diaminopimelic acid decarboxylases (DapDC) from *E. coli* and *Corynebacterium glutamicum* (Fig. 7). This conserved region appears in the same relative position in each enzyme (53 to 57% towards the carboxy terminus) and is located eight amino acids from the presumed pyridoxal phosphate binding site in ADC, H-K-L. Both *E. coli* and *C. glutamicum* DapDCs were approximately 67% identical in this sequence. Yeast (D-V-G-G-G-F), mouse (D-I-G-G-G-F) and trypanosome (D-I-G-G-G-F) ornithine decarboxylase also appear to contain sequences with homology to ADC (D-V-G-G-G-L). These regions occur from 53 to 61% towards the carboxy end of

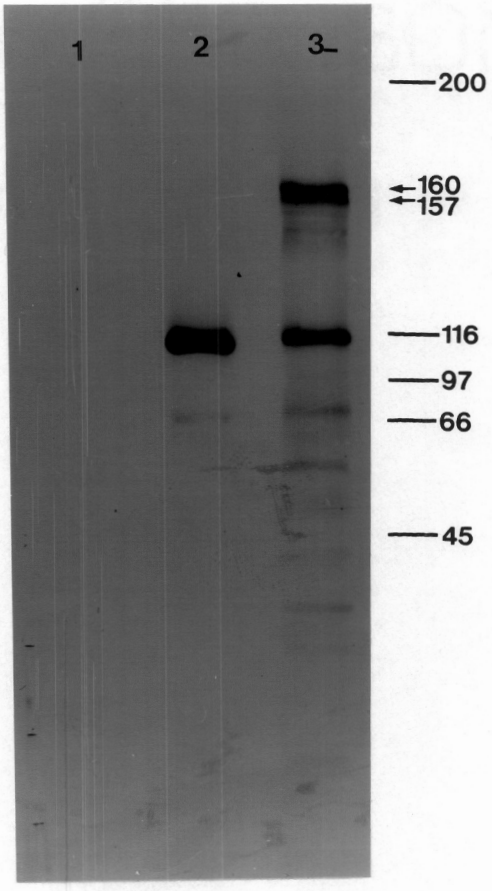


Figure 6. Identification of ADC::ß-galactosidase fusion proteins from *E. coli* CB806 transformed with pRM65. Immunoblots were carried out as described in Materials and Methods. Protosorb column flow-through (lane 1), ß-galactosidase (lane 2), and ß-galactosidase and ADC::ß-galactosidase fusion proteins (lane 3). Sizes (kilodalton) of molecular mass standards are indicated.

Table 2. Comparison of amino acid composition of predicted and purified *E. coli* arginine decarboxylase. *Taken from Wu and Morris (1973).

	Amino acid analysis*		DNA Sequence	
Amino acid	No.	%	No.	%
Ala	52	7.6	48	7.3
Arg	37	5.4	38	5.8
Asx	72	10.5	66	10.0
Asn			23	3.5
Asp			43	6.5
Cys	7	1.0	8	1.2
Glx	99	14.4	90	13.7
Gln			36	5.5
Glu			54	8.2
Gly	53	7.7	51	7.8
His	17	2.5	21	3.2
Ile	28	4.1	33	5.0
Leu	68	9.9	67	10.2
Lys	19	2.7	18	2.7
Met	25	3.6	24	3.7
Phe	21	3.1	20	3.0
Pro	33	4.8	28	4.3
Ser	43	6.3	41	6.2
Thr	27	3.9	24	3.7
Trp	12	1.7	9	1.4
Tyr	27	3.9	25	3.8
Val	47	6.8	47	7.1
Total	687		658	
MW	74,000 Da		73,980 Da	

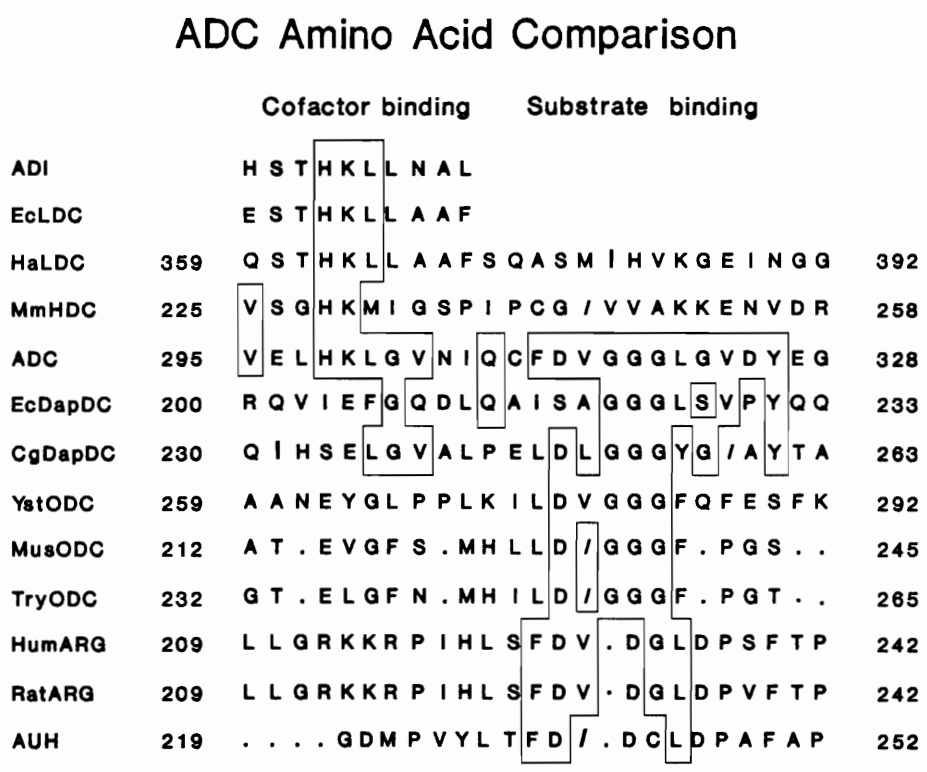


Figure 7. Comparison of ADC and other enzymes which recognize structurally similar substrates. Gaps (.) have been introduced to achieve the optimum alignment. Amino acids matching ADC are boxed, conservative changes relative to ADC (|), other matching residues (:), and mismatches (*) are indicated. *E. coli* ADI (inducible), LDC (ECLDC), DapDC (ECDapDC), *Hafnia alvei* LDC (HALDC), *Morganella morgenii* HDC (MMHDC), *Corynebacterium glutamicum* DapDC (CGDapDC), yeast (YstODC), mouse (MusODC) and trypanosome (TryODC) ODCs, human arginase (HumARG), rat arginase (RatARG), and *E. coli* AUH.

each enzyme and were 83, 67, and 67% identical to the ADC sequences, respectively. Human and rat arginase both contain the sequence F-D-V-D-G-L near their carboxyl terminus. This arginase sequence is 71% identical to the region conserved in ADC (F-D-V-G-G-G-L).

Mapping of the *speA* gene within the *E. coli* chromosome.

The *speA* gene has been mapped by cotransduction to 62.8 (Hafner et al., 1977) minutes on the *E. coli* chromosome. The *speB, speA* and *metK* genes, in this gene order, have been identified on plasmid pKA5, which contains an 8.0 kb *Eco*RI fragment derived from the *E. coli* chromosome (Boyle et al., 1984). The DNA sequence reported in this paper contains the *speA* gene, intergenic regions, as well as partial sequences of the *speB* and *metK* genes. Since *speB* and *metK* have been sequenced (Markham et al., 1984; Szumanski and Boyle, 1990), it was possible to produce a 6,530 bp continuous DNA sequence of this region in pKA5. A restriction map of this 6,530 bp sequence was generated and compared to the restriction map of the *E. coli* chromosome constructed by Kohara (1987). The *speA* gene is located at 62.9 minutes on Kohara's map and is contained in lambda clones 1H10 and 23G45. The presence of the *speA* gene on these lambda clones has been confirmed by Southern analysis using a *speA* probe (C. Satishchandran et al., 1990).

DISCUSSION

This paper describes the subcloning and nucleotide sequence of the *speA* gene encoding *E. coli* ADC. When ADC was originally purified and analyzed by Wu and Morris (Wu and Morris, 1973), they reported that purified ADC resolved into two major and three minor bands when electrophoresed on a native gel. On a denaturing gel these two major bands migrated with a molecular mass corresponding to 74,000 and 70,000 Da. When either the

74 or 70,000 Da band were assayed for ADC activity, each species decarboxylated arginine. Isolation of ADC by immunoprecipitation of pulse-labeled *E. coli* also demonstrated two major bands corresponding to 74,000 and 70,000 Da, as well as minor bands in the 30- to 40,000 Da range (Buch and Boyle, 1985a). These minor bands are probably nascent chains of ADC because they disappear when the label was chased. The DNA sequence of the 3.2 kb *BalI-AccI* fragment in pRM15 revealed only one ORF (ORF1) capable of encoding a 74,000 or 70,000 Da polypeptide.

The loss of approximately 4,000 Da from a precursor is characteristic of the processing of a signal peptide during translocation (Oliver, 1985; Randall et al., 1987). The presence of a signal peptide in ADC was supported by the finding that the 74,000 Da species, but not the 70,000 Da species, accumulated in an *E. coli* strain unable to process signal sequences (Buch and Boyle, 1985a). Furthermore, selective disruption of the cell envelope showed that the 70,000 Da species is localized within the inner periplasmic space (Buch and Boyle, 1985a). A review of prokaryotic signal peptides characteristics (Oliver, 1985; Randall et al., 1987) indicates that the predicted amino terminus of ADC does not possess a typical signal sequence. Signal peptides usually contain three distinct regions; a positively charged amino terminus, a core of 12 to 20 primarily hydrophobic amino acid residues, followed by a signal peptidase processing site. Kyte-Doolittle hydropathy plots (not shown) indicate that a signal peptide starting at the beginning of ORF1 would have a hydrophobic core comprised of two short hydrophobic regions, but would possess a negatively charged amino terminus. However, a net positive charge on a signal peptide is not an absolute requirement for export to the periplasm (Iino et al., 1987; Inouye et al., 1982; Puziss et al., 1989; Vlasuk et al., 1983).

The rate of processing of precursor ADC to the mature form occurs with kinetics

atypical for most signal sequences (Buch and Boyle, 1985a). Signal peptides are usually processed very rapidly, usually within 30 to 60 seconds. Studies using site-directed mutagenesis on various *E. coli* signal peptides have shown that progressively decreasing the net charge on the amino terminus results in a corresponding decrease in the efficiency of translocation and a reduced rate of synthesis (Inouye et al., 1982; Puziss et al., 1989; Vlasuk et al., 1983). When the net charge of the signal peptide of *E. coli* prolipoprotein was decreased from +2 to -2, 12 min were required to completely convert prolipoprotein into lipoprotein, resulting in an accumulation of the precuror in the cytolasm. Pulse-chase experiments in *E. coli* showed that the ADC precursor requires approximately 6 min for ADC precursor to be converted into mature ADC (Buch and Boyle, 1985a). The slow processing rate suggests that ADC accumulates in the cytoplasm following synthesis and is processed posttranslationally. This appears to be the case as Morris et. al. (Morris and Koffron, 1969) found that 20% of the putrescine synthesized in an *E. coli* K-12 strain grown in the absence of arginine utilized the ADC-AUH pathway. Thus, a distribution of active ADC in the periplasm versus the cytoplasm might explain the partitioning of endogenously synthesized arginine between polyamine and protein synthesis.

Most decarboxylases require pyridoxal phosphate as a cofactor for catalytic activity and many of these enzymes contain a pyridoxal phosphate binding consensus sequence, S-X-H-K. *E. coli* ADC, LDC and *Morganella morganii* HDC contain either the amino acid sequence S-X-H-K or simply H-K in their pyridoxal phosphate binding sites (Boeker et al., 1971; Fecker et al., 1986; Vaaler and Snell, 1989). The function of the histidine and lysine residues in the pyridoxal phosphate binding consensus sequence has been investigated in *M. morganii* HDC using site-directed mutagenesis (Vaaler and Snell, 1989). Studies indicated that Lys-232, homologous to Lys-299 in ADC, was involved in decarboxylation, but not in

pyridoxal phosphate or histidine binding. His-231, homologous to His-298 in ADC, appeared to play a role in hydrogen bonding of HDC to histidine or pyridoxal phosphate. ADI and LDC are similar in subunit size, molecular weight and their pyridoxal phosphate binding sequences (Boeker et al., 1971; Morris and Boeker, 1983). In these enzymes pyridoxal phosphate binds to the ϵ-amino group of the lysine residue within the conserved region, S-T-H-K-L-L (Morris and Boeker, 1983). The predicted pyridoxal phosphate binding site of *H. alvei* LDC is similar (nine of ten residues match) to the *E. coli* LDC pyridoxal phosphate binding site and also contains the amino acid sequence S-T-H-K-L-L (Fecker et al., 1986)(Fig. 7). Three of these conserved amino acids, H-K-L, are retained in biosynthetic ADC. The conservation of the amino acid sequence H-K-L in the pyridoxal phosphate binding sites of several amino acid decarboxylases and their similar relative positions within each enzyme (45 to 49% towards the carboxyl end) suggests that H-K-L serves as the pyridoxal phosphate binding site for biosynthetic ADC.

Pyridoxal phosphate binding sites are usually followed by a hydrophobic sequence that is thought to form a hydrophobic pocket in which the cofactor or the substrate binds (Morris and Boeker, 1983). Eight residues from the presumed pyridoxal phosphate binding site of ADC begins an amino acid sequence, D-V-G-G-G-L, which is conserved (67 to 71% identical) in *E. coli* and *Corynebacterium* DapDCs and three eukaryotic ODCs. Hydropathy plots of each of these amino acid sequences (data not shown) reveals that all are predicted to produce a hydrophobic region. The possibility that this region is involved in substrate binding is supported by the observation that ADC, DapDCs and ODCs each recognize structurally similar substrates and catalyze similar reactions (each removes the C1 carboxyl group, Fig. 8). In contrast, *H. alvei* LDC and *M. morganii* HDC pyridoxal phosphate binding sites are followed by a hydrophobic sequence, but neither LDC or HDC show any

homology with ADC in this region (Fig. 5). Human and rat arginases, which also use arginine as a substrate, contain a similar sequence, F-D-V-D-G-L, that is 71% identical to the conserved region of ADC. Interestingly, this sequence is within one of the most highly conserved regions between arginase and *E. coli* AUH, the second enzyme in the ADC pathway (Fig. 7, Fig. 8).

The *speA* -10 and -35 promoter regions are separated by 21 bp, suggesting that the ADC promoter is weakly transcribed (Hawley and McClure, 1983). Preliminary experiments indicate that the *speA* promoter is indeed weakly transcribed since a *speA-lacZ* fusion borne on a multicopy plasmid produces less ß-galactosidase activity than does a single copy chromosomal *lacZ* gene in *E. coli.*

A polycistronic mRNA containing *speA* and *speB* is produced from the *speA* promoter. The *speB* gene is also capable of transcription from an additional promoter located within the 5'-end of the *speA* ORF to produce a monocistronic *speB* mRNA (Szumanski and Boyle, 1990). A classical rho-independent terminator structure begins 69 bp downstream from the end of the *speA* ORF and ends 34 bp upstream of the *speB* ORF. This 37 bp structure has a 15-bp GC-rich stem (with one mismatch), a 7 base loop, and ends with seven consecutive U residues. The structure does not appear to function as an attenuator as it lacks a suitable ribosome binding site and contains a translation stop codon within the stem. How transcription of the *speB* gene occurs if this terminator-like structure exists *in vivo* is unknown, although it may play a role in regulating the expression of the *speB* gene. The *speB* gene, but not *speA*, is induced by agmatine, while both the *speA* and *speB* genes are repressed by cAMP.

The presence of a CRP binding consensus sequence in the promoter region of *speA* is consistent with its inhibition by cAMP (Wright and Boyle, 1982). Presumably the

Structurally Related Substrates

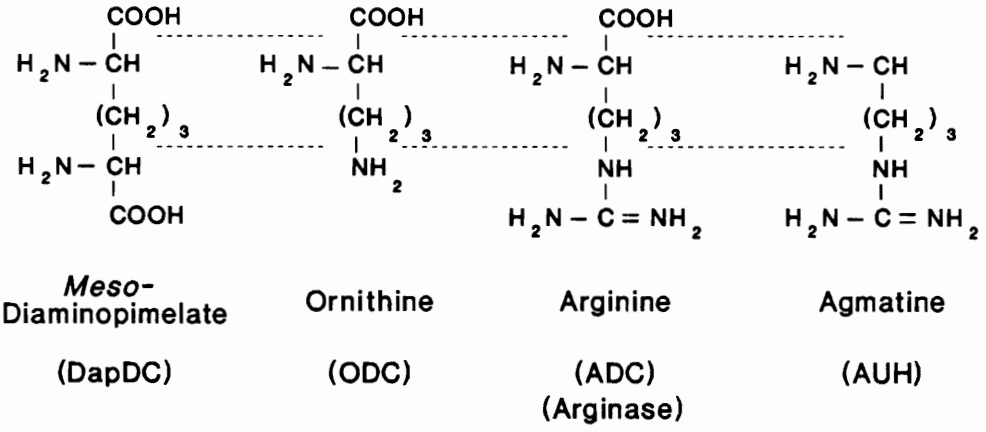


Figure 8. Structurally similar substrates used by enzymes containing the conserved sequence F-D-V-G-G-G-L (see Fig. 5). Dashed lines indicates the common structural skeleton recognized by each enzyme. Enzymes are in parentheses. All decarboxylases remove the carboxyl group in the C1 position. Arginase and agmatine ureohydrolase (AUH) remove the urea group from their respective substrates. ADC is biosynthetic arginine decarboxylase; DapDC is diaminopimelic acid decarboxylase; ODC is ornithine decarboxylase.

cAMP-CRP complex would bind to this site and interfere with the initiation of transcription of the *speA* gene. However, preliminary experiments of *E. coli* bearing the *speA-lacZ* translational fusion plasmid pRM65 demonstrate that the production of ADC::β-galactosidase fusion protein is not inhibited by cAMP, in contrast to the induction of β-galactosidase synthesis by cAMP in control cells. Additional experiments indicate that two factors contribute to this apparent insensitivity of the plasmid borne *speA-lacZ* fusion to cAMP-mediated regulation. First, the high copy number of plasmid pRM65 influences the regulation of the *speA* gene by cAMP. Second, the degree of repression of ADC by cAMP appears to be strain dependent. This finding is not surprising, as the *speA* gene of some strains of *E. coli* are known to be insensitive to cAMP-mediated inhibition (Shaibe et al., 1985a). We are currently attempting to clarify the role of cAMP-mediated regulation of the *speA* gene.

CHAPTER II

Regulation of the *speA* gene encoding biosynthetic arginine decarboxylase of *Escherichia coli* by cAMP, and putrescine

INTRODUCTION

Putrescine (diaminobutane) is an aliphatic cation that is required for optimum growth of all cells. Putrescine and other polyamines are involved in many biological processes, but their physiological role(s) remains unclear. Most eucaryotes possess a single putrescine biosynthetic pathway (Pathway I), in which ornithine decarboxylase (ODC) converts ornithine to putrescine. Bacteria and plants also have a second putrescine biosynthetic pathway (Pathway II). In bacteria, arginine is converted to agmatine by biosynthetic arginine decarboxylase (ADC), encoded by the *speA* gene. Agmatine is hydrolyzed to putrescine and urea by agmatine ureohydrolase (AUH), encoded by the *speB* gene.

The presence of two parallel putrescine biosynthetic pathways is necessary in *Escherichia coli* because lacks arginase and therefore, cannot convert arginine to ornithine. In the presence of exogenous arginine, as in the intestine, the intracellular pool of ornithine becomes limiting due to arginine feedback repressing arginine biosynthesis (Tabor and Tabor, 1969a). Consequently, putrescine synthesis from Pathway I would decrease. Thus, Pathway II ensures that putrescine is produced as nutritional conditions change (Morris et al., 1970).

Native ADC is a tetramer, with a molecular size of 280,000 Da. The enzyme requires magnesium and pyridoxal phosphate for activity (Morris and Fillingame, 1974; Wu and Morris, 1973). Monomers of ADC are synthesized as 74,000 Da precursor polypeptides that

are post-translationally processed to a 70,000 Da form during translocation to the inner periplasmic space (Buch and Boyle, 1985). This compartmentalization of ADC explains the finding that exogenous arginine is preferentially channeled into putrescine synthesis; approximately 20% of exogenous arginine is converted to putrescine, with the remainder being incorporated into protein (Tabor and Tabor, 1969b). In the periplasm, ADC is able to decarboxylate arginine to agmatine as the arginine is imported into the cell. Agmatine induces AUH activity in the cytoplasm, resulting in increased putrescine production from Pathway II. The *speA* and *speB* genes have been cloned (Tabor et al., 1983) and sequenced (Moore and Boyle, 1990; Szumanski and Boyle, 1990). A polycistronic messenger RNA (mRNA) is produced from the *speA* promoter, but the *speB* gene also produces a moncistronic mRNA (Szumanski and Boyle, 1990). The open reading frames (ORF) for ADC and AUH are separated by 140 bp that contains a rho-independent terminator (Moore and Boyle, 1990). It is not clear how a polycistronic mRNA containing *speB* is transcribed in the presence of this structure.

The putrescine biosynthetic genes (*speA*, *speB*, *speC*) are subject to metabolic controls. All three genes are negatively regulated by a mechanism involving cAMP and cAMP receptor protein (CRP)(Wright and Boyle, 1982). In bacteria, the greater the limitation of a carbon source, the greater the intracellular concentration of cAMP (Alpher and Ames, 1977; Buettner et al., 1973; Epstein et al., 1975; Pastan and Adhya, 1976). As the growth rate of E. coli increases, so does the bacterium's need for putrescine. Thus, the level of cAMP acts as a signal to coordinate putrescine production with the growth rate of the bacterium. The amount and activity of ADC and ODC are also controlled by the putrescine concentration in *E. coli*, suggesting both inhibition and repression of the enzymes (Tabor and Tabor, 1969a). The experiments in this paper show that cAMP-CRP controls ADC

expression indirectly by inhibiting the activity of the enzyme and that putrescine represses the *speA* gene at the level of transcription. The inhibition of ADC activity by cAMP-CRP and putrescine is additive, indicating these effectors regulate ADC by independent regulatory mechanisms.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are described in Table 3. *E. coli* strains were grown in either 3-(N-morpholino)-propanesulfonic acid (MOPS) medium (Neidhardt et al., 1974) supplemented with 0.2% glucose, 1 ug/ml thiamine, and 50 ug/ml amino acids except arginine, Luria broth (LB)(1% tryptone, 0.5% yeast extract, and 1% NaCl)(Maniatis et al., 1982) or Terrific broth (TB) (1.4% tryptone, 2.7% yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4)(Tarrof and Hobbs, 1987). *E. coli* used for lysogen construction were grown in λYM medium (1% tryptone, 1% yeast extract, 0.25% NaCl, and 0.2% maltose) (Simmons et al., 1987) and plated on MacConkey's agar supplemented with ampicillin. Lambda infections used λTB top agar (1% tryptone, 0.5% NaCl, 10 mM $MgSO_4$ and 0.7% agar) and plates (Silhavy et al., 1984). *E. coli* used for P1 infections used LGC broth (1% tryptone, 0.5% yeast extract, 1% NaCl, 0.2% glucose, 5 mM $CaCl_2$ and 10 mM $MgCl_2$) and plates. Antibiotics were used at the following concentrations; ampicillin at 100 ug/ml, kanamycin at 20 ug/ml, and tetracycline at 12 ug/ml.

Enzymes and reagents

Restriction endonucleases, T4 DNA ligase, isopropylthio-β-galactopyranoside (IPTG), halogenated indolyl-β-D-galactopyranoside (Bluo-Gal), and NACS columns were purchased from Bethesda Research Laboratories, Inc. (BRL)(Gaithersburg, MD).

Table 3. *E. coli* strains, plasmids, and bacteriophage used in Chapter II.

Strain, plasmid, or bacteriophage	Description	Reference or source
KC14	*gal*	Kline
KC14-1	Δ(*argF-lac*)*U169 zah*-735::Tn10 derivative of KC14	This study
KCRM161	KC14-1 (λRM161)	This study
KCRM65	KC14-1 (λRM65)	This study
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Casadaban
MCRM161	MC4100 (λRM161)	This study
MCRM65	MC4100 (λRM65)	This study
CB806	F⁻ Δ*lacZ lacY*$^+$ *galK rpsL thi recA56 phoA8*	Schneider
CBRM161	CB806 (λRM161)	This study
CBRM65	CB806 (λRM65)	This study
BD1412	Δ*lac ara thi strA sup lac-pro*	Duncan
SH205	Hfr Δ(*argF-lac*)*U169* (*zah-735*::Tn*10*)	Schweizer
SH305	MC4100 Δ*glpD102 recA1 srl*::Tn*10*	Schweizer
Plasmids		
pCB267	*lacZ* transcriptional fusion vector	Schneider
pMC1403	*lacZ* translational fusion vector	Casadaban
pRM15	pGEM-3Z with a 3.2 kb *Acc*I-*Bal*I fragment	Moore
pRM65	pMC1403 with a 2.1 kb *Bam*HI fragment	Moore
pRM160	*lacZ* transcriptional fusion vector	This study
pRM161	pRM160 with a 2.1 kb *Bam*HI fragment	This study
pRM162	pCB267 with a 2.1 kb *Bam*HI fragment	This study
pRM200	pBR322 with a 884 bp *Pst*I fragment	This study
Bacteriophage		
λRZ5	λ *lacZYA* transcriptional fusion vector for recombination with plasmids pRM161 and pMC1403	Gunsalus
λRM65	λRZ5 X pRM65 recombinant, Amp^r	This study
λRM161	λRZ5 X pRM161 recombinant, Amp^r	This study
P1::Tn*5*	Kan^r Amp^s	Kuner

Ortho-nitrophenyl-β-D-galactopyranoside (ONPG) was purchased from Sigma Chemical Co. (St. Louis, MO). 1-^{14}C-L-arginine was from Dupont, NEN Research Products (Boston, MA). Nu-Sieve low-melt agarose was from FMC BioProducts (Rockland, ME). Southern hybridizations used the Genius hybridization kit from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Construction of a *lacZ* transcriptional fusion plasmid

A transcriptional fusion vector for producing lambda lysogens was constructed by digesting plasmids pMC1403 (Casadaban et al., 1980) and pCB267 (Schneider and Beck, 1986) with *Sma*I and *Sac*I (Fig. 9). Each digest produced an approximately 1,930 bp *Sma*I-*Sac*I restriction fragment and either a 7,970 bp or 6,170 bp fragment, respectively. The 1,930 bp from pCB267 carries a multiple cloning site, the *lacZ* ribosome binding site and 1,951 nucleotides of the *lacZ* ORF. Following digestion of pMC1403 and pCB267 with *Sma*I and *Sac*I, restriction fragments were separated on a 1.0 % Nu-Sieve gel. The gel bands containing the 7,970 bp fragment from pMC1403 and the 1,930 bp fragment from pCB267 were excised, melted, ligated and transformed into *E. coli* DH5α. Exchanging this *Sma*I-*Sac*I carrying the *lacZ* ribosome binding site with the *Sma*I-*Sac*I fragment from pMC1403 produced the transcriptional fusion vector, pRM160. Clones were selected that produced white, ampicillin resistant colonies on LB plates spread with 100 ul of Bluo-Gal (20 mg/ml). Recombinant plasmids were isolated and restriction sites were mapped to confirm their identity.

Construction of transcriptional and translational *speA-lacZ* fusions

Transcriptional *speA-lacZ* fusions were constructed in plasmids pCB267 and pRM160, a derivative of pCB267. A 2,119 bp *Bam*HI fragment containing the *speA* promoter and 1,389 bp of the 5'-end of the *speA* gene was cloned into pRM160 and pCB267. Plasmid

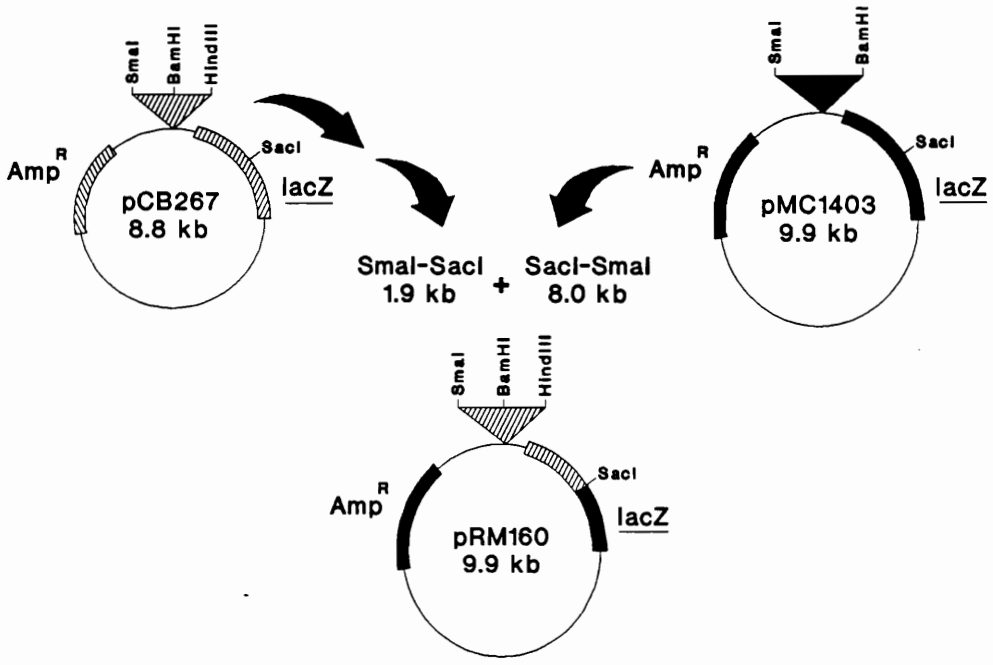


Figure 9. Construction of the transcriptional *lacZ* fusion plasmid pRM160. Plasmids pCB267 and pMC1403 were digested with *Sma*I and *Sac*I. A 1.9 kb *Sma*I-*Sac*I restriction fragment from pMC1403 was removed and replaced by a 1.9 kb *Sma*I-*Sac*I fragment from pCB267 to produce pRM160.

pRM15 was digested with *Bam*HI and the products were separated in a 1.0% Nu-Sieve gel. The band containing the 2,119 bp fragment was excised, melted, ligated into the *Bam*HI site of pRM160 or pCB267, and transformed into *E. coli* DH5α. Recombinant clones were selected on LB plates supplemented with ampicillin and spread with 100 ul of Bluo-Gal (20 mg/ml). Plasmids were isolated from clones which produced blue, ampicillin resistant colonies. The identity and orientation of the 2,119 bp *Bam*HI fragment in pRM161 or pRM162 was confirmed by restriction analysis. A translational *speA-lacZ* fusion carrying the same 2,119 bp *Bam*HI fragment in plasmid pMC1403 has been previously described (see Fig. 3)(Moore and Boyle, 1990).

P1 transduction of (*arg*F-*lac*)U169 into *E. coli* KC14

E. coli SH205 (*zah-735*::Tn*10*) carries Δ(*argF-lac*)*U169* tightly linked with transposon Tn*10* (Schweizer and Boos, 1983). The selection of transductants is convenient due to the tetracycline resistance gene carried by Tn*10*. The Δ(*argF-lac*)*U169* (*zah-735*::Tn*10*) fragment was transduced to *E. coli* KC14 using a kanamycin resistant P1::Tn*5* according to standard methods (Silhavy et al., 1984). Transductants were plated onto LB plates containing tetracycline and spread with Bluo-Gal. White, tetracycline resistant colonies were screened on LB or LB kanamycin plates spread with 100 ul of Bluo-Gal (20 mg/ml) to ensure that the P1 phage nor Tn*5* had not integrated into the *E. coli* chromosome. *E. coli* KC14-1 was Lac$^-$, tetracycline resistant and kanamycin sensitive and was chosen for studies of cAMP- and putrescine-mediated regulation of ADC.

Construction of *speA-lacZ* lysogens

E. coli MC4100 was transformed with either *speA-lacZ* fusion plasmid pRM161 or pRM65 and infected with lambda RZ5 (Jones and Gunsalus, 1987). Phage were harvested by treatment of the culture with chloroform and the cell debris was removed by

centrifugation. Lambda lysates were titered and used to produce lysogens in *E. coli* strains CB806, KC14-1, and MC4100. Lambda was preadsorbed to the bacteria for 20 minutes at room temperature and shaken for 2 h at 37°. Bacteria were plated on MacConkey lactose plates supplemented with 100 ug/ml ampicillin. Approximately ten Lac^+, ampicillin resistant clones were selected and assayed for ß-galactosidase activity. A lysogen containing a single *speA-lacZ* fusion was chosen based on its ß-galactosidase activity; the presence of a single lambda prophage was confirmed by Southern analysis. The presence of prophage in each *E. coli* strain was confirmed by UV induction of lambda phage. *E. coli* strains bearing transcriptional or translational *speA-lacZ* fusions were chosen for further study.

Southern hybridizations

Genomic DNA was isolated from *E. coli* strain KC14 and lysogens using CTAB (Ausubel et al., 1989). The DNA was digested with restriction enzyme *Xho*I and fragments separated on a 0.7% agarose gel. DNA bands were transferred to a Nytran nylon membrane by capillary blotting and cross-linked for 30 sec at 100 Watts (UV Stratolinker 2400, Stratagene). Plasmid pRM15 was grown in TB, isolated, and purified on CsCl gradients. DNA restriction fragments for making probes for the *speA* and *bla* genes were obtained by digesting pRM15 with *Hin*cII or with *Bgl*I and *Sca*I, respectively. The appropriate restriction fragments were separated on a 1% Nu-Sieve agarose gel and DNA extracted from the agarose using a NACS column. Probe DNA was labeled and hybridized according to the manufactors instructions. After the membrane was probed with *bla* DNA, the probe was removed, and the membrane was reprobed with a *speA* probe.

Regulation of *speA-lacZ* by cAMP and putrescine

A 5 ml culture of each *E. coli* strain was grown shaking overnight at 37° in MOPS medium supplemented with 0.2% glucose, 1 ug/ml thiamine, and 50 ug/ml amino acids

except arginine. *E. coli* strain KC14 was supplemented with IPTG (5 mM final concentration) to induce the lactose operon. The cultures were diluted into fresh media that either contained or lacked the appropriate effector; 5 mM or 10 mM (final concentration) cAMP or putrescine or 5 mM cAMP and 10 mM putrescine. Cells were grown for a minimum of three generations to a density of approximately 80 Klett Units and chilled on ice. Aliquots were removed from each culture, the absorbance at 600 nm recorded, and β-galactosidase activity determined. The remaining cells were harvested by centrifugation, washed with saline, and sonicated.

Enzymes Assays and protein determination

ADC activity was determined by decarboxylation of ^{14}C-arginine as previously described (Wright and Boyle, 1982). β-galactosidase activity was assayed by the method of Miller (1972). Protein concentrations were determined using the Bradford method (Bradford, 1976).

RESULTS

Strain-specific repression of ADC by cAMP

Because Shaibe et al. (1985a) had shown that ADC in certain strains of *E. coli* was not inhibited by cAMP supplementation, four strains of *E. coli* were tested. The amount of repression in these *E. coli* strains was found to be strain-specific. The specific activity of ADC from *E. coli* strains KC14, BD1412, CB806, and MC4100 grown in the presence of 5 mM cAMP was approximately 62, 92, 84, and 81%, respectively, of the ADC activity of bacteria grown in the absence of cAMP. This reduction in ADC activity was reproducible and did not vary significantly among experiments. The lactose operon of strain KC14 (cultured in 5 mM IPTG) was induced 1.5- to two-fold by the addition of 5 mM cAMP.

Addition of 10 mM cAMP to cultures of *E. coli* KC14 increased the induction of the lactose operon of strain KC14 to 2.4-fold.

Construction of a *lac* mutant of *E. coli* strain KC14

The Lac⁻ *E. coli* KC14-1 was used as a host for *speA-lacZ* lambda lysogens in *speA* regulatory studies. All of the 34 tetracycline resistant clones recovered produced white colonies on LB plates spread with Bluo-Gal and were kanamycin sensitive, indicating that neither the P1::Tn*5* or Tn*5* had integrated into the *E. coli* chromosome. Six of these clones were grown to early log phase and assayed for β-galactosidase activity in LB media supplemented with ampicillin. None of the transductants produced any detectable β-galactosidase activity compared to 1,220 units of β-galactosidase produced by the parental strain, *E. coli* KC14.

The *lacZ* and *speA* genes are well separated on the *E. coli* K-12 chromosome, located at 6 and 62.9 min, respectively. The ability of cAMP and CRP to repress the ADC activity in *E. coli* KC14-1 was tested using the parental strain KC14 as a control. Cultures of *E. coli* KC14 and KC14-1 grown in MOPS minimal medium supplemented with 5 mM cAMP produced 65% and 62% of the ADC activity of control cells. The lactose operon of *E. coli* KC14 expressed 1,225 units of ß-galactosidase when induced by IPTG and 5 mM cAMP, but KC14-1 produced no detectable ß-galactosidase activity. Furthermore, *E. coli* KC14 and KC14-1 grew at the same rate and the growth rates were not affected by the addition of 5 mM cAMP to the medium.

β-galactosidase activities of *speA-lacZ* fusions

To determine whether β-galactosidase is being expressed from the *speA* promoter β-galactosidase activities of plasmid borne *speA-lacZ* fusions was assayed. *E. coli* CB806 was transformed with one of the following plasmids; pRM160, pMC1403, pRM161, pRM162 or

pRM65 (Table 3). Bacteria were grown to mid-log phase, harvested and assayed for β-galactosidase activity (Table 4). The transcriptional *speA-lacZ* fusions pRM161 and pRM162 produced 124 and 98 units of β-galactosidase, respectively. The translational *speA-lacZ* fusion pRM65 produced 1,192 units of β-galactosidase. The 10-fold increase in β-galactosidase activity produced by the translational *speA-lacZ* fusion appears to be an artifact of the transcriptional fusion vectors pCB267 and pRM160. The translational termination codons preceeding the *lacZ* gene and the *lacZ* ribosome binding site in pRM160 were derived from pCB267. Plasmids pRM161 and pRM162 each carry identical 2.1 kb *Bam*HI fragments in which the *speA* reading frame uses a translation termination codon (TAG) that overlaps the *lacZ* ribosome binding site (AGAGGG). Apparently a ribosome that stalls at the TAG translation termination codon hinders intitiation of translation of the *lacZ* mRNA, resulting in a decrease in the β-galactosidase activity.

Plasmid pCB267 contains a pair of divergently oriented indicator genes, *lacZ* and *phoA*, so that divergent promoter activity can be monitored. Plasmid pRM162 contains a *speA-lacZ* fusion in pCB267 using a 2.1 kb *Bam*HI fragment identical to those carried by pRM65 and pRM161. When pRM162 was assayed for promoter activity divergent to *speA* none was found. It is not known whether any *metK* regulatory sequences are contained in the 2.1 kb *Bam*HI fragment.

In order to accurately examine the influences of effectors on the *speA* promoter, single copies of the transcriptional and translational *speA-lacZ* fusions were transfered to the chromosomes of *E. coli* strains KC14-1, CB806, and MC4100. When each strain carrying either a transcriptional or a translational *speA-lacZ* fusion was assayed for β-galactosidase activity, fusions in *E. coli* CB806 produced the highest level of β-galactosidase and fusions in strain KC14-1 produced the least (Table 5, Fig. 10).

Table 4. β-galactosidase activities of transcriptional and translational *speA-lacZ* fusion carried on plasmids. Units of β-galactosidase are according to Miller (1972). * 2.1 kb *Bam*HI fragment in pRM160; ** 2.1 kb *Bam*HI fragment in pCB267.

Strain	Plasmid	Type	β-Galactosidase Activity
KC14	none	*lac* operon	1,990
CB806	pRM160	vector	0
CB806	pRM161*	transcriptional	124
CB806	pRM162**	transcriptional	98
CB806	pMC1403	vector	0
CB806	pRM65	translational	1,192

Table 5. ADC and β-galactosidase activities of *E. coli* strains grown in MOPS-glucose medium. ADC activity expressed as picomoles CO_2 formed per hour per mg protein. β-galactosidase activity is calculated according to Miller (1972). TS = transcriptional; TL = translational.

Strain	Fusion	ADC*	β-Galactosidase*
KC14	none	253 ± 15.9	2235** ± 165.5
KC14-1	none	246 ± 9.2	0
λKCRM161	TS	264 ± 18.0	8-16
λKCRM65	TL	246 ± 7.9	66 ± 7.2
CB806	none	141 ± 6.4	0
λCBRM161	TS	157 ± 15.8	7-12
λCBRM65	TL	143 ± 9.8	433 ± 35.4
MC4100	none	280 ± 5.1	0
λMCRM65	TL	315 ± 22.9	180 ± 28.2

* = Specific activity ± standard deviation.
** = lactose operon induced with 5 mM IPTG.

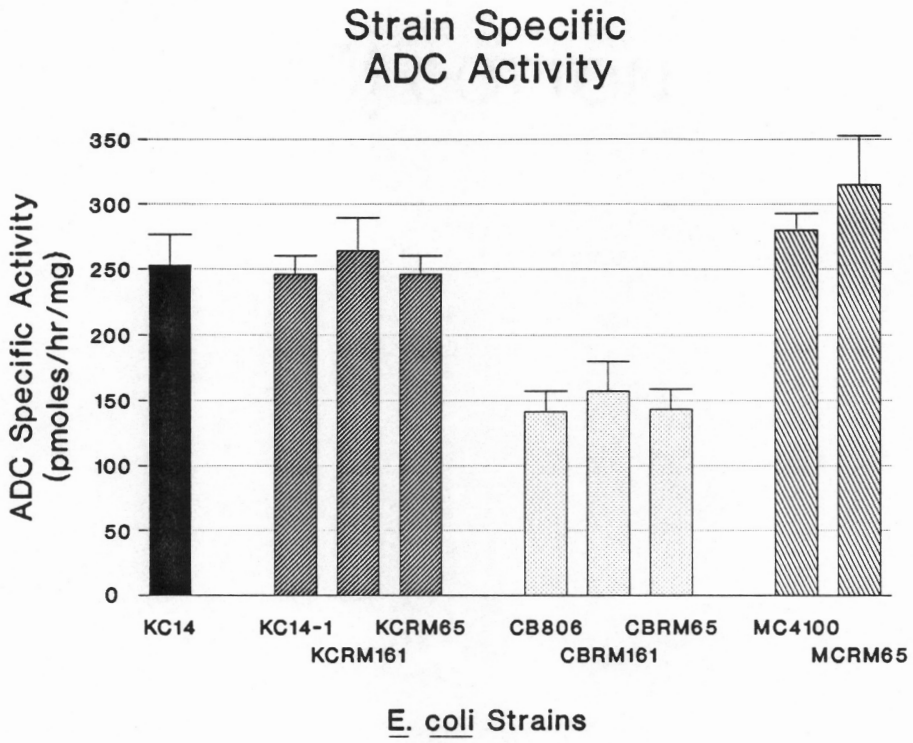


Figure 10. ADC activities of *E. coli* strains with or without a lambda lysogen *speA-lacZ* fusion.

The *speA* promoter appears to be weakly expressed. Although the translational *speA-lacZ* fusion in *E. coli* lysogen strain λKCRM65 produced more β-galactosidase activity than did the corresponding transcriptional fusion, the level of β-galactosidase expression remained lower than that produced by the lactose operon in strain KC14.

Southern hybridization

The presence of the β-lactamase gene in the chromosomes of *E. coli* strains bearing lambda lysogens was confirmed using a labeled 360 bp *Bgl*I-*Sca*I restriction fragment derived from the *bla* gene of pRM15 (Fig. 11). Each of bands corresponding to a *bla* gene also contained *speA* DNA as determined by a *speA* probe (Fig. 11). DNA fragments that hybridized to the the *speA* probe, but not to the *bla* probe were considered to be derived from the *speA* gene instead of the *speA-lacZ* fusion. The presence of more than one signal from each *Xho*I digest appeared to be due to incomplete digestion of the genomic DNA.

Regulation of ADC and *speA* by cAMP and putrescine

The presence of a second copy of the *speA* promoter in the chromosome of *E. coli* strains containing *speA-lacZ* fusions did not produce any detectable effect on the *speA* regulation by 5 mM cAMP or 10 mM putrescine. The addition of 5 mM cAMP to cultures of *E. coli* strains carrying single copies of *speA-lacZ* fusions resulted in a strain-specific reduction in ADC activity (Table 6, Fig. 12). While the reduction varied with the strain tested, the β-galactosidase activity of both transcriptional and translational *speA-lacZ* fusions was not repressed. In contrast, the β-galactosidase activity of the lactose operon of strain KC14 increased 1.5-fold. The generation time of each strain remained constant when the bacteria were grown either in the presence or absence of 5 mM cAMP or 10 mM putrescine (Table 7). Thus, the changes in activity can not be attributed to a reduction in the growth rate. Increasing the cAMP concentration to 10 mM did not effect the growth of *E. coli*

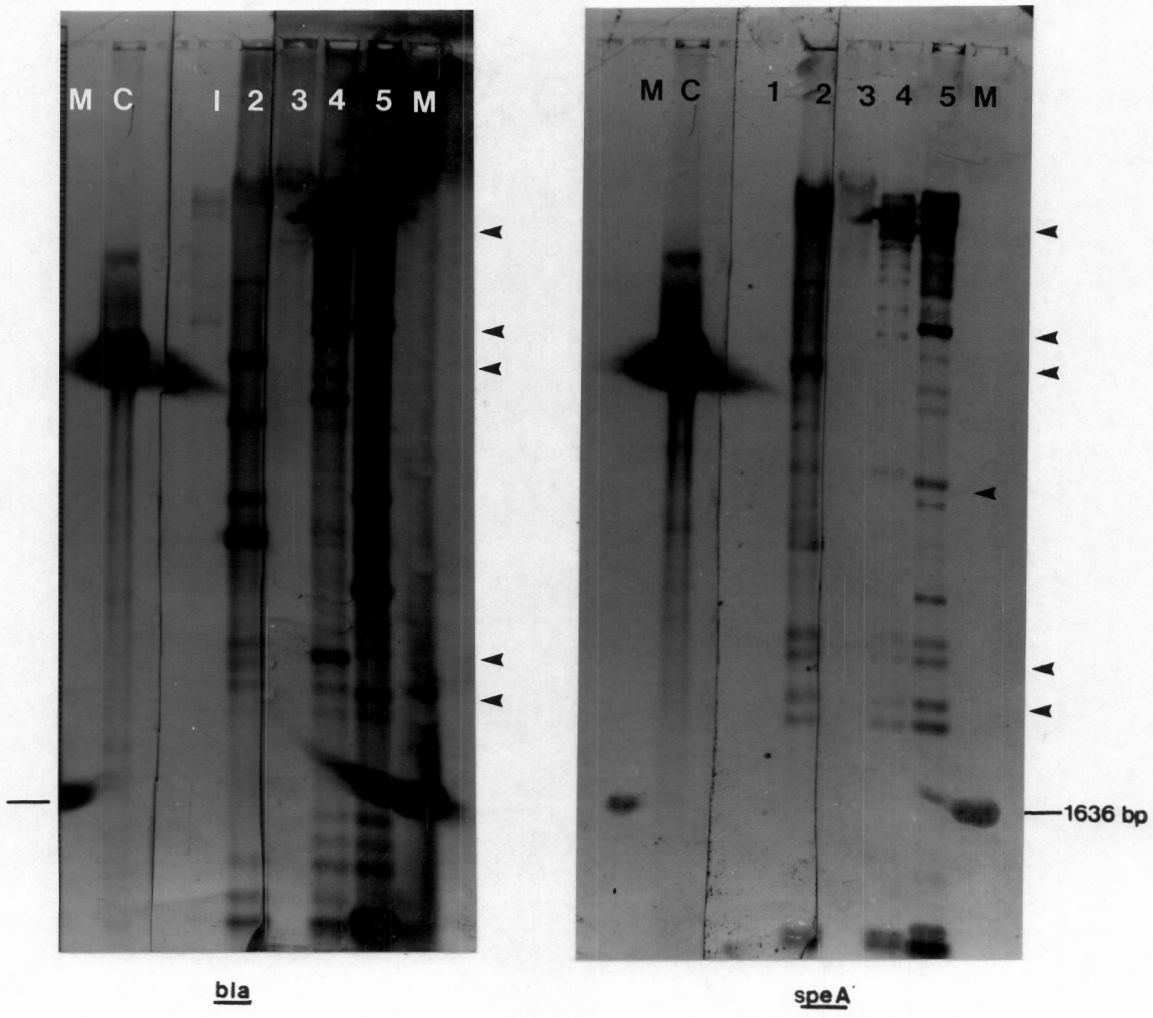


Figure 11. Southern blots of *E. coli* λlysogen genomic DNA. Approximately 0.2 to 2.0 μg of genomic DNA was digested with 30 units of *Xho*I and separated on a 0.7% agarose gel. The membrane on the left was probed with a labeled 360 bp *Bgl*I-*Sca*I restriction fragment (*bla*) and the membrane on the right was probed with a *1,077 bp Hin*cII restriction fragment (*speA*). Lane M is molecular weight standards; lane C is positive control (linear pRM15); lane 1 is *E. coli* λCBRM65; lane 2 is λCBRM161; lane 3 is λKCRM65; lane 4 is λKCRM161; lane 5 is λMCRM65. Bands appearing in both Southern blots (►) and size standards (bp) are indicated.

VITA

I was born on Febuary 1, 1952 in Morristown, New Jersey. I grew up in a U.S. Navy family; I lived in Hawaii and Alaska before attending elementary school in California, Turkey, and Germany. After graduating from Surrattsville Senior High School in Clinton, Maryland, I received a Bachelor of Science in Environmental Sciences from the University of Virginia in June, 1976. I worked as a technician in the Clinical Chemistry Laboratory at the University of Virginia Medical Center from September, 1978 to November, 1981. My training in research began when I transfered into Dr. John Dunn's laboratory in the Department of Internal Medicine where I worked on the structure and biosynthesis of thyroid hormone until September, 1984. In 1984 I entered the graduate program at Virginia Polytechnic Institute and State University in the Department of Plant Pathology, Plant Physiology, and Weed Science. In 1986 I transfered to Dr. Stephen Boyle's laboratory in the Virginia-Maryland Regional College of Veterinary Medicine where I studied gene regulation in *E. coli.*

TABLE 6. ADC and β-galactosidase activities of *E. coli* strains grown in MOPS-glucose with cAMP or putrescine or both. Relative ADC activities are expressed as specific activity relative to specific activity in cells grown in unsupplemented MOPS-glucose medium (see Table 5). The strains were grown for 3 to 4 generations and extracts assayed as described in Methods. The activities are the averages of three assays from one to four experiments. TS = transcriptional; TL = translational; N.D. = not determined; * = low activity.

		Relative Enzyme Activities									
		cAMP				cAMP + Putr.		Putrescine			
		5 mM		10 mM		5 mM + 10mM		5 mM		10 mM	
Strain	Fusion	ADC	β-Gal	ADC	β-Gal	ADC	β-Gal	ADC	β-Gal	ADC	β-Gal
KC14	none	0.64	1.56	0.46	2.80	0.48	2.26	0.79	1.05	0.78	1.05
λKCRM161	TS	0.62	0.94	N.D.	N.D.	0.49	0.69	N.D.	N.D.	0.56	0.75
λKCRM65	TL	0.64	0.90	0.40	0.89	0.39	0.48	0.80	1.08	0.76	0.59
λCBRM161	TS	0.76	1.07	N.D.	N.D.	0.51	0.56	N.D.	N.D.	0.51	0.37*
λCBRM65	TL	0.80	1.08	0.81	1.41	0.54	0.59	0.80	1.22	0.69	0.60
λMCRM65	TL	0.81	1.20	lysis	lysis	lysis	lysis	0.83	0.96	0.53	0.56

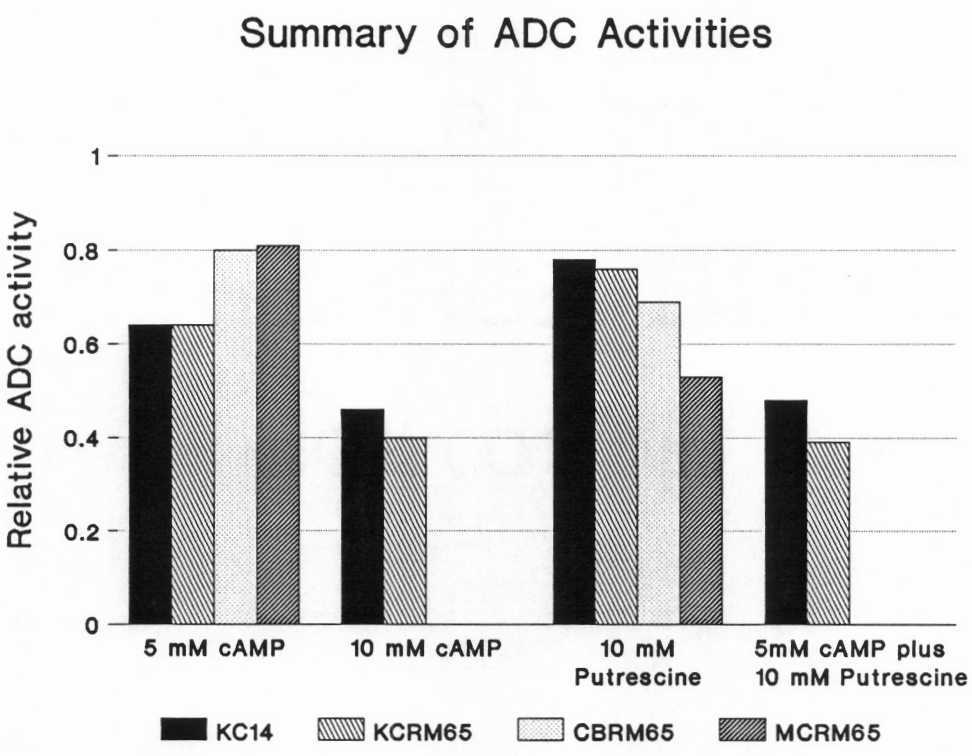


Figure 12. Summary of relative ADC activities of *E. coli* strains carrying a translational *speA-lacZ* fusion.

Table 7. Generation times (min) of various *E. coli* strains grown for three to four generations in MOPS-glucose medium. PG indicates poor growth due to inhibited growth rate.

		cAMP		Putrescine		cAMP+Putrescine
Strain	None	5 mM	10mM	5 mM	10 mM	5 mM+10 mM
KC14	62	62	64	60	62	64
λKCRM161	66	66	--	--	64	65
λKCRM65	66	66	60	66	60	62
λCBRM161	60	--	--	--	60	58
λCBRM65	55	50	PG	58	60	48
λMCRM65	42	34	lysis	40	50	lysis

KC14, λKCRM161 or λKCRM65, but increased the induction of the lactose operon of strain KC14 from the *speA-lacZ* fusions of strain λKCRM161 or λKCRM65 changed. These results indicate that cAMP and CRP do not repress the *speA* gene directly, but act indirectly to inhibit the activity of ADC.

Addition of 10 mM putrescine to *E. coli* KC14 did not effect expression of β-galactosidase from the lactose operon. The ADC activity of cultures of strains λKCRM65 and λCBRM65 were reduced 24% and 31% while the β-galactosidase activity was reduced 41% and 40%, respectively (Table 6, Fig. 13). When cultures were grown in the presence of both 5 mM cAMP and 10 mM putrescine, the lactose operon of *E. coli* strain KC14 was induced 2.3-fold. Strain λMCRM65 was induced to lytic growth when grown under these conditions. The ADC activity of *E. coli* λKCRM65 and λCBRM65 was reduced 61% and 46% and β-galactosidase activity was reduced 52% and 41%, respectively. These results suggest that cAMP does not directly repress *speA* transcription. Therefore, cAMP and putrescine reduce ADC activity via independent mechanisms because (i) ADC, but not the *speA-lacZ* fusions respond to cAMP, (ii) both ADC and the *speA-lacZ* fusions do respond to putrescine, and (iii) the effects of cAMP and putrescine on ADC and β-galactosidase activities are additive.

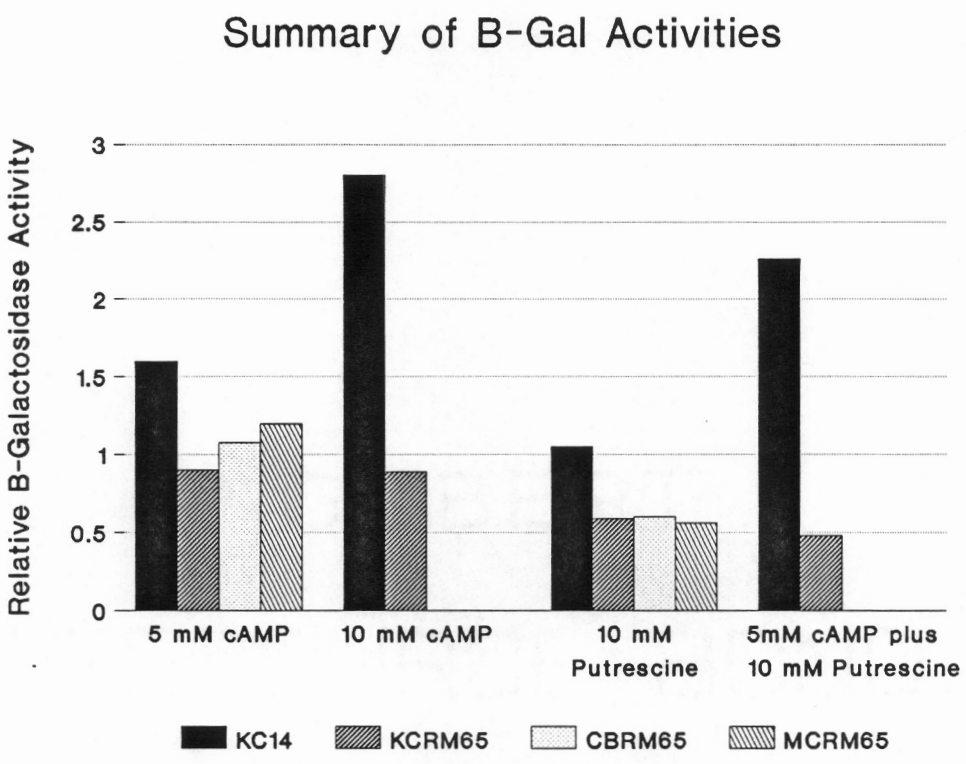


Figure 13. Summary of relative β-galactosidase activities of *E. coli* strains carrying a translational *speA-lacZ* fusion.

DISCUSSION

The cAMP-CRP complex regulates gene expression by a number of mechanisms. cAMP-CRP can act directly by binding near promoters of regulated genes to alter the rate of of transcription (de Crombrugghe et al., 1984; Ullmann et al., 1983) or the complex can inhibit the activity of an enzyme, as in the case of adenylate cyclase (*cya*) (Majerfeld et al., 1981). cAMP-CRP could also control *speA* transcription indirectly by controlling the synthesis of a repressor protein or an antisense (divergent) RNA, as in *crp* gene (Okamoto and Freundlich, 1986). As a first step to determine whether transcriptional or post-translational mechanisms were operative in regulating ADC, we constructed transcriptional and translational *speA-lacZ* fusions and transfered single copies of either type of fusion to the chromosomes of several strains of *E. coli*. The presence of a second copy of the *speA* promoter in the *E. coli* chromosome did not alter either cAMP or putrescine mediated regulation of ADC activity. *E. coli* strains carrying *speA-lacZ* fusions as lysogens produced the same ADC specific activity and grew at the same rate as cells without the fusion. Therefore, any effector that is required for regulation of *speA* is not limiting in lysogenized cells (ie. diploid for *speA* promoter).

The ADC::β-galactosidase hybrid protein contains 70% of the *speA* ORF beginning with the 5'-end (Moore and Boyle, 1990). The ADC::β-galactosidase fusion protein does not produce an active ADC in addition to β-galactosidase since the specific activity of ADC in cells bearing a translational *speA-lacZ* fusion remains the same as the host cells without the fusion. This finding is not unexpected as the deletion of 355 nucleotides (18% of *speA*) of the 3'-end of the *speA* ORF results in the loss of ADC activity (Moore and Boyle, 1990). Since the expression of β-galactosidase from *E. coli* strains carrying translational *speA-lacZ* fusions are not effected by cAMP, the regulation of ADC activity by cAMP-CRP does not

appear to be at the transcriptional or translational level. It is still possible, however, that the 30% of the 3'-end of the *speA* mRNA missing in the *speA-lacZ* fusion is required for some type of translational regulation of ADC.

In vitro and *in vivo* studies have shown cAMP-CRP regulates the activity of adenylate cyclase (*cya*) post-translationally, at the enzyme level (Majerfeld et al., 1981; Ullmann and Danchin, 1983). It appears that the activity of ADC is also regulated post-translationally either directly or indirectly by the cAMP-CRP complex. This was unexpected as the region upstream of *speA* contains a CRP-like binding sequence, 5'-TGTGC-3' (consensus: 5'-TGTGA-3'), located near the *speA* promoter (Moore and Boyle, 1990). Gel retardation assays showed that changing the CRP binding sequence of the lactose operon from 5'-TGTGA-3' to 5'-TGTGC-3' reduced, but did not abolish, CRP binding (Jansen, 1987). Although this site may bind cAMP-CRP complex in *speA*, results of *speA-lacZ* fusion experiments indicate that cAMP-CRP does not control the expression of ADC at the transcriptional level. Neither the β-galactosidase activity of transcriptional or translational *speA-lacZ* fusions significantly decreased in response to cAMP, in contrast to the reduction in the ADC specific activities in the same cells.

Because cAMP supplementation is known to alter the growth rate of *E. coli*, a number of *E. coli* strains were tested for their responsiveness to cAMP. The effects on the growth rate and degree of regulation of *speA* expression by cAMP was determined to be strain-specific. The decrease in ADC specific activity varied from 9 to 40% depending on the strain of *E. coli* tested. This finding supports the observation by Shaibe et al. (Shaibe et al., 1985a) that the activities of ADC and ODC from some *E. coli* strains are not regulated by cAMP. Shaibe et al. also noted that the cAMP-mediated regulation of ADC and ODC occurred in strains with a slow growth rate. The ability of *E. coli* strains to regulate ADC

may be related to the intracellular level of cAMP in the cells before supplementation with cAMP. *E. coli* strain KC14 and its lysogenized derivatives growing in MOPS-glucose minimal medium have a slow generation time (62 min) that was not affected by the addition of 10 mM cAMP in the cultures. In contrast, lysogenized *E. coli* MC4100 had a fast generation time (42 min) in the same medium, but lambda phage were induced when the bacteria were grown with 10 mM cAMP. The slow growth rate of *E. coli* KC14 and its derivatives may reflect low intracellular levels of cAMP as *cya* mutants have been shown to have a slower growth rate compared to wild type bacteria (D'Ari, 1988). Addition of exogenous cAMP to the growth medium would be expected to have a greater effect on the ADC activity of *E. coli* strains containing low levels of cAMP.

Addition of putrescine to the growth medium also results in reduced ADC expression. Our results support the observation of Tabor and Tabor (1969) that putrescine caused inhibition of ADC and repression of *speA*. The specific activity of ADC from *E. coli* grown in a chemostat decreased approximately 45% following the addition of 10 mM putrescine. The specific activity of ADC and β-galactosidase in *E. coli* strains carrying *speA-lacZ* fusions decreased 24 to 31% and 41 to 47%, repectively, in cultures containing 10 mM putrescine. The amount of repression of the *speA* gene by putrescine was determined to be independent of the strain tested.

Based on *in vitro* studies, putrescine can competitively inhibit ADC activity (Tabor and Tabor, 1969). A possible means by which putrescine could negatively regulate ADC activity post-translationally would be if putrescine induced the synthesis of two basic proteins, called antizymes, that are known to inhibit the activity of ADC (Panagiotidis and Canellakis, 1984). These ADC antizymes have been identified as ribosomal proteins S20/L26 and L34 (Panagiotidis and Canellakis, 1984). However, there are no published reports in which

cAMP-CRP induces the synthesis of ribosomal proteins.

The addition of 5 mM cAMP to cultures supplemented with 10 mM putrescine did not further decrease the β-galactosidase activity of *speA-lacZ* fusions. This result supports the conclusion that cAMP does not regulate *speA* transcription. The decrease in ADC activity in *E. coli* strains supplemented with 5 mM cAMP and 10 mM putrescine was additive, indicating independent regulatory mechanisms. The same observation was observed for ADC activity in *E. coli* KC14 (Leigh Busse, M.S. thesis, 1988, VPI and SU).

SUMMARY / FUTURE DIRECTIONS

The following conclusions can be drawn from the experiments presented in this thesis:

1. The *speA* gene encoding ADC is comprised of a 1974 bp ORF.
   a. The predicted amino acid composition and molecular size of the ORF, 73,980 Da, closely resembles that of purified ADC.
   b. The 5'-end of the ORF contains a signal sequence with a net negative charge
   c. The *speA* gene is separated from the *speB* gene by 140 bp that contains a rho-independent terminator.
   d. Restriction analysis of *speA* maps the gene to 62.9 minutes on the Kohara physical *E. coli* map.
2. ADC has a consensus pyridoxal phosphate binding site as well as additional conserved amino acid sequences of enzymes which bind similar substrates.
3. Transcriptional and translational *speA-lacZ* fusions indicate that the *speA* promoter is at least 10-fold weaker than the *lacZ* promoter.
4. cAMP and CRP inhibit ADC activity either indirectly or directly at the post-translational level.
5. Putrescine represses *speA* expression at the transcriptional level.
6. cAMP-CRP complex and putrescine negatively control ADC through independent regulatory mechanisms.

The translation of *speA* mRNA could initiate at either of three possible sites in the *speA* ORF. The amino acid sequence of the amino terminus of ADC has not been determined

because the ADC amino acid terminus is blocked. Thus, it is not clear at this time which of these sites actually functions *in vivo*. In an attempt to predict which of these sites actually functions in *speA*, the three potential signal peptides were searched for characteristics typical of *E. coli* signal peptides and signal peptidase cleavage sites. The amino terminus of each of the possible signal peptides carries a different charge (-2, -1, or +1), so the kinetics of translocation into the periplasm of wild type and mutant signal peptides was compared to the rate of transport of ADC.

The first possible translation initiation site (site 1) would use a four bp Shine-Dalgarno sequence (GAGG), begin at the 5'-end of the *speA* ORF (Met), and would produce an ADC signal peptide with a net negative charge (-2). Most bacterial signal peptide contain a positively charged amino terminus and are rapidly transproted, usually within one min. Mutant *E. coli* maltose binding protein or lipoprotein signal peptides containing a negatively charged amino acid terminus are still translocated to the periplasm, but at a slower rate than the wild type. The kinetics of translocation of ADC closely resembles those of mutant signal peptides carrying a -2 charge, requiring six and eight min, respectively, to transport all precursor forms into the periplasm. The reduced rate of transport in signal peptide mutants does not appear to be dependent on the type of signal peptidase used to remove the signal peptide; maltose binding protein uses signal peptidase I (Lep) and lipoprotein uses signal peptide II (Lsp)(von Heijne, 1990). The predicted amino acid sequence of the *speA* ORF was searched for possible signal peptidase I cleavage sites using the so-called "(-3,-1)-rule" and for signal peptidase II cleavage sites using the consensus sequence, Leu-Ala-(Gly, Ala) ↓ Cys. Processing of the ADC signal peptides does not appear to involve signal peptidase II as none of the potential ADC signal peptides contains a signal peptide consensus sequence in the appropriate location. No obvious signal peptidase I was identified for signal

peptides originating at the 5'-end of the *speA* ORF.

A second potential translation initiation site (site 2) begins with Met, 26 amino acids into the *speA* ORF. This site contains a larger, six bp Shine-Dalgarno sequence (AGGAGG) and would produce a signal peptide with a -1 charge. Attempts to identify a signal peptide processing site using the above criteria were unsuccessful. Finally, a signal peptide beginning at a Met, 35 amino acids into the *speA* ORF (site 3) would use a 6 bp Shine-Dalgarno sequence (AGGAAG) and possess a positively charged signal peptide (+1) characterisitic of most bacterial signal peptides. Postively charged signal peptides are usually translocated very quickly, usually within 30 to 60 seconds. Pulse chase experiments have shown that the ADC requires at least six min for the signal peptide to be processed. It is not clear how the rate of translocation of a positively charged ADC signal peptide is significantly reduced compared to other positively charged *E. coli* signal peptides. Site 3 does not contain an obvious signal peptide I processing site. In summary, site 1 would produce a signal peptide with transport kinetics resembling that of ADC, while site 3 would produce a signal peptide with a characteristic postively charged amino terminus. It may be possible to determine the amino acid seqeunce of the precursor form of ADC. This would be possible by purifying ADC from *E. coli* grown in the presence of an inhibitor of signal peptidase, such as globomycin C, or *E. coli* mutants defective in signal peptidase I and signal peptidase II.

The results in this dissertation have set the stage for defining the exact nature of the post-translational mechanism by which cAMP-CRP regulates ADC activity and putrescine represses *speA* expression. In the *E. coli* strains KC14, CB806, and MC4100, there is a correlation between their ability to grow in high levels of cAMP (10 mM) and their and its derivatives grow in MOPS-glucose minimal medium at a relatively slow rate, with a

generation of approximately 62 min. Derivatives of *E. coli* CB806 and MC4100 grow in the same medium at a faster rate, with generation times of approximately 40 and 50 min, respectively. The growth rates of *E. coli* KC14 and its derivatives were not affected, but growth of *E. coli* CB806 and MC4100 derivatives were severely inhibited by 10 mM cAMP. *E. coli* MC4100 appears to be the most sensitive to cAMP since the prophage in λMCRM65 was induced, followed by lysis of the cells. Presumably, the intracellular level of cAMP in *E. coli* KC14 derivatives remains relatively low following the addition of exogenous cAMP. These results suggests that *E. coli* KC14 either produces high levels of phosphodiesterase or transports cAMP inefficiently.

The amount of repression of ADC activity by cAMP-CRP and the ability to grow in the presence of high levels of cAMP was determined to be strain-specific. The ADC activity of *E. coli* KC14 grown in MOPS-glucose minimal medium supplemented with 5 mM cAMP was reduced approximately 35 to 40% while the ADC activity in strains CB806 and MC4100 was reduced 19 to 24%. The strain-specific response to exogenous cAMP may be due to different intracellular concentrations of cAMP in each strain. *E. coli* KC13 is deleted in the *cya* gene encoding adenylate cyclase and is unable to synthesize cAMP. When *E. coli* KC14 (wild type) or KC13 (*cya*) are grown in the presence of 5 mM cAMP, the activity of ODC is repressed 40% and 60%, respectively (Leigh Busse, M.S. thesis, VPI and SU, 1988). The 50% increase in ADC repression in *E. coli* KC13 is probably due to a lower initial concentration of cAMP. If the initial concentration of cAMP was lower in *E. coli* KC14 and its derivatives, those strains should show a greater response to supplemented cAMP. Thus, the negative effects on ADC activity would also be greater. In order to validate these possibilities, it will be necessary to measure cAMP levels and phosphodiesterase activity in the various *E. coli* strains. It would even be possible to assess this genetically by introducing

cya and *cpd* mutations into the strains.

Whether cAMP-CRP regulates the activity of ADC directly or indirectly can be tested *in vitro* by adding cAMP to cell extracts containing low levels of cAMP and assaying ADC activity. *E. coli* KC13 cannot synthesize cAMP due to a mutated adenylate cyclase gene (*cya*). In the absence of cAMP, the *crp* gene is derepressed, and cellular CRP levels will be high. Thus, a cell lysate from *E. coli* KC13 grown in MOPS-glucose medium should contain relatively high ADC activities compared to the wild type (*E. coli* KC14) due to the lack of cAMP-CRP complex. The addition of cAMP to this lysate will result in a decrease in the specific activity of ADC only if cAMP-CRP regulates ADC directly.

Putrescine regulates ADC expression primarily at the transcriptional level. When bacteria were grown in the presence of 10 mM putrescine the decrease in β-galactosidase activity from the *speA-lacZ* fusions matched or was slightly greater than the decrease in the ADC activity. The increase in repression exhibited by β-galactosidase is probably due to the enzyme having a slower turnover rate or increased enzyme stablity compared to ADC. There was little inhibition of ADC by putrescine unless it also binds to the ADC::β-galactosidase hybrid protein to regulate β-galactosidase activity. This does not seem likely as the cells were washed free of putrescine prior to lysis and the concentration of intracellular putrescine was diluted at least 1,000-fold in the process of making cell extracts. If the synthesis of ADC antizymes are induced by putrescine, they did not significantly contribute to post-translational regulation of ADC activity.

Putrescine could regulate *speA* directly by interacting with the *speA* promoter or indirectly by interacting with a regulatory protein. The direct interaction of putrescine with the *speA* promoter could be tested using an *in vitro* transcription assay. A purified DNA fragment containing the *speA* gene and 5'-regulatory region could be transcribed in the

presence or absence of putrescine and the levels of *speA* mRNA measured. If the addition of putrescine results in a decrease in the rate of *speA* mRNA synthesis, then putrescine acts directly at the *speA* promoter, either by interacting with RNA polymerase or an initiation factor.

Putrescine could also regulate ADC indirectly, by interacting with a repressor protein. The existence of a *speA* repressor protein could be tested using a gel-retardation assay or DNaseI protection experiment. Crude lysates from *E. coli* grown in MOPS-glucose medium in the presence of putrescine would be incubated with a DNA fragment containing the *speA* promoter and 5'-regulatory region. If a putrescine interacts with a *speA* repressor protein to enhance its binding to the *speA* regulatory region, the mobility of the DNA fragment containing the *speA* operator site will be reduced in a gel-retardation assay. A DNAaseI footprinting experiment using the same cell extract would reveal the site at which the regulatory protein binds.

LITERATURE CITED

Abraham, K.A. 1968. Studies on DNA-dependent RNA polymerase from *Escherichia coli*. I. The mechanism of polyamine induced stimulation of enzyme activity. Eur. J. Biochem. 5:143-146.

Aiba, H., and J. Krakow. 1981. Isolation and characterization of the amino and carboxy proximal fragments of the adenosine cyclic 3',5'-phosphate receptor protein of *Escherichia coli*. Biochemistry 20:4774-4780.

Aiba, H., S. Fujimo, and N. Ozaki. 1982. Molecular cloning and nucleotide sequence of the gene for *E. coli* cAMP receptor protein. Nuc. Acids Res. 10:1345-1362.

Aiba, H. 1983. Autoregulation of the *Escherichia coli crp* gene: CRP is a transcriptional repressor for its own gene. Cell 32:141-149.

Aiba, H. 1985. Transcription of the *Escherichia coli* adenylate cyclase gene is negatively regulated by cAMP-cAMP receptor protein. J. Biol. Chem. 260:3063-3070.

Aiba, H., T. Nakamura, H. Mitani, and H. Mori. 1985b. Mutations that alter the allosteric nature of cAMP receptor protein of *Escherichia coli*. EMBO J. 4:3329-3331.

Algranati, I.D., and S.H. Goldemberg. 1977. Polyamines and their role in protein synthesis. Trends Biochem. Sci. 2:272-274.

Alpher, M.D., and B.N. Ames. 1978. Transport of antibiotics and metabolic analogs by systems under cyclic AMP control: positive selection of *Salmonella typhimurium cya* and *crp* mutants. J. Bacteriol. 133:149-157.

Ames, B.N., and T.D. Dubin. 1960. The role of polyamines in neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. 235:769-775.

Anderson, W.B., A.B. Schneider, M. Emmer, R.L. Perlman, and I. Pastan. 1971. Purification of and properties of the cyclic adenosine-3',5'-monophosphate receptor protein which mediates cyclic adenosine-3',5'-monophosphate-dependent gene transcription in *Escherichia coli*. J. Biol. Chem. 246:5929-5937

Aono, R., M. Yamasaki, and G. Tamura. 1978. Changes in composition of envelope proteins in adenylate cyclase- or cyclic AMP receptor protein-deficient mutants of *Escherichia coli*. J. Bacteriol. 136:812-814.

Applebaum, D., D.L. Sabo, E.H. Fischer, and D.R. Morris. 1975. Biodegradative ornithine decarboxylase of *Escherichia coli*. Purification, properties, and pyridoxal 5'-phosphate binding site. Biochemistry 14:3675-3681.

Applebaum, D.M., J.C. Dunlap, and D.R. Morris. 1977. Comparison of the biosynthetic and biodegradative ornithine decarboxylases of *Escherichia coli*. Biochemistry 16:1580-1584.

Atkins, J.F., J.B. Lewis, C.W. Anderson, and R.F. Gesteland. 1975. Enhanced differential synthesis of proteins in a mammalian cell-free system by addition of polyamines. J. Biol. Chem. 250:5688-5695.

Auger, E.A., K.E. Redding, T.B. Plumb, L.C. Childs, S.Y. Meng, and G.N. Bennett. 1989. Construction of *lac* fusions to the inducible arginine- and lysine decarboxylase genes of *Escherichia coli* K-12. Mol. Microbiol. 3:609-620.

Ausubel, F.M., R. Brent, R.F. Kingston, D.D. Moore, J.G. Siedman, J.A. Smith, and K. Struhl. 1989. Current Protocols in Molecular Biology. Volume. 1. Green Publishing Assoc. and Wiley-Interscience.

Bachmann, B.J. 1987. Linkage map of *Escherichia coli* K-12. *In*: *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. Eds. Ingraham, J.L., K. Brooks Low, B. Magasanik, M. Schaechter, and H. E. Umbarger. American Society for Microbiology, Washington, D.C. p. 807-876.

Bachrach, U. 1973. *Function of naturally occurring polyamines*. American Press, Inc., New York.

Bankaitis, V.A., and P.J. Bassford, Jr. 1982. Regulation of adenylate cyclase synthesis in *Escherichia coli*: studies with *cya-lac* operon and protein fusion strains. J. Bacteriol. 151:1346-1357.

Barroso, L., J.M. Wright, R.C. Moore, T. Patel, and S.M. Boyle. 1990. Analysis and sequence of the *speC* gene encoding biosynthetic ornithine decarboxylase in *Escherichia coli* (in preparation; see accession number M33766 in GenBank).

Betoni, A.J., P.P. McCann, and A. Sjoerdsma. 1982. Restriction of bacterial growth by inhibitors of polyamine biosynthesis by using monofluoromethylornithine, difluoromethylarginine and dicyclohexylammonium sulfate. Biochem. J. 208:435-441.

Blethen, S.L., E.A. Boeker, and E.E. Snell. 1968. Arginine decarboxylase from *Escherichia coli*. I. Purification and specificity for substrates and coenzyme. J. Biol. Chem. 243:1671-1677.

Boeker, E.A., and E.E. Snell. 1968. Arginine decarboxylase from Escherichia coli. II. dissociation and reassociation of subunits. J. Biol. Chem. 243:1678-1684.

Boeker, E.A., E.H. Fischer, and E.E. Snell. 1969. Arginine decarboxylase from *Escherichia coli*. III. Subunit structure. J. Biol. Chem. 244:5239-5245.

Boeker, E.A., E.H. Fischer, and E.E. Snell. 1971. Arginine decarboxylase from *Escherichia coli*. IV. Structure of the pyridoxal phosphate binding site. J. Biol. Chem. 246:6776-6781.

Botsford, J.L., and M. Drexler. 1978. The cyclic 3',5'-adenosine monophosphate receptor protein and regulation of 3',5'-adenosine monophosphate synthesis in *Escherichia coli*. Mol. Gen. Genet. 165:47-56.

Bowman et al. et al., W.H., C.W. Tabor, and H. Tabor. 1973. Spermidine biosynthesis. Purification and properties of propylamine transferase from *Escherichia coli*. J. Biol. Chem. 248:2480-2486.

Boyle, S. M., M.F. MacIntyre, and B.H. Sells. 1977. Polyamine levels in *Escherichia coli* during nutritional shift-up and exponential growth. Biochim. Biophys. Acta. 477:221-227.

Boyle, S.M., G.D. Markham, E.W. Hafner, J.M. Wright, H.Tabor, and C.W. Tabor. 1984. Expression of the cloned genes encoding the putrescine biosynthetic enzymes and methionine adenosyltransferase of *Escherichia coli (speA, speB, speC* and *metK*). Gene 30:129-136.

Boyle, S.M., J.M. Wright, and C. Satishchandran. 1985. Analysis *in vitro* and *in vivo* of the role of cyclic AMP on *speC* expression in *Escherichia coli*. Recent Progress in Polyamine Research. L. Selmeci, M.E. Brosnan, N. Seiler, Eds. p. 3-11.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Buch, J.K., and S.M. Boyle. 1985a. Biosynthetic arginine decarboxylase in *Escherichia coli* is synthesized as a precursor and located in the cell envelope. J. Bacteriol. 163:522-527.

Buch, J., and S.M. Boyle. 1985b. Analysis *in vivo* of *speA* (arginine decarboxylase) gene expression by gene fusion in *Escherichia coli*. Recent Progress in Polyamine Research. L. Selmeci, M.E. Brosnan, N. Seiler, Eds. p. 13-19.

Buettner, M.J., E. Spitz, and H.V. Rickenberg. 1973. Cyclic adenosine 3',5'-monophosphate in *Escherichia coli*. J. Bacteriol. 114:1068-1073.

Busse, L.A. 1988. Characterization of the interaction of putrescine and the adenosine-3',5'-cyclic monophosphate-cAMP receptor protein complex in the regulation of the *speC* gene encoding ornithine decarboxylase in *Escherichia coli*. M.S. Thesis, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Casadaban, M. J., J. Chou, and S. Cohen. 1980. *in vitro* fusions that join an enzymatically active ß-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. 143:971-980.

Cataldi, A.A., and I.D. Algranati. 1989. Polyamines and regulation of ornithine biosynthesis in *Escherichia coli*. J. Bacteriol. 171:1998-2002.

Clarke, L., and J. Carbon. 1976. A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire *E. coli* genome. Cell 9:91-99.

Cohen, S.S. 1971. Introduction to the polyamines. Prentice-Hall, Inc., New Jersey.

Cohn, M.S., C.W. Tabor, and H. Tabor. 1980. Regulatory mutations affecting ornithine decarboxylase activity in *Saccharomyces cerevisiae*. J. Bacteriol. 142:791-799.

Cossart, P., and B. Gicquel-Sanzey. 1982. Cloning and sequence of the *crp* gene of *E. coli* K-12. Nuc. Acids Res. 10:1363-1378.

Cossart, P. and B. Gicquel-Sanzey. 1985. Regulation of expression of the *crp* gene of *Escherichia coli* K-12: *in vivo* study. J. Bacteriol. 161:454-457.

Cunin, R., N. Glansdorff, A. Piérard, and V. Stalon. 1986. Biosynthesis and metabolism of arginine in bacteria. Micro Reviews 50:314-352.

Cunningham-Rundles, S., and W.K. Maas. 1975. Isolation, characterization, and mapping of *Escherichia coli* mutants blocked in the synthesis of ornithine decarboxylase. J. Bacteriol. 124:791-799.

D'Ari, R., A. Jaffé, P. Bouloc, and A. Robin. 1988. Cyclic AMP and cell division in *Escherichia coli*. J. Bacteriol. 170:65-70.

de Cromrrugghe, B.S., S. Busby, and H. Buc. 1984. Cyclic AMP receptor protein: role in transcription activation. Science 224:831-838.

Deters, J., J. Miskimen, and K.J. Dougall. 1974. A putrescine requiring mutant of *Neurospora crassa*. Genetics 77:s16-17 abstract.

Dills, S.S., A. Apperson, M.R. Schmidt, and M.H. Saier. 1980. Carbohydrate transport in bacteria. Microbiol. Rev. 44:385-418.

Dion, A.S., and S.S. Cohen. 1972a. Polyamines and the delay in deoxyribonucleic acid synthesis in some bacteriophage T4 infections. J. Virol. 8:925-927.

Dion, A.S., and S.S. Cohen. 1972b. Polyamine stimulation of nucleic acid synthesis in an uninfected and phage-infected polyamine auxotroph of *Escherichia coli* K-12. Proc. Nat. Acad. Sci. 69:213-217.

Ebright, R.H., P. Cossart, B. Gicquel-Sanzey, and J. Beckwith. 1984. Mutations that alter the DNA sequence specificity of the catabolite gene activator protein of *E. coli*. Nature 311:232-235.

Echandi, G., and I.D. Algranati. 1975a. Protein synthesis and ribosomal distribution in a polyamine auxotroph of *Escherichia coli*: studies in cell-free systems. Biochem. Biophys. Res. Commun. 62:313-319.

Echandi, G., and I.D. Algranati. 1975b. Defective 30S ribosomal particles in a polyamine auxotroph of *Escherichia coli*. Biochem. Biophys. Res. Commun. 67:1185-1191.

Eilen, E., C. Pampeno, and J. Krakow. 1978. Production, and properties of the alpha core derived from the cyclic adenosine monophosphate receptor protein of *Escherichia coli*. Biochemistry 17:2569-2473.

Epstein, W., L.B. Rothman-Denes, and J. Hesse. 1975. Adenosine 3',5'-cyclic monophosphate as mediator of catabolic repression in *Escherichia coli*. Proc. Nat. Acad. Sci. 72:2300-2303.

Fecker, L.F., H. Beier, and J. Berlin. 1986. Cloning and characterization of a lysine decarboxylase gene from *Hafnia alvei*. Mol. Gen. Genet. 203:177-184.

Fong, W.F., J.S. Heller, and E.S. Canellakis. 1976. The appearance of an ornithine decarboxylase inhibitory protein upon the addition of putrescine to cell cultures. Biochem. Biophys. Acta. 428:456-465.

Fowler, A.V., and I. Zabin. 1978. Amino acid sequence of ß-galactosidase. XI. Peptide ordering and the complete sequence. J. Biol. Chem. 253:5521-5525.

Fuchs, E., R.L. Millette, W. Zillig, and G. Walter. 1967. Influence of salts on RNA synthesis by DNA-dependent RNA-polymerase from *Escherichia coli*. Eur. J. Biochem. 3:183-193.

Fujita, K., S. Matsufuji, Y. Murakami, and S. Hayashi. 1984. Antizyme to ornithine decarboxylase is present in the liver of starved rats. Biochem. J. 218:557-562.

Gale, E.F. 1940. The production of amines by bacteria. Biochem. J. 34:392-413.

Gale, E.F. 1946. The bacterial amino acid decarboxylases. Adv. Enzymol. 6:1-32.

Garges, S., and S. Adhya. 1985. Sites of allosteric shift in the structure of the cyclic AMP receptor protein. Cell 41:745-751.

Garges, S., and S. Adhya. 1988. Cyclic AMP-induced conformational change of cyclic AMP receptor protein (CRP): Intragenic suppressors of cyclic AMP-independent CRP mutations. J. Bacteriol. 170:1417-1422.

Geiger, L.E., and D.R. Morris. 1978. Polyamine deficiency reduces the rate of DNA replication fork movement in *Escherichia coli*. Nature (London) 272:730-732.

Geiger, L.E., and D.R. Morris. 1980. Stimulation of deoxyribonucleic acid replication fork movement by spermidine analogs in polyamine-deficient *Escherichia coli*. J. Bacteriol. 141:1192-1198.

Glansdorff, N. 1987. Biosynthesis of arginine and polyamines. *In*: *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. Eds. Ingraham, J.L., K. Brooks Low, B. Magasanik, M. Schaechter, and H. E. Umbarger. American Society for Microbiology, Washington, D.C. p. 321-344.

Glaser, G., S. Kobi, and A.B. Oppenheim. 1980. Fusion of the promoter region of rRNA operon *rrn*B to *lacZ* gene. Nuc. Acids Res. 8:4327-4345.

Goldemberg, S.H., and I.D. Algranati. 1977. Polyamines and protein synthesis. Studies in various polyamine-requiring mutants of *Escherichia coli*. Mol. Cell. Biochem. 16:71-77.

Grodzicker, T., R.R. Arditti, and H. Eisen. 1972. Establishment of repression by lambdoid phages in catabolite activator protein and adenylate cyclase mutants of *Escherichia coli*. Proc. Nat. Acad. Sci. USA. 69:366-370.

Guiso, N., and B. Blazy. 1980. Regulatory aspects of the cyclic AMP receptor protein in *E. coli* K-12. Biochem. Biophys. Res. Commun. 94:278-283.

Gunther, T. and H.W. Peter. 1979. Polyamines and osmoregulation in *Escherichia coli*. FEMS Micro. Lett. 5:384-389.

Hafner, E.W., C.W. Tabor, and H. Tabor. 1977. Isolation of a *metK* mutant with a temperature-sensitive S-adenosylmethionine synthetase. J. Bacteriol. 132:832-840.

Hafner, E.W., C.W. Tabor, and H. Tabor. 1979a. Mutants of *Escherichia coli* that do not contain 1,4-diaminobutane (putrescine) or spermidine. J. Biol. Chem. 254:12419-12426.

Hafner, E.W., C.W. Tabor, and H. Tabor. 1979b. Identification of 3 classes of ColE1-DNA (*E. coli*) plasmids (Clarke-Carbon Bank) carrying either (*speA*$^+$, *speB*$^+$, *metK*$^+$), (*speC*$^+$), or (*speD*$^+$): the gene products are 6- to 10-fold overproduced. Fed. Proc. 38:395.

Hanahan, D. 1983. Studies on transformation or *Escherichia coli* with plasmids. J. Mol. Biol. 166:557-580.

Hannonen, P., J. Jänne, and R. Raine. 1972. Separation and partial purification of S-adenosyl-methionine decarboxylase and spermidine and spermine synthetases from rat liver. Biochem. Biophys. Res. Commun. 46:341-348.

Harwood, J.P., and A. Peterkofsky. 1975. Glucose-sensitive adenylate cyclase in toluene-treated cells of *Escherichia coli* B. J. Biol. Chem. 250:4656-4662.

Harwood, C.R., and E. Meynell. 1975. Cyclic AMP and the production of sex pili by *E. coli* K-12 carrying derepressed sex factors. Nature 254:628-630.

Harman, J.G., K. McKenny, and A. Petrekofsky. 1986. Structure-function analysis of three cAMP-independent forms of the cAMP receptor protein. J. Biol. Chem. 261:16332-16339.

Hawley, D.K., and W.R. McClure. 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nuc. Acids Res. 11:2237-2255.

Heller, J.S., W.F. Fong, and E.S. Canellakis. 1976. Induction of a protein inhibitor to ornithine decarboxylase by the end products of its reaction. Proc. Nat. Acad. Sci. USA. 73:1858-1862.

Heller, J.S., R. Rostomily, D.A. Kyriakidis, and E.S. Canellakis. 1983. Regulation of polyamine biosynthesis in *Escherichia coli* by basic proteins. Proc. Nat. Acad. Sci. USA. 80:5181-5184.

Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28:351-359.

Herbst, E.J., and E.E. Snell. 1948. Putrescine as a growth factor for *Haemophilus papainfluenzae*. J. Biol. Chem. 176:989-990.

Hirschfield, I.N., H.J. Rosenfeld, Z. Leifer, and W.K. Maas. 1970. Isolation and characterization of a mutant of *Escherichia coli* blocked in the synthesis of putrescine. J. Bacteriol. 101:725-730.

Höltje, J.V. 1978. Streptomycin uptake via an inducible polyamine transport system in *Escherichia coli*. Eur. J. Biochem. 86:1165-1171.

Höltta, E. J. Jänne, and J. Pispa. 1974. The regulation of polyamine synthesis during the stringent control in *Escherichia coli*. Biochem. Biophys. Res. Commun. 59:1104-1111.

Hong, J.-S., G.R. Smith, and B.N. Ames. 1971. Adenosine 3',5'-cyclic monophosphate concentration in the bacterial host regulates the viral decision between lysogeny and lysis. Proc. Nat. Acad. Sci. USA. 68:2258-2262.

Horwitz, S.Z, and L.A. Loeb. 1988. An *E. coli* promoter that regulates transcription by DNA superhelix-induced cruciform extrusion. Science 241:703-705.

Igarashi, K. K. Kashiwagi, K. Kishida, T. Kakegawa, and S. Hirose. 1981. Decrease in the S1 protein of 30-S ribosomal subunits in polyamine requiring mutants of *Escherichia coli* grown in the absence of polyamines. Eur. J. Biochem. 114:127-131.

Iino, T., M. Takahashi, and T. Sako. 1987. Role of amino-terminal positive charge on signal peptide in staphylokinase export across the cytoplasmic membrane of *Escherichia coli*. J. Biol. Chem. 262:7412-7417.

Inouye, S., X. Soberon, T. Franceschini, K. Nakamura, K. Itakura, and M. Inouye. 1982. Role of positive charge on the amino-terminal region of the signal peptide secretion across the membrane. Proc. Natl. Acad. Sci., U.S.A. 79:3438-3441

Irwin, N., and M. Ptashne. 1987. Mutants of the catabolite activator protein of *Escherichia coli* that are specifically deficient in the gene-activation function. Proc. Nat. Acad. Sci. 84:8315-8319.

Ish-Horowicz, D., and J. F. Burke. 1981. Rapid and efficient cosmid cloning. Nuc. Acids Research 9:2989-2998.

Janecek, J., J. Naprstek, Z. Dobrova, M. Jiresova, and J. Spizek. 1979. Adenylate cyclase activity in *Escherichia coli* cultured under various conditions. FEMS Microbiol. Lett. 6:305-307.

Jansen, C., A.M. Gronenborn, and G.M. Clore. 1987. The binding of the cyclic AMP receptor protein to synthetic DNA sites containing permutations in the consensus sequence TGTGA. Biochem. J. 246:227-232.

Jones, H.M., and R.P. Gunsalus. 1987. Regulation of *Escherichia coli* fumerate reductase (*fdrABCD*) operon expression by regulatory electron acceptors and the *fnr* gene product. J. Bacteriol. 169: 3340-3349.

Jorstad, C.M., and D.R. Morris. 1974. Polyamine limitation of growth slows the rate of polypeptide chain elongation in *Escherichia coli*. J. Bacteriol. 119:857-860.

Joseph, E. C. Bernsley, N. Guiso, and A. Ullman. 1982. Multiple regulation of the adenylate cyclase in *Escherichia coli*. Mol. Gen. Genet. 185:262-268.

Jovanovich, S.B. 1985. Regulation of a *cya-lac* fusion by cyclic AMP in *Salmonella typhimurium*. J. Bacteriol. 161:641-649.

Kallio, A., P.P. McCann, and P. Bey. 1981. DL-α(Difluoromethyl)arginine: A potent enzyme-activated irreversible inhibitor of bacterial arginine decarboxylases. Biochemistry 20:3163-3166.

Kameji, T., and A.E. Pegg. 1987. Inhibition of translation of mRNAs for ornithine decarboxylase and S-adenosylmethionine decarboxylase by polyamines. J. Biol. Chem. 262:2427-2430.

Kashiwagi, K., H. Kobayashi, and K. Igarashi. 1986. Apparently unidirectional polyamine transport by proton motive force in polyamine-deficient *Escherichia coli*. J. Bacteriol. 165:972-977.

Kashiwagi, K., and K. Igarashi. 1987. Nonspecific inhibition of *Escherichia coli* ornithine decarboxylase by various ribosomal proteins: detection of a new ribosomal protein possessing strong antizyme activity. Biochem. Biophys. Acta 911:180-190.

Kawamukai, M., J. Kishimoto, R. Utsumi, M. Himeno, T. Komano, and H. Aiba. 1985. negative regulation of adenylate cyclase gene (*cya*) expression by cyclic AMP-cyclic AMP receptor protein in *Escherichia coli*: studies with *cya-lac* protein and operon fusion plasmids. J. Bacteriol. 164:872-877.

Kline, E.L., V. Bankaitis, C.S. Brown, and D. Montefiari. 1979. Imidazole acetic acid as a substitute for cAMP. Biochem. Biophys. Res. Commun. 87:566-574.

Kohara, Y., K. Akiyama, and K. Isono. 1987. The physical map of the whole *E. coli* chromosome: Application of a new strategy for the paired analysis and sorting of a large genomic library. Cell 50:495-508.

Kuner, J.,L. Avery, D.E. Berg, and D. Kaiser. 1981. *In*: Microbiology 1981, Ed. Schlessinger, D.. American Society for Microbiology.

Kyriakidis, D.A., J.S. Heller, and E.S. Canellakis. 1978. Modulation of ornithine decarboxylase activity in *Escherichia coli* by positive and negative effectors. Proc. Nat. Acad. Sci. 75:4699-4703.

Kyriadidis, D.A. 1983. Effect of plant growth hormones and polyamines on ornithine decarboxylase activity during the germination of barley seeds. Physiol. Plant. 57:499-504.

Ladenburg, A. and J. Abel. 1888. J. Ber. dt. chem. Ges. 21:758.

Leeuwenhoek, A. van. 1678. Observationes D. Anthinii Leeuwenhoek, de Natis e semine genitali Aminalculis. Phil. Trans. R. Soc. 12: 1040-1043.

Lipetz, P.D., C.S. Lowney, D. Mhasker, and R.W. Hart. 1980. Gene structure and function. *In:* D.H. Dean, L.F. Johnson, T.C. Kimball and P.S. Pertman (Eds.). Ohio State University Press, Columbus, Ohio.

Liu, L.F., and J.C. Wang. 1978. *Micrococcus luteus* DNA gyrase: active components and a model for its supercoiling of DNA. Proc. Nat. Acad. Sci. USA. 75:2098-2102.

Maas, W.K. 1961. Studies on repression of arginine biosynthesis in *Escherichia coli*. Cold Spring Harbor Symp. Quant. Biol. 26:183-191.

Maas, W.K., Z. Leifer, M.A. Poindexter, and J. Poindexter. 1970. Studies with mutants blocked in the synthesis of polyamines. Ann. New York Acad. Sci. 171:957-967.

Majerfeld, I.H., D. Miller, E. Spitz, and H.V. Rickenberg. 1981. Regulation of the synthesis of adenylate cyclase in *Escherichia coli* by the cAMP-cAMP receptor protein complex. Mol. Gen. Genet. 181:470-475.

Mallick, V., and P. Herrlich. 1979. Regulation of synthesis of a major outer membrane protein: cyclic AMP represses *Escherichia coli* protein III synthesis. Proc. Nat. Acad. Sci. 76:5520-5523.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

March, S., and S.M. Boyle. 1981. The biochemical basis of the putrescine growth requirement of *Haemophilus parainfluenzae*. 81st Ann. Meeting of Amer. Soc. for Microbiol., Abstract #I113.

Markham, G.D., C.W. Tabor, and H. Tabor. 1982. S-Adenosylmethionine decarboxylase of *Escherichia coli*. Studies on the covalently linked pyruvate required for activity. J. Bacteriol. 257:12063-12068.

Markham, G.D., E.W. Hafner, C.W. Tabor, and H. Tabor. 1983. S-Adenosylmethionine synthetase (methionine adenosyltransferase)(*Escherichia coli*). Methods Enzymol. 94:219-222.

Markham, G.D., J. DeParasis, and J. Gatmaitan. 1984. The sequence of *metK*, the structural gene for S-adenosylmethionine synthetase in *Escherichia coli*. J. Biol. Chem. 259:14505-14507.

McKay, D., I. Weber, and T. Steitz. 1982. Structure of catabolite gene activator protein at 2.9 Å resolution. J. Biol. Chem. 257:9518-9524.

McMurry, L.M., and I.D. Algranati. 1986. Effect of polyamines on the translation fidelity *in vivo*. Eur. J. Biochem. 155:383-390.

Menzel, R., and M. Gellert. 1987. Modulation of transcription by DNA supercoiling: A deletion analysis of the *Escherichia coli gyrA* and *gyrB* promoters. Proc. Nat. Acad. Sci. USA. 84:4185-4189.

Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Moore, R.C., and S.M. Boyle. 1990. Nucleotide sequence and analysis of the *speA* gene encoding biosynthetic arginine decarboxylase in *Escherichia coli*. J. Bacteriol. 172:4631-4640.

Mori, K, and H. Aiba. 1985. Evidence for negative control of *cya* transcription by cAMP and cAMP receptor protein in intact *Escherichia coli* cells. J. Biol. Chem. 260:14838-14843.

Morris, D.R., and A.B. Pardee. 1965. A biosynthetic ornithine decarboxylase in *Escherichia coli*. Biochem. Biophys. Res. Commun. 20:697-702.

Morris, D.R., and A.B. Pardee. 1966. Multiple pathways for putrescine biosynthesis in *Escherichia coli*. J. Biol. Chem. 241:3129-3135.

Morris, D.R., and K.L. Koffron. 1967. Urea production and putrescine biosynthesis by *Escherichia coli*. J. Bacteriol. 94:1516-1519.

Morris, D.R., and K.L. Koffron. 1969. Putrescine biosynthesis in *Escherichia coli*. (Regulation through pathway selection). J. Biol. Chem. 244:6094-6099.

Morris, D.R., and C.M. Jorstad. 1970. Isolation of conditionally putrescine-deficient mutants of *Escherichia coli*. J. Bacteriol. 101:731-737.

Morris. D.R., W.H. Wu, D. Appelbaum, and K.L. Koffron. 1970. Regulatory pattern in putrescine biosynthesis in *Escherichia coli*. Ann. N.Y. Acad. Sci. 171:968-976.

Morris, D.R. 1973. RNA and protein synthesis in a polyamine- requiring mutant of *Escherichia coli*. *In:* D.H. Russell (Ed.), *Polyamines in normal and neoplastic growth*, Raven Press, New York. pp. 111-122.

Morris, D.R., and M.T. Hansen. 1973. Influence of polyamine limitation on the chain growth rates of β-galactosidase and its messenger ribonucleic acid. J. Bacteriol. 116:588-592.

Morris, D.R., and C.M. Jorstad. 1973. Growth and macromolecular composition of a mutant of *Escherichia coli* during polyamine limitation. J. Bacteriol. 113:271-277.

Morris, D.R., and R.H. Fillingame. 1974. Regulation of amino acid decarboxylation. Ann. Rev. Biochem. 43:303-325

Morris, D.R., and E.A. Boeker. 1983. Biosynthetic and biodegradative ornithine and arginine decarboxylases from *Escherichia coli*. Methods Enzymol. 94:125-134.

Munro, G.F., K. Hercules, J. Morgan, and W. Sauerbier. 1972. Dependence of the putrescine content of *Escherichia coli* on the osmotic strength of the medium. J. Biol. Chem. 247:1272-1280.

Munro, G.F., and W. Sauerbier. 1973. Osmotically induced excretion of putrescine by mutants of *Escherichia coli* defective in potassium transport. J. Bacteriol. 116:488-490.

Musso, R.E., R. DiLauro, S. Adhya, and B. de Crombrugghe. 1977. Dual control for transcription of the galactose operon by cyclic AMP and its receptor protein at two interspersed promoters. Cell 12:847-854.

Niedhardt, F. C., P. L. Bloch, and D. F. Smith. 1974. Culture medium for Enterobacteria. J. Bacteriol. 119:736-747.

Nielson, L.D., D. Monard, and H.V. Rickenberg. 1973. Cyclic 3',5'-adenosine monophosphate phosphodiesterase of *Escherichia coli*. J. Bacteriol. 116:857-866.

Okamoto, K., and M. Freundlich. 1986. Mechanism for the autogenous control of the *crp* operon: Transcriptional inhibition by a divergent RNA transcript. Proc. Nat. Acad. Sci. USA. 83:5000-5004.

Oliver, Donald. 1985. Protein secretion in *Escherichia coli*. Ann. Rev. Microbio. 39:615-648.

Panagiotidis, C.A., and E.S. Canellakis. 1984. Comparison of the basic *Escherichia coli* antizyme 1 and antizyme 2 with the ribosomal proteins S20/L26 and L34. J. Biol. Chem. 259:15025-15027.

Pastan, I, and R. Perlman. 1970. Cyclic adenosine monophosphate in bacteria. Science 169:339-344.

Pastan, I., and S. Adhya. 1976. Cyclic adenosine 5'-monophosphate in *Escherichia coli*. Bacteriol. Rev. 142:527-551.

Paulin, L., J. Vehmaanperä, I. Nykänen, and H. Pösö. 1983a. GTP insensitive ornithine decarboxylase in acetobacteria able to synthesize spermine. Biochem. Biophys. Res. Commun. 114:779-784.

Paulin, L., H. Ruohola, I. Nykänen, and M. Pösö. 1983b. The incorporation of 1-3 diaminopropane into thermine by an extreme thermophile: a novel route for the biosynthesis of polyamines. FEMS Microbiol. Lett. 19:299-302.

Pegg, A.E. 1986. Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J. 234:249-262.

Pruisiner, S., R.E. Miller, and R.C. Valentine. 1972. Adenosine 3',5'-cyclic monophosphate control of the enzymes of glutamine metabolism in *Escherichia coli*. Proc. Nat. Acad. Sci. 69:2922-2926.

Puziss, J. W., J. D. Fikes, and P. J. Bassford, Jr. 1989. Analysis of mutational alterations in the hydrophilic segment of the maltose-binding protein signal peptide. J. Bacteriol. 171:2303-2311.

Raina, A., and P. Hannonen. 1971. Separation of enzyme activities catalysing spermidine and spermine synthesis in rat brain. FEBS Lett. 16:1-4.

Randall, L.L., S.J.S. Hardy and J. R. Thom. 1987. Export of protein: a biochemical view. Annu. Rev. Microbiol. 41:507-541.

Recsei, P.A., and E.E. Snell. 1972. Histidine decarboxylaseless mutants of *Lactobacillus* 30a: isolation and growth properties. J. Bacteriol. 112:624-626.

Rickenberg, H.V. 1974. Cyclic AMP in prokaryotes. Ann. Rev. Microbio. 28:353-369.

Ronson, C.W., B.T. Nixon, and F.M. Ausubel. 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell 49:579-581.

Rubenstein, K.E., E. Streibel, S. Massey, L.Lapi, and S.S. Cohen. 1972. Polyamine metabolism in potassium-deficient bacteria. J. Bacteriol. 112:1213-1221.

Sahagan, B.G., and J.E. Dahlberg. 1979. A small, unstable RNA molecule of *Escherichia coli*: spot 42 RNA: II. accumulation and distribution. J. Mol. Biol. 131:593-605.

Sanger. F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Nat. Acad. Sci. USA. 74:5463-5467.

Satishchandran, C. and S.M. Boyle. 1984. Antagonistic transcriptional regulation of the putrescine biosynthetic enzyme agmatine ureohydrolase by cyclic AMP and agmatine in *Escherichia coli*. J. Bacteriol. 157:552-559.

Satishchandran, C., G.D. Markham, R.C. Moore and S.M. Boyle. 1990. Location of *speA*, *speB*, *speC*, and *metK* genes on the physical map of *Escherichia coli*. J. Bacteriol. 172:4748.

Saxe, S., and A. Revzin. 1979. Cooperative binding to DNA of catabolite activator protein of *Escherichia coli*. Biochemistry 18:255-263.

Schneider, K., and C. F. Beck. 1986. Promoter-probe vectors for the analysis of divergently arranged promoters. Gene 42:37-48.

Schneider, K., and C.F. Beck. 1987. New expression vectors for identifying and testing signal structures for initiation and termination of transcription. Meth. Enzymol. 153:452-461.

Schweizer, H., and W. Boos. 1983. Transfer of the Δ(*argF-lac*)U169 mutation between *Escherichia coli* strains by selection for a closely linked Tn10 insertion. Mol. Gen. Genet. 192:293-294.

Schweizer, H., W. Boos, and T.J. Larson. 1985. Repressor for the *sn*-glycerol-3-phosphate regulon of *Escherichia coli* K-12: cloning of the *glpR* gene and identification of its product. J. Bacteriol. 161:563-566.

Schwimmer, S. 1968. Differential effects of putrescine, cadaverine and glyoxal-bis-(guanylhydrasone) on DNA- and nucleohistone-supported DNA synthesis. Biochem. Biophys. Acta 166:251-254.

Seyfried, C.E., and D.R. Morris. 1979. Relationships between inhibition of polyamine biosynthesis and DNA replication in activated lymphocytes. Cancer Res. 39:4861-4867.

Shaibe, E., E. Metzer, and Y.S. Halpern. 1985a. Control of utilization of L-arginine, L-ornithine, agmatine, and putrescine as nitrogen sources in *Escherichia coli* K-12. J. Bacteriol. 163:938-942.

Shaibe, E., E. Metzer, and Y.S. Halpern. 1985b. Metabolic pathway for the utilization of L-arginine, L-ornithine, agmatine, and putrescine as nitrogen sources in *Escherichia coli*. J. Bacteriol. 163:933-937.

Silhavy, T.J., M.L. Bergman, and L.W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Simmons, R.W., F. Houman, and N. Kleckner. 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene 53:85-96.

Smith, T.A. 1972. The physiology of the polyamines and related compounds. Endeavour 31:22-28.

Smith, T.A. 1985. Polyamines. Ann. Rev. Plant Physiol 36:117-43.

Sneath, P.H.A. 1955. Putrescine as an essential growth factor for a mutant of *Aspergillus nidulans*. Nature (London) 175:818.

Speranza, A., and N. Bagni. 1977. Putrescine biosynthesis in *Agrobacterium tumefaciens* and in normal and crown gall tissues of *Scorzonera hispanica* L. Z. Pflanzenphysiol. 81:S226-S233.

Srinivasan, P.R., D.V. Young, and W. Maas. 1973. Stable ribonucleic acid synthesis in stringent (*rel*$^+$) and relaxed (*rel*$^-$) polyamine auxotrophs of *Escherichia coli* K-12. J. Bacteriol. 116:648-655.

Stevens, L., and M.D. Winther. 1979. Spermine, spermidine and putrescine in fungal development. Adv. Microb. Physiol. 19:63-148.

Stoltzfus, L., and G. Wilcox. 1989. Effect of mutations in the cyclic AMP receptor protein-binding site on araBAD and araC expression. J. Bacteriol. 171:1178-1184.

Struhl, K. 1986. A rapid method for creating recombinant DNA molecules. BioTechniques 3:452-453.

Szumanski, M.B.W., and S.M. Boyle. 1990. Analysis and sequence of the *speB* gene encoding agmatine ureohydrolase, a putrescine biosynthetic enzyme in *Escherichia coli*. J. Bacteriol. 172:538-547.

Tabor, H, S.M. Rosenthal, and C.W. Tabor. 1958. The biosynthesis of spermidine and spermine from putrescine and methionine. J. Biol. Chem. 233:907-914.

Tabor, C.W., and H. Tabor. 1966. Transport systems for 1,4-diaminobutane, spermidine, and spermine in *Escherichia coli*. J. Biol. Chem. 241:3714-3723.

Tabor, H., and C.W. Tabor. 1969a. Formation of a 1,4-diaminobutane and of spermidine by an ornithine auxotroph of *Escherichia coli*. J. Biol. Chem. 244:2286-2292.

Tabor, H., and C.W. Tabor. 1969b. Partial separation of two pools of arginine in *Escherichia coli*:preferential use of exogenous rather than endogenous arginine for the biosynthesis of 1,4-diaminobutane. J. Biol. Chem. 244:6383-6387.

Tabor, C.W., and H. Tabor. 1976. 1,4-Diaminobutane (putrescine), spermidine, and spermine. Ann. Rev. Biochem. 45:285-306.

Tabor, C.W., H. Tabor, and E.W. Hafner. 1978. *Escherichia coli* mutants completely deficient in adenosylmethionine decarboxylase and in spermidine biosynthesis. J. Biol. Chem. 253:3671-3676.

Tabor, H.C., C.W. Tabor, M.S. Cohn, and E.W. Hafner. 1981. Streptomycin resistance (*rpsL*) produces and absolute requirement for polyamines for growth of an *Escherichia coli* strain unable to synthesize putrescine and spermidine [Δ(*speA-speB*)Δ*speC*]. J. Bacteriol. 147:702-704.

Tabor, C.W., H. Tabor, E.W. Hafner, G.D. Markham, and S.M. Boyle. 1983. Cloning of the *Escherichia coli* genes for the biosynthetic enzymes for polyamines. Methods Enzymol. 94:117-121.

Tabor, C.W., and H. Tabor. 1985. Polyamines in microorganisms. Microbiol. Rev. 49:81-99.

Tabor, C.W., and H. Tabor. 1987. The *speEspeD* operon of *Escherichia coli*. Formation and processing of a proenzyme form of S-adenosylmethionine decarboxylase. J. Biol. Chem. 262:16037-16040.

Takahashi, M., B. Blazy, and A. Baudras. 1979. Non-specific interactions of CRP from *E. coli* with native and denatured DNAs: control of binding by cAMP and cGMP and by cation concentration. Nuc. Acids Res. 7:1699-1712.

Takahashi, M., B. Balzy, A. Baudras, and W. Hillen. 1983. On the origin of selectivity in recognition by cyclic adenosine-3',5'-monophosphate receptor protein of its specific binding site of the lactose promoter region. J. Mol. Biol. 167:895-899.

Tarrof, C.W., and C.A. Hobbs. 1987. Improved media for growing plasmid and cosmid clones. Focus 9(2):12

Travers, A.A. 1984. Conserved features of coordinately regulated *E. coli* promoters. Nuc. Acids Res. 12:2605-2618.

Ullmann, A., E. Joseph, and A. Danchin. 1979. Cyclic AMP as a modulator of polarity in polycistronic transcriptional units. Proc. Nat. Acad. Sci. USA. 76:3194-3197.

Ullmann, A., and A. Danchin. 1983. Role of cyclic AMP in bacteria. Adv. Cyclic Nuc. Res. 15:1-53.

Utsumi, R., H. Tanabe, Y. Nakamoto, M. Kawanukai, H. Sakai, M. Himeno, T. Komano, and Y. Hirota. 1981. Inhibitory effect of adenosine 3',5'-phosphate on cell division of *Escherichia coli* K-12 mutant derivatives. J. Bacteriol. 147:1105-1109.

Vaaler, G.L., and E. E. Snell. 1989. Pyridoxal 5'-phosphate dependent histidine decarboxylase: Overproduction, purification, biosynthesis of soluble site-directed mutant proteins, and replacement of conserved residues. Biochemistry 28:7306-7313.

Vlasuk, G. P., S. Inouye, H. Ito, K. Itakura, and M. Inouye. 1983. Effects of the complete removal of basic amino acid residues from the signal peptide on secretion of lipoprotein in *Escherichia coli*. J. Biol. Chem. 258:7141-7148.

von Heijne, G. 1990. The signal peptide. J. Memb. Biol. 115:195-201.

Wang, J.Y.J., D.O. Clegg, and D.E. Koshland, Jr. 1981. Molecular cloning and amplification of the adenylate cyclase gene. Proc. Nat. Acad. Sci. USA. 78:4684-4688.

Weber, I.T., K. Takio, K. Titani, and T.A. Steitz. 1982. The cAMP-binding domains of the regulatory subunit of cAMP-dependent protein kinase and the catabolite gene activator protein are homologous. Proc. Nat. Acad. Sci. USA. 79:7679-7683.

Whitney, P.A., and D.R. Morris. 1978. Polyamine auxotrophs of *Saccharomyces cerevisiae*. J. Bacteriol. 134:214-220.

Wickner, R.B., C.W. Tabor, and H. Tabor. 1970. Purification of adenosylmethionine decarboxylase from *Escherichia coli* W: evidence for covalently bound pyruvate. J. Biol. Chem. 245:2132-2139.

Wright, J.W., and S. M. Boyle. 1982. Negative control of ornithine decarboxylase and arginine decarboxylase by adenosine-3':5'-cyclic monophosphate in *Escherichia coli*. Mol. Gen. Genetics 186:482-487.

Wright, J.M., C. Satishchandran, and S.M. Boyle. 1986. Transcription of the speC (ornithine decarboxylase) gene of *Escherichia coli* is repressed by cyclic AMP and its receptor protein. Gene 44:37-45.

Wu, W.H., and D.R. Morris. 1973. Biosynthetic arginine decarboxylase from *Escherichi coli* (Purification and properties). J. Biol. Chem. 248:1687-1695.

Xie, Q, C.W. Tabor, and H. Tabor. 1989. Spermidine biosynthesis in *Escherichia coli*: promoter and termination regions of the *spe*ED operon. J. Bacteriol. 171:4457-4465.

Young, D.V., and P.R. Srinivasan. 1972. Regulation of macromolecular synthesis by putrescine in a conditional *Escherichia coli* putrescine auxotroph. J. Bacteriol. 112:30-39.

Zubay, G. 1980. The isolation and properties of CAP, the catabolite gene activator. Methods Enzymol. 65:856-877.