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ANALYSIS OF PATTERN FORMATION IN THE HETEROCYST-FORMING FILAMENTOUS CYANOBACTERIUM *Anabaena* SP. STRAIN PCC 7120

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ABSTRACT

ANALYSIS OF PATTERN FORMATION IN THE HETEROCYST-FORMING FILAMENTOUS CYANOBACTERIUM *Anabaena* SP. STRAIN PCC 7120

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Anabaena sp. strain PCC 7120 is a photoautotrophic organism that utilizes oxygen-evolving photosynthesis for growth. In *Anabaena* sp., oxygen-intolerant nitrogenase activity is segregated to specialized cells, called heterocysts, in which the concentration of O_2 is kept very low. In nitrogen-deficient media, heterocysts are formed by the differentiation of semi-regularly spaced vegetative cells.

Transposon insertions within the *hetR* gene prevented the evident differentiation of heterocysts (Het^- phenotype). Transcriptional fusions with the reporter genes, *luxAB*, that encode luciferase, a protein complex that can produce light, showed that *hetR* is induced within two hours following the removal of nitrate from the medium. Photon-accumulating microscopy showed that by 3.5 hours, the induced expression of *hetR* is limited to spaced groups

of cells. The cells with enhanced luminescence are more narrowly delimited by 6 hours; and by 24 hours, cells midway between two mature heterocysts are most luminescent. The induction of *hetR* was found to require a functional copy of the *hetR* gene, indicating that *hetR* is autoregulatory.

Transposon-generated mutant N10 is also Het$^-$. The N10 mutant tends to change to a mutant that forms multiple contiguous heterocysts (Mch phenotype). Other insertions into the site of transposition typically directly generated the Mch phenotype. When borne on a plasmid, the wild-type gene, *hetN*, does not complement the Het$^-$ phenotype; rather, *hetN* suppresses heterocyst differentiation in wild-type cells. The suppression requires an intact copy of *hetN*. The sequences of the proteins encoded by *hetN* and by a large *orf*, the 5′ *orf*, found upstream from *hetN*, resemble the sequences of proteins that are involved in the syntheses of fatty acids and secondary metabolites. A third *orf*, *hetI*, is transcribed from the opposite strand of DNA and lies just 42 bp 3′ from *hetN*. The protein encoded by *hetI* shows sequence similarity to *Bacillus* spp. proteins that are required for the syntheses of secondary metabolites. The *hetI* gene may be required for vegetative growth.

A physical and immunological approach was also undertaken in an attempt to study how cells within a filament communicate. Intercellular movement of fluorescent dyes was not observed. However, a protein associated with the plasma membranes of cells was identified that cross-reacts with antibodies raised against a gap junction protein. This observation is consistent with the possibility that aqueous channels provide cytosolic continuity between cells.

To Laura, For Her Love and Support

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wealth of information on most matters and his unexpected outbursts of song would always bring a smile to my face.

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Chris Bauer at the University of Chicago pointed out some errors within the DNA sequence of the 5' *orf* using data from Jim Golden's laboratory at Texas A&M University. Accordingly, I have added a T at bp 941, CA at bps 946 and 947, a C at bp 961, and an A at bp 1476; removed a T from between bp 952 and 953 and a G from between bp 1490 and 1491; and converted a T to a G at bp 1045 and a C to a T at bp 1473. I thank my colleagues for sharing their data with me.

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4.8 A single filament of *Anabaena* sp. PCC 7120, grown in nitrogen-free medium and containing a heterocyst, which was then labeled with the lipophilic dye NBD-PC 122

LIST OF ABBREVIATIONS AND SYMBOLS

+	Presence of a functional gene* or of a phenotype**
-	Lack of a functional gene or of a phenotype
Δ	Deletion
λ	Bacteriophage lamda
σ	Sigma factor
AA	Allen and Arnon medium
ACP	Acyl carrier protein
Ap	Ampicillin
ATP	Adenosine 5′-triphosphate
Bm	Bleomycin
bp	Basepair(s)
BLOS	A gene cassette that contains a ***bom*** (*oriT*) region, the ***luxAB*** a transcriptional reporter genes, an ***oriV*** region, and the ***aad*** gene conferring **S**m^r/Sp^r
Bom	Basis of mobilization during conjugation
BSA	Bovine serum albumin
cAMP	Adenosine 3′:5′-cyclic monophosphate
Chl	Chlorophyll

Cm	Chloramphenicol
cpm	Counts per minute
DNA	Deoxyribonucleic acid
DR	Double recombinant
EDTA	Ethylenediaminetetraacetic acid
Em	Erythromycin
FA	Fatty acid
FAS	Fatty acid synthase
FRAP	Fluorescence redistribution after photobleaching
g	Gravity
GTP	Guanosine 5′-triphosphate
HEPES	N-(hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
IPTG	Isopropyl-thiogalactoside
kb	Kilobasepair(s)
kD	Kilodalton(s)
Km	Kanamycin
LB	Luria-Bertani medium
MES	2-(N-morpholino)ethanesulfonic acid
NAD(P)H	reduced form of nicotinamide adenine dinucleotide (3′-phosphate)
Nm	Neomycin
orf	Open reading frame
PAGE	Polyacrylamide gel electrophoresis

PHB	Poly-ß-hydroxybutyrate
PK	Polyketide
PKS	Polyketide synthase
PMSF	Phenylmethanesulfonyl fluoride
ppGpp	Guanosine tetraphosphate
r	Resistant
s	Sensitive
SDS	Sodium dodecyl sulfate
Sm	Streptomycin
Sp	Spectinomycin
SR	Single recombinant
Suc	Sucrose
TBS	Tris buffered saline
TES	N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid
Tn	Transposon
Tris	Tris(hydroxymethyl)aminomethane

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* Names of genes and genetic loci are italicized

** Names of phenotypes are capitalized

Chapter 1

GENERAL INTRODUCTION

INTRODUCTION

Research on procaryotic development has focused on the mechanisms by which the cells sense environmental cues and thereupon initiate and coordinate the processes required for cellular differentiation. An in-depth understanding requires that the procaryotes whose development is studied can be analyzed both biochemically and genetically. The development of some procaryotes, whose development leads to a state that is distinct from that of vegetatively growing cells, has been subject to extensive investigation. Examination of the literature shows that even in these 'simple' organisms, complex regulatory networks are utilized to achieve developmental transitions. In fact, numerous signals that are known to be used by eucaryotes to regulate differentiation and metabolism, including the production of extracellular peptidic factors, steroid hormone-like molecules, CAMP and ion gradients, are also utilized by procaryotes. In procaryotes, as in eucaryotes, specific receptors or sensors for the various signals regulate transcriptional responses through a complex of response regulator proteins that often have a variety of kinase and phosphatase activities.

In both procaryotic and eucaryotic organisms, temporally controlled processes are the most easily studied and best understood aspects of differentiation, whereas spatial control, which is required for pattern formation, is more difficult to approach experimentally and is poorly understood. All of the procaryotic developmental systems studied display spatial regulation that includes the controlled expression of genes within specific cells or compartments. In most cases it is unclear what generates the spatial differences, but the genetic manipulability of the procaryotic genome allows for a rapid dissection of the mechanisms involved in pattern formation. In this introduction, I will present a general description of the types of development that occur in some of the most extensively studied procaryotic developmental systems. Because processes that are known to control the initiation of, and spatially regulated gene expression during, development probably exert the greatest influence on pattern formation, I will give a more detailed account of these processes.

GENERAL DESCRIPTION OF DEVELOPMENT IN SOME PROCARYOTES

When filaments of the cyanobacterium *Anabaena* are placed in media that lack a source of fixed nitrogen, a small fraction of the vegetative cells differentiates into specialized cells called heterocysts (Figure 1.1). The primary known function of heterocysts is the fixation of dinitrogen (Wolk, 1982). Differentiation into a heterocyst appears to be a terminal stage of development because i) heterocysts do not undergo cell division and ii) there are no

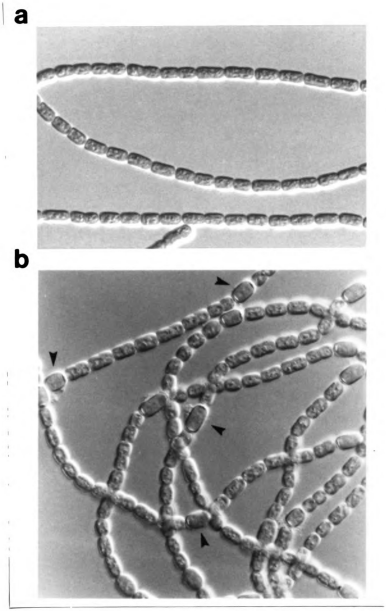


Figure 1.1 Photomicrographs displaying filaments of *Anabaena* sp. strain PCC 7120 grown in the presence of nitrate (a) and 24 hours following the removal of nitrate from the medium (b). In (b), the arrows indicate the positions of some of the heterocysts. Magnification = 1000x

confirmed accounts of the germination of mature heterocysts.

Intercellular exchange of metabolites, nitrogenous compounds from the heterocysts and, it is thought, carbohydrates from the vegetative cells, is required for continued growth in media depleted of fixed nitrogen. Therefore, *Anabaena* has a truly multicellular nature in the sense that cells of different types interact to maintain growth. The only other free-living procaryote in which a similar type of metabolic exchange is known is the actinomycete, *Frankia*, in which nitrogen-fixing vesicles form at the tips of mycelia (Benson and Silvester, 1993). Very little information is available that concerns the regulation of development in *Frankia*; however, some of the morphological features of its development are similar to sporulation in another genus of the actinomycetes, *Streptomyces*, an organism that will be discussed below. The hallmark of heterocyst differentiation in *Anabaena* is that heterocysts arise non-randomly along the length of the filaments (Wolk and Quine, 1975). Therefore, *Anabaena* is a 'simple' organism in which to study a process that forms a one-dimensional pattern. Some strains of *Anabaena* also form spores, called akinetes. Akinetes also appear to arise in a non-random pattern when heterocysts are present (Wolk, 1964). The pattern of akinete formation varies among strains. Some strains form akinetes adjacent to heterocysts whereas other strains form akinetes, initially, midway between two heterocysts. Because the patterns of both heterocyst formation and akinete formation are thought to depend on intercellular interactions, *Anabaena* may produce two distinct types of pattern-forming signals.

Sporulation of *Bacillus subtilis* is the most extensively studied and best characterized procaryotic developmental process. The great amount of knowledge of *Bacillus* sporulation that has been attained has made it the model system on which much research on other procaryotic systems is based. This fact will become apparent throughout this introduction. When *Bacillus* encounters nutrient limitation, cells undergo an asymmetric invagination of their membranes to form an internalized cell. The internal cell, the forespore, is transformed into a spore, while the surrounding cell, the mother cell, produces an environmentally resistant coat around the spore. During nutrient deprivation, *B. subtilis* also undergoes other types of development: the process of gaining genetic competence (the ability to take up exogenous DNA), and the production of antibiotics and extracellular proteases. Sporulation and competence development are clearly interrelated but also have unique and separable properties; competence development appears to be more closely associated with the production of antibiotics.

The developmental biology of spore formation in the *Streptomyces* has not been studied as extensively; however, because of the commercial importance of the *Streptomyces*, a great deal has been learned about their biology. Sporulation of *Streptomyces* resembles, in many ways, the formation of conidia in filamentous fungi such as the well studied *Aspergillus nidulans* (Timberlake, 1991). In the *Streptomyces*, aseptate, filamentous, multinucleoid vegetative cells grow along a solid surface as substrate mycelia. When nutrients become limiting, the mycelia branch upward to produce aerial mycelia (Figure 1.2).

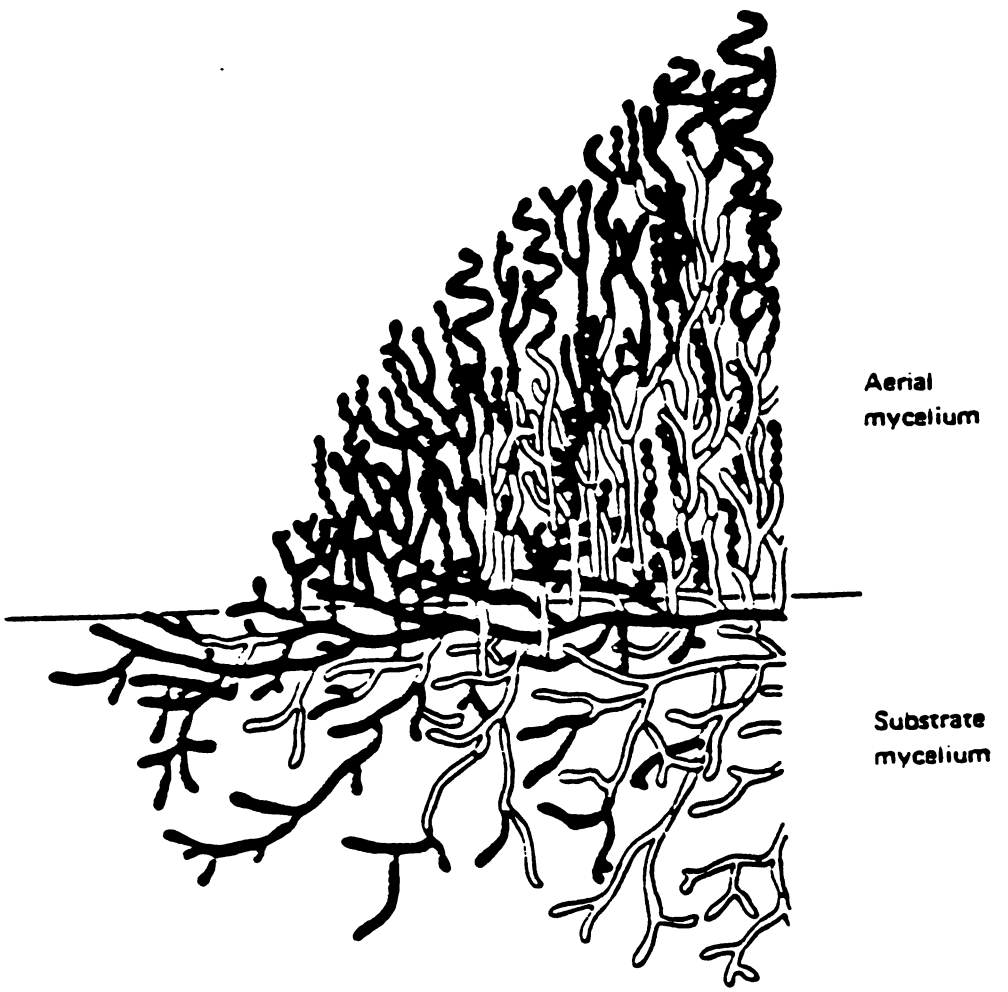


Figure 1.2 An idealized diagram of a vertical section through the center of a sporulating colony of *Streptomyces coelicolor*. Black areas represent intact cells and the white areas represent cells that are lysing. Reproduced, with permission, from Figure 6-4 in M. Dworkin. 1985. *Developmental Biology of the Bacteria*. p.90. Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA.

The aerial mycelia, deriving nutrients from the substrate mycelia (Mendez et al., 1985), undergo spatially regulated differentiation and septation into spores (Figure 1.2). The most extensively studied aspect of *Streptomyces* sporulation is the developmentally regulated induction of biosythesis of antibiotics. Interestingly, the antibiotics or metabolites produced are also, in some cases, required for morphological differentiation and sporulation.

The developmental pattern of the myxobacteria mimics that of other lower eucaryotes, the slime molds, such as *Dictyostelium* (Loomis, 1982). Single *Myxococcus* cells can have an 'adventurous' life style that is terminated in response to environmental cues, particularly nutrient limitation in conjunction with a previously established high cell density, that cause the cells to aggregate into macroscopic structures called fruiting bodies. Within the resulting aggregates of cells, the internal compartment appears to contain the spores that are formed, perhaps at the expense of the life of the outermost cells. During differentiation, *Myxococcus*, like *Streptomyces*, produces extracellular factors that are required for the completion of development.

PROCESSES INVOLVED IN THE INITIATION OF DIFFERENTIATION

B. subtilis

Seven genetic loci have been found in *B. subtilis* that are required for the initiation of sporulation: *spo0A*, *spo0B*, *spo0E*, *spo0F*, *spo0H*, *spo0J*, and *spo0K* (Hoch, 1976). Spo0A belongs to the response regulator family of procaryotic regulatory proteins that are activated by phosphorylation (Ferrari et

al., 1985b; Stock et al., 1989, 1990). Spo0A binds to DNA and can activate or repress transcription depending on the target (Strauch et al., 1990). Spo0A is also autoregulatory, repressing its own transcription during vegetative growth while inducing its own transcription when activated (Yamashita et al., 1986). A multicomponent phosphorelay system has been found to control the phosphorylation of Spo0A (Burbulys et al., 1991). The major components of this system include the proteins KinA, Spo0F and Spo0B (Figure 1.3). KinA is an autophosphorylating transmitter kinase; however, KinA does not appear to be an integral membrane protein that senses external stimuli (Perego et al., 1989). Spo0F is the principal substrate for phosphorylation by KinA (Burbulys et al., 1991; Perego et al., 1989). Although Spo0F is another response regulator-like protein, and contains a phosphate-accepting domain, it lacks a DNA-binding regulatory domain (Trach et al., 1985). Spo0B appears to be a phosphotransferase that mediates the relocation of the phosphate group from spo0F to Spo0A. The phosphotransferase reaction carried out by Spo0B is reversible, and Spo0B can therefore also specifically remove the Spo0A phosphate group (Burbulys et al., 1991).

The eventual activation of Spo0A appears to be the decisive step in the initiation of sporulation. Whereas activated Spo0A can directly induce sporulation-specific genes, perhaps its most important effect is the inhibition of transcription of *abrB* (Perego et al., 1988; Zuber and Losick, 1987). The AbrB protein binds cooperatively to DNA and represses many operons that are induced at the onset of stationary growth, including some required for

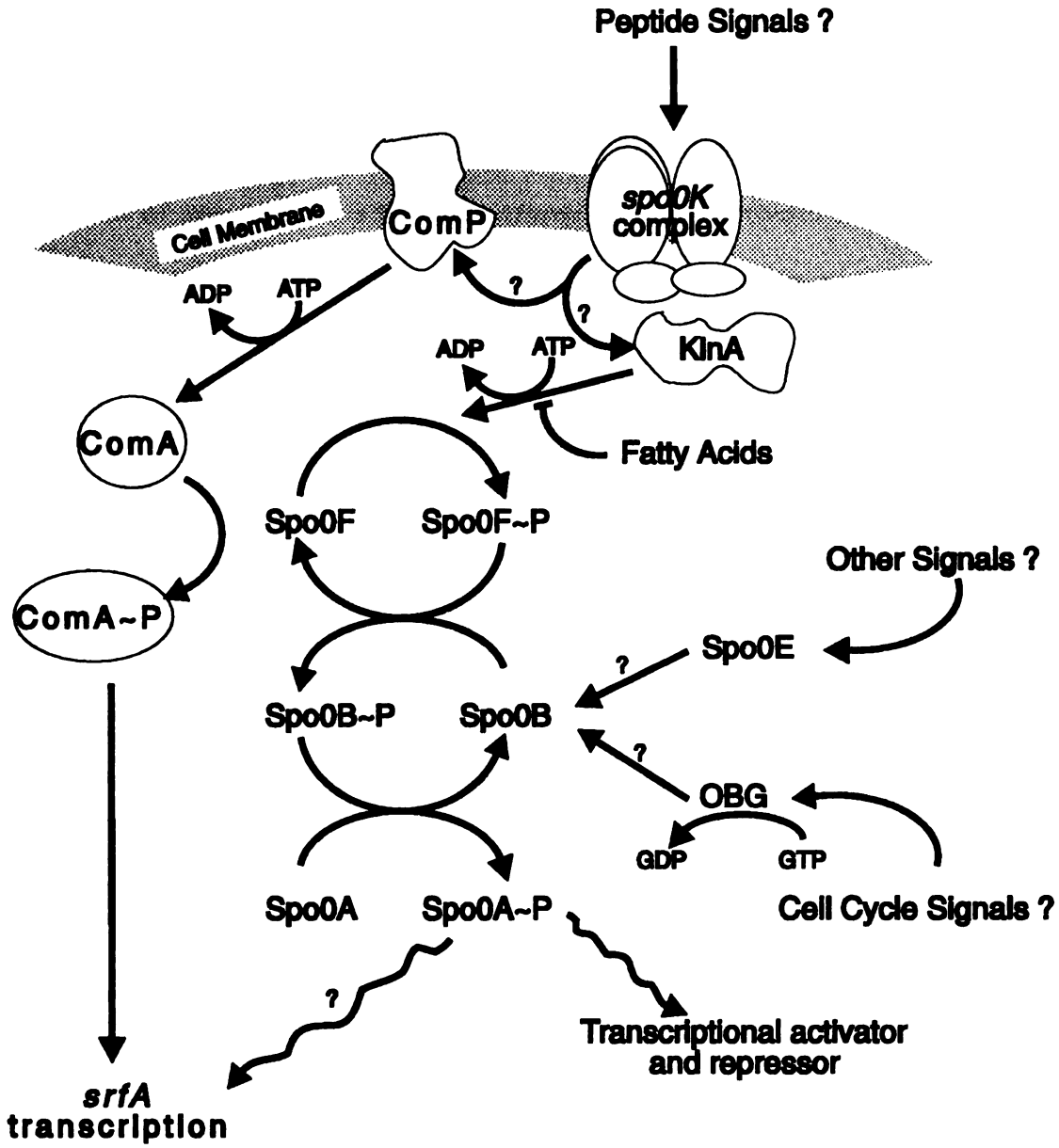


Figure 1.3 Diagram showing the proposed pathways of regulation for the activation of Spo0A and ComA. Adapted from Burbulys et al., 1991.

sporulation (Strauch et al., 1989). Binding by AbrB reinforces other regulatory mechanisms to prevent the onset of sporulation during vegetative growth. By repressing the transcription of *abrB*, Spo0A effectively amplifies its regulatory domain by derepressing operons that are controlled by AbrB. The final step in the initiation of sporulation is the post-transcriptional activation of the earliest sporulation-specific sigma factor, σ^H (Healy et al., 1991), encoded by *spo0H* (Carter and Moran, 1986; Dubnau et al., 1988; Zuber et al., 1989). σ^H directs the initiation of transcription of i) the sporulation-specific genes that are required to initiate septum formation as well as ii) a sigma factor, σ^F, that is needed for the following stages of development (Wu et al., 1989). The increase in activity of σ^H appears to result from both increased stability of mRNA and decreased turnover of the σ^H-protein (Healy et al., 1991). It is not known how the activation of Spo0A accomplishes the increase in activity of σ^H.

Most response regulators are directly phosphorylated by their cognate kinase (Stock et al., 1989, 1990); the numerous intermediates between KinA and Spo0A provide possible regulatory points at which multiple environmental signals may be integrated to control the initiation of sporulation. In some *spo0A* missense mutants, and in strains that overexpress KinA, the multicomponent phosphorelay is bypassed (Perego et al., 1989; Speigelman et al., 1990). It has been suggested that the presence of the multicomponent phosphorelay system in *Bacillus* reflects the "seriousness of the commitment to shut down cell division and initiate sporulation" (Burbulys et al., 1991). Spo0E appears to inhibit the activation of Spo0A by negatively regulating the phosphorelay

system (Perego et al., 1991a; Burbulys et al., 1991). Overexpression of *spo0E* inhibits sporulation while most mutations within *spo0E* cause hypersporulation (Perego et al., 1991a). However, some *spo0E* nonsense mutations inhibit sporulation, a phenomenon that is explicable if the carboxyl-terminus of the Spo0E protein can relieve an inhibition of sporulation mediated by the amino-terminus. Since all *spo0A* missense mutations that bypass the phosphorelay components, and some *spo0A* non-bypass missense mutations (Olmedo et al., 1990), can relieve the inhibition caused by the *spo0E* nonsense mutants, Spo0E appears to exert its control within the phosphorelay pathway, possibly by affecting the transfer of phosphate from Spo0B to Spo0A (Burbulys et al., 1991). Another protein that may regulate the phosphorelay pathway is Obg, which is encoded in the same operon as Spo0B (Ferrari et al., 1985; Trach and Hoch, 1989). Obg is a GTP-binding protein that is required for vegetative growth. Obg, like G-proteins, displays GTPase activity (Fohger and Hoch, unpublished; cited in Burbulys et al., 1991). It has been proposed that Obg may affect the phosphotransferase activity of Spo0B in response to the levels of GTP. Growth conditions that lower endogenous levels of GTP (reviewed by Freese et al., 1985) or direct lowering of levels of GTP by the addition of the drug decoyinine (Mitani et al., 1977), which inhibits GMP synthetase, can induce sporulation. Therefore, Obg-GTP may block Spo0B phosphotransferase activity while Obg-GDP stimulates Spo0B and allows sporulation to begin. Early experiments showed that sporulation is limited to a short phase of the cell cycle (Mandelstam and Higgs, 1974). Therefore, the similarity of Obg to the Ras

proteins, which regulate the cell cycle of eucaryotes, suggests that Obg may respond to cell cycle signals (Trach and Hoch, 1989). It is interesting that even when cells are grown in enriched media, lowering of the endogenous levels of GTP can initiate sporulation (Freese et al., 1985; Mitani et al., 1977), suggesting that GTP levels play the dominant role in signalling the onset of stationary growth. However, addition of decoyinine, which specifically inhibits GTP synthesis, does not induce the other late growth responses (i.e., competence development and production of antibiotics; Freese, 1981); therefore, divergent pathways may induce these responses. Additionally, the induction of sporulation by either nutrient stepdown or decoyinine is not very effective before cultures have achieved a high cell density, suggesting that an additional factor is involved in the induction of sporulation (Grossman and Losick, 1988). This matter will be discussed below.

Induction of competence development also utilizes a signal transduction pathway. The *srfA* operon encodes the structural proteins required for the production of the cyclic lipopeptide antibiotic surfactin (Nakano et al., 1991a). Induction of *srfA* occurs at the onset of stationary phase, as does the induction of sporulation, and usually requires *comA*, *comP* and *spo0K* (Figure 1.3; Hahn and Dubnau, 1991; Nakano et al., 1991b; Nakano and Zuber, 1991). ComA is a response regulator protein (Weinrauch et al., 1989, 1990) that, when activated by phosphorylation, binds to specific sites upstream from the *srfA* promoter to activate *srfA* transcription (Roggiani and Dubnau, unpublished; cited in Marahiel et al., 1993). ComP shows sequence similarity to the histidine

kinase signal transmitters; ComA is the primary substrate for phosphorylation (Weinrauch et al., 1990). ComP-independent induction of *srfA* can occur, but still requires ComA and *spo0K* as well as Spo0A, Spo0B, Spo0F and KinA. ComP-independent induction of *srfA* is independent of AbrB function (Marahiel et al., 1993); it is unknown how ComP-independent induction of *srfA* occurs. The induced expression of *srfA* has been determined to be the primary function of ComA and ComP in competence development. Placing *srfA* under the control of an IPTG-inducible promoter allows for IPTG-induced competence development, even during exponential cell growth (Hahn and Dubnau, 1991; Nakano and Zuber, 1991).

Another factor that is required for competence development and efficient sporulation is encoded by *sfp* (Nakano et al., 1992). Both inactivation and overexpression of *sfp* lead to reduced transcription of *srfA* and to a reduction in competence and sporulation. It follows that normal expression of *srfA* requires a specific level of expression of *sfp*. Some other genes that are induced late in growth, including a gene that encodes a protein similar to nitrite reductase, also require *sfp* for induced expression (Marahiel et al., 1993). That *sfp* strains can be complemented by growth in the presence of *sfp*$^+$ strains suggests that Sfp is involved in the regulation or production of an extracellular factor. This observation is highly intriguing because evidence is accumulating that extracellular peptidic factors, produced by cells that have grown to a high density, are required for both efficient sporulation (Grossman and Losick, 1988) and induction of *srfA* (Magnusson and Grossman, unpublished; cited in Marahiel

et al., 1993). Furthermore, the *spo0K* operon, which is required for efficient initiation of sporulation as well as for the induction of *srfA* and competence development, has been found to contain five genes that encode proteins highly similar to oligopeptide permeases (Perego et al., 1991; Rudner et al., 1991). Surprisingly, disrupting the last gene of the *spo0K* operon does not affect sporulation but does prevent the development of competence (Rudner et al., 1991). Therefore, *spo0K* appears to play a role in sensing extracellular factors and may either sense different signal molecules or affect different signal transduction pathways that are used for efficient initiation of sporulation and competence development. The observation that overexpression of *kinA* alleviates the effect of *spo0K* mutations on sporulation lends support to the possibility that *spo0K* gene products may regulate the activity of KinA (Figure 1.3; Rudner et al., 1991).

Although numerous factors appear to be required for the initiation of sporulation in *Bacillus*, nutrient limitation is evidently the major catalyzer (Freese et al., 1985), but how is nutrient limitation sensed? The molecule ppGpp has long been considered a signal for the onset of metabolic stress or stationary growth. In *E. coli*, the cellular levels of ppGpp are inversely correlated with the growth rate (Lazzarini et al., 1971). It is generally considered that ppGpp synthesis is caused by uncharged tRNAs during amino acid starvation, a process that is referred to as the stringent response. The stringent response is mediated by the *relA* locus (Dennis, 1977; Dennis and Nomura, 1974); however, both *E. coli* (Atherly, 1979) and *Bacillus* (Fehr and Richter, 1981;

Price and Brown, 1981) also show *relA*-independent ppGpp synthesis in response to carbon or energy limitation. The levels of ppGpp have been shown to increase in *B. subtilis* in response to nutrient limitation (Lopez et al., 1991). ppGpp inhibits the activity of IMP dehydrogenase, which converts IMP to GMP. Therefore, ppGpp may directly regulate the intercellular levels of GTP; alternatively, ppGpp may have a more direct role in the initiation of sporulation. Recent evidence suggests that KinA activity is repressed by free *cis*-unsaturated fatty acids (Strauch et al., 1992). In *E. coli*, the stringent response blocks an early step in the synthesis of fatty acids (Lueking and Goldfine, 1975; Nunn and Cronan, 1976; Spencer et al., 1977). If ppGpp also blocks fatty acid synthesis in *B. subtilis*, then inhibition of fatty acid production by ppGpp could derepress the activity of KinA.

It is curious that lowering GTP levels with decoyinine does not induce competence development or production of antibiotics. Perhaps ppGpp is required to induce these late growth responses, and directly lowering GTP levels with decoyinine prevents both *relA*-dependent and -independent ppGpp synthesis. It is apparent that extracellular peptide-like factors also have some control over both sporulation and competence development (Grossman and Losick, 1988). The exact nature of these factors and how they influence differentiation is, at present, unknown.

Streptomyces

In the *Streptomyces*, increases in ppGpp levels also occur at the onset of the stationary or slow growth phase, and precede the induction of antibiotic production and sporulation. A decrease in the GTP pool also precedes and may also initiate differentiation in some species of *Streptomyces*. As in *Bacillus*, decoyinine can induce sporulation in the *Streptomyces* (reviewed by Ochi, 1988).

Sporulation deficient mutants of some species of *Streptomyces* can be rescued by the addition of an exogenous factor. In *S. griseus*, the factor was found to be 2-isocapryloyl-3R-hydroxymethyl-*r*-butyrolactone (A-factor) (Khokhlov, 1985). Following the inception of nutrient limitation, A-factor is produced rapidly and diffuses from the cells, and thus appears to play an integral role in the initiation of sporulation. A receptor specific for A-factor has been identified as a soluble protein that represses antibiotic production and sporulation (Miyake et al., 1989, 1990). Binding of A-factor relieves the repression, as do mutations in the gene that encodes the A-factor-binding protein (Miyake et al., 1990).

The regulation of biosynthesis of A-factor and antibiotic production share a feature with *Bacillus* sporulation in that the phosphorylation of DNA-binding proteins, such as AfsR (Hong et al., 1991; Horinouchi and Beppu, 1992; Horinouchi et al., 1990), is required for the production of A-factor and antibiotics (Hara et al., 1983; Horinouchi et al., 1983; Stein and Cohen, 1989). Overexpression of AfsR suppresses the phenotype of a mutation in another

gene required for antibiotic production, *afsB* (Horinouchi and Beppu, 1984; Horinouchi et al., 1983; Stein and Cohen, 1989). Therefore, sensor/transmitter kinases and response regulator proteins may also be required for the initiation of sporulation in the *Streptomyces*.

At least seven genetic loci have been identified, mutations in which block the formation of aerial mycelia in *S. coelicolor*. These mutants have been called bald (*bld*) because colonies lack the furry appearance produced by aerial mycelia. All of the *bld* mutations except *bldC* also adversely affect antibiotic production. Sporulation in most of the *bld* mutants is conditional, depending upon the carbon source that is supplied to the hyphae, whereas the defect in antibiotic production is not affected by the carbon source (Chater, 1989). Generally, the presence of glucose is required for expression of the Bld phenotype. Therefore, a catabolite repression mechanism may be involved in the conditional nature of the Bld mutants. The *bldC* mutants, like A-factor mutants, can be induced to sporulate in the presence of an unknown exogenous factor produced by sporulating colonies (Chater, 1989).

Surprisingly, the entire pathway for the initiation of sporulation appears to be bypassed by the overexpression of *whiG* (Chater et al., 1989). The WhiG protein is a sigma factor whose overexpression causes formation of aerial mycelia and spores under typically vegetative conditions. Overexpression of WhiG also causes spores to form within the substrate mycelia, apparently circumventing the requirement for formation of aerial mycelia. If WhiG is inactivated, sporulation is completely blocked, although aerial mycelia still form

(Mendez and Chater, 1987).

Myxococcus xanthus

In *M. xanthus*, nutrient limitation is again correlated with an increase in the cellular levels of ppGpp (Manoil and Kaiser, 1980), although very little is known about any signal transduction pathway that may initiate the developmental response. It has been found that high concentrations of glycerol (0.5M) can effectively induce sporulation (Dworkin and Gibson, 1964). Similar levels of glycerol are found to inhibit the methylation of the methyl-accepting chemotaxis proteins that regulate chemotaxis in *E. coli* (Oosawa and Imae, 1983), which suggests that a signal transduction pathway may be required for the initiation of sporulation in *Myxococcus*. Accordingly, a gene encoding a serine/threonine kinase has been shown to be required for the initiation of sporulation (Munoz-Dorado et al., 1991; 1993). In fact, as many as 26 putative serine/threonine kinases, now identified in *M. xanthus*, may control a variety of environmental and developmental responses.

Besides nutrient limitation, a high cell density (ca. 5×10^8 cells ml^{-1}) is required for initiation of sporulation (reviewed by Shimkets, 1990). The cell density requirement is related to the observation that extracellular factors are required for all of the stages of development (Hagen et al., 1978; Kroos and Kaiser, 1987; Kuspa et al., 1986). The requirement for extracellular factors became apparent when four classes of conditional mutants were found. A mutant from one class was able to complement any of the other classes of

mutants in co-culture, and wild type cells could complement mutants from each class (Hagen et al., 1978). The complementation was shown to be due to the exchange of extracellular factors and not to genetic recombination (LaRossa et al., 1983). The genetic loci for the complementation groups have been designated *asg, bsg, csg* and *dsg* for A, B, C and D signalling. The gene *bsgA* has been found to affect the expression of genes that are normally induced at the onset of, or shortly after, the initiation of differentiation (Figure 1.4; Gill and Cull, 1986; Kroos and Kaiser, 1987). The sequence of the protein encoded by the *bsgA* gene is similar to the sequence of ATP-dependent proteases (Gill et al., 1993), such as La from *E. coli* (Amerik et al., 1988). In *E. coli*, the *lon*-encoded protein, La, activates transcription of genes by degrading the LexA repressor protein (reviewed by Goldberg, 1992). A second-site mutation has been isolated that alleviates the effect of the *bsgA* mutation and also causes the premature expression of genes that are normally expressed early in development (Gill et al., 1993), suggesting that BsgA may have a function similar to La. Since BsgA is an intracellular protein, it may control the synthesis of the extracellular factor involved in B-signalling. Because it had previously been suggested that the cell density signal required to initiate sporulation is an adenosine-containing factor (Shimkets and Dworkin, 1979), it is interesting that what appears to be an ATP-dependent protease is required early in differentiation.

The other early-acting extracellular signalling factor, the A factor, is required for the induction of genes that are expressed approximately one hour after

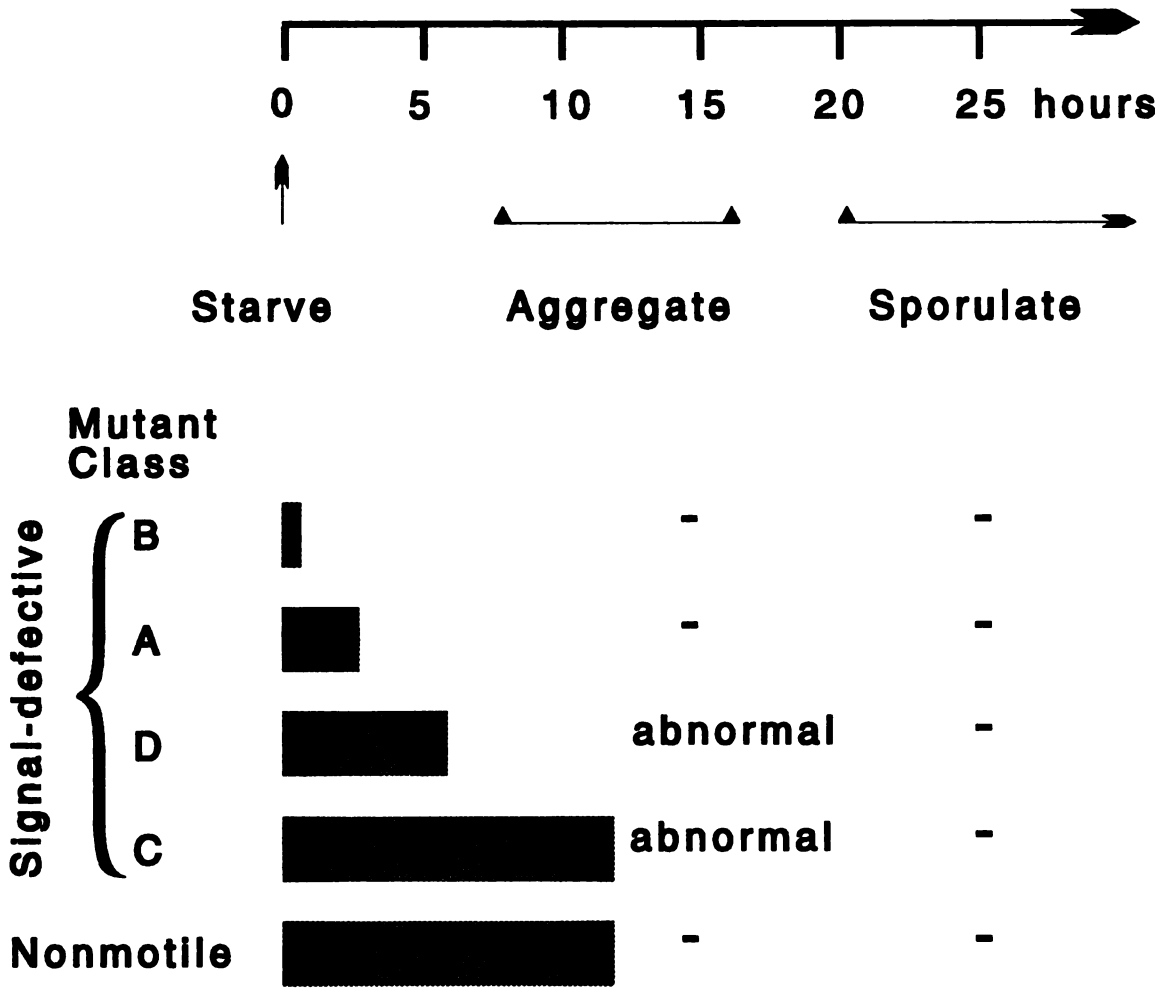


Figure 1.4 Classes of developmental mutants of *Myxococcus xanthus*. The thin lines below the time line indicate the periods during which distinguishable phases of development take place. The wide grey horizontal bars show the extent of development that occurs prior to the expression of the developmental defect. The columns to the right indicate the phenotypic defects with respect to aggregation and sporulation. Adapted from Kaiser and Losick, 1993.

development has begun (Kroos and Kaiser, 1987; Kroos et al., 1986; Kuspa et al., 1986). Half of the A factor activity has been attributed to extracellular proteases that can be functionally replaced by trypsin or other proteases (Plamann et al., 1992). The other half of the A factor activity has been attributed to a mixture of eight amino acids (Tyr, Pro, Phe, Trp, Leu, Ile, Ala and Ser; [Kuspa et al., 1992a]). The amount of A factor released into the medium is proportional to the cell density, implicating A factor as a cell density signal (Kuspa et al., 1992b). Suppressor mutations have been isolated that allow induction of some A-dependent genes in an *asg* background. Because the suppressor mutations are recessive to the wild-type allele, they are thought to be the result of inactivation rather than enhancement of function (Kaplan et al., 1991). Therefore, the inactivated suppressor genes may encode negative regulators of the A-dependent genes examined. However, the suppressor mutations did not restore fruiting body formation or sporulation in the *asg* mutants, suggesting that not all A-dependent genes are regulated in a similar manner (Kaplan et al., 1991).

Anabaena

The initiation of heterocyst differentiation in *Anabaena* is also poorly understood. There are conflicting reports about whether ppGpp accumulates in filaments of *Anabaena* that are deprived of fixed nitrogen (Adams et al. 1977; Rogerson et al., 1978; Akinyanju and Smith, 1979). However, the level of cAMP, another molecule known to stimulate signal transduction pathways,

does increase following nitrogen stepdown (Hood et al., 1979) and other environmental perturbations (Ohmori, 1989). Furthermore, the addition of exogenous cAMP can induce formation of multiple contiguous heterocysts (Smith and Ownby, 1981). Because the activity of adenylate cyclase, which produces cAMP, is regulated by intracellular calcium ion concentrations (Bianchini et al., 1990), calcium ion concentrations may play a role in regulating the initiation of heterocyst differentiation.

The NtcA protein, found in a unicellular cyanobacterium, is similar in sequence to cAMP-binding proteins and is required for the regulation of genes that are involved in nitrogen metabolism (Vega-Palas et al., 1990, 1992). NtcA apparently binds to specific DNA sequences in the promoters of regulated genes (Flores et al., 1993). A DNA-binding protein, BifA, found in *Anabaena*, is also similar to cAMP-binding bacterial regulatory proteins and is 77% identical to NtcA (Frías et al., 1993; Wei et al., 1993). The binding sites that have been characterized for BifA are near genes that are expressed during vegetative growth or late in heterocyst development, and inactivation of *bifA* prevents growth on nitrate as well as on N_2 (Golden et al., personal communication).

Transcriptional reporters within the nitrate reductase operon of *Anabaena* show an induction of transcription within 20 minutes following removal of nitrate from the medium (Wolk et al., 1991; Y. Cai, personal communication). The rapid induction of transcription suggests that sensory responses to the removal of nitrate from the medium may be mediated by signal transduction

pathways. Rapid changes in patterns of protein phosphorylation following nitrogen deprivation (Mann et al., 1991) are also consistent with the supposition that signal transduction pathways are activated by removal of fixed nitrogen from the medium.

Characterization of the *patA* gene of *Anabaena* provided evidence that a signal transduction pathway also affects the positioning of heterocysts. Mutations within *patA* cause heterocysts to develop almost exclusively at terminal cells in the filament. The *patA* gene encodes a protein that resembles CheY (Liang et al., 1992), a phosphate-accepting response regulator protein that controls chemotaxis in *E. coli*. Although CheY does not bind to DNA but instead activates the flagellar motor apparatus (Matsumura et al., 1984), it has been proposed that the PatA protein is a transcriptional regulator (Liang et al., 1992).

The clearest example of early developmental control in *Anabaena* comes from studies on the *hetR* gene. Inactivation of *hetR* blocks evident heterocyst differentiation, whereas multiple copies of *hetR* induce heterocyst formation in nitrogen-containing media and multiple contiguous heterocysts in N_2-grown filaments (Black et al., 1993; Buikema and Haselkorn, 1991a, 1991b). Although the behavior of *hetR* is analogous to that described for *whiG* in the *Streptomyces* (Chater et al., 1989), HetR bears no significant similarity to other characterized proteins and contains no previously identified DNA-binding motifs. A point mutation in *hetR* that converts a serine codon to an asparagine codon causes a loss of HetR function (Buikema and Haselkorn, 1991a). Conceivably,

the serine residue could be a phosphate acceptor. The recent identification of a tyrosine/serine phosphatase in another filamentous, heterocyst-forming cyanobacterium, *Nostoc commune* (Potts et al., 1993), enhances the possibility that HetR is phosphorylated at a serine residue. Data presented in this dissertation (and in Black et al., 1993) show that *hetR* is induced within 2 hours following the removal of nitrate from the medium, and that an intact copy of *hetR* is required for the induction. *hetR* is the earliest induced gene known to be involved in heterocyst differentiation, and also regulates its own expression. Following 3.5 hours of nitrogen deprivation, only small groups of cells show enhanced levels of *hetR* expression. Therefore, the positions within the filaments where *hetR* is induced may be preconstrained or, alternatively, may be the result of regulation that acts during the induction of *hetR*.

The temporal and spatial regulation of *hetR* may be directly related to the mechanism(s) of pattern formation because: i) the transcription of *hetR* is induced within two hours following the removal of nitrate from the medium, long before proheterocysts can be distinguished by microscopy, but at about the same time at which the development of proheterocysts can no longer be suppressed by the addition of ammonia and certain inhibitors (Bradley and Carr, 1977); ii) *hetR* appears to become fully induced only within cells that are to differentiate into heterocysts; and iii) *hetR* is required in order for heterocysts to form. Hence, the induction of transcription of *hetR* may mark the initiation of differentiation. A possible role for *hetR* in pattern formation will be discussed further in the following section of the introduction and in Chapter 2.

The production of, and requirement for, extracellular factors during the initiation of development of *B. subtilis*, *Streptomyces* and *M. xanthus* implies that a selective advantage is gained by the intrapopulation coordination of development. An extracellular factor has also been shown to stimulate akinete formation in the filamentous cyanobacterium, *Cylindrospermum licheniforme* (Fisher and Wolk, 1976), whereas no such factor has been found to affect heterocyst differentiation. In contrast to the cell density requirement for development displayed by *B. subtilis* and *M. xanthus*, the only cell density effect on heterocyst differentiation is a decrease in heterocyst frequency in dense cultures where light limitation causes a slower rate of growth. However, filaments of *Anabaena* require a certain minimum number of vegetative cells before heterocysts can develop (Wilcox et al., 1973b; Wolk, 1967). Moreover, mutants that grow in nitrogen-containing media as short filaments, and other mutants that fragment following nitrogen stepdown, fail to form heterocysts (Buikema and Haselkorn, 1991; Ernst et al., 1992). In some cases, these mutants show no induction of *hepA* (my unpublished observations), a gene that is normally induced within differentiating cells ca. 7 hours after the initiation of nitrogen deprivation (Holland and Wolk, 1990; Wolk et al., 1993). It is not known whether the requirement for a minimum filament length occurs prior to, or following, the initiation of differentiation.

It is possible that there may be a relationship between filament length and the expression of *hetR*. Since a very short filament does not differentiate, if *hetR* is not induced in very short filaments, then a requirement for a minimum

filament length for differentiation may be expressed prior to the induction of *hetR*, possibly prior to the initiation of differentiation. On the other hand, if the timing and level of induction of *hetR* are the same in short filaments as they are in long filaments, then i) a requirement for a minimum filament length for differentiation is expressed following the induction of *hetR*, or may be expressed prior to the induction of *hetR* but does not affect the expression of *hetR*; and ii) the activation of *hetR*, alone, is not sufficient to induce heterocyst formation. It is also possible that within short filaments, *hetR* would be initially induced, but is then either re-repressed, or never achieves the level of induction that is obtained in long filaments. In this case, it is possible that *hetR* has a direct role in preventing differentiation in short filaments.

If *hetR* is not induced in short filaments, the same mechanism(s) that spatially restricts the expression of *hetR*, perhaps the production of an inhibitor of differentiation (see below), may also prevent differentiation in short filaments. Therefore, unlike *M. xanthus*, *S. griseus*, and *B. subtilis*, which produce *extracellular* cell density signals that *induce* differentiation (e.g., A signal, A-factor, and possibly peptides, respectively), *Anabaena* may produce an *intercellular* signal that *inhibits* differentiation. Chapter 3 presents evidence that *Anabaena* may synthesize a metabolite that can suppress heterocyst differentiation and may regulate heterocyst spacing. Furthermore, a cell that has visibly begun to differentiate, forming a proheterocyst, may regress to a vegetative state if a cell that is very close to the proheterocyst is destroyed (Wilcox et al., 1973b). The regression of the proheterocyst may be a

consequence of wounding of the filament (Wolk and Quine, 1975) or, alternatively, sensing of filament length may control differentiation beyond the time of initiation of differentiation, a premise that is consistent with the notion that sensing itself occurs after initiation. The reversibility of the early stages of heterocyst differentiation may also be important for controlling the positions at which heterocysts differentiate, as will be discussed in the following section, and is in contrast to the irreversibility of sporulation in *B. subtilis* once sporulation is initiated by the activation of Spo0A.

Another recently characterized mutation, P6, also blocks the evident differentiation of heterocysts (Ernst et al., 1992). The presence of the wild type gene, *hetP*, on a replicating plasmid causes multiple clustered heterocysts to form when filaments are grown on N_2 (F. Fernández-Piñas, personal communication). The function of the protein encoded by *hetP* is unknown. Whereas HetR has been found to be required for both heterocyst and akinete differentiation in *Nostoc ellipsosporum*, HetP is required only for heterocyst differentiation (Leganés et al., personal communication). Therefore, if HetR and HetP act within the same pathway of developmental regulation, HetP must act after HetR, and must be within a branch of the pathway that is specific to the regulation of heterocyst differentiation, whereas HetR would act prior to a branchpoint that separates the regulation of development of heterocysts and of akinetes.

PROCESSES LEADING TO LOCALIZED GENE EXPRESSION DURING DEVELOPMENT

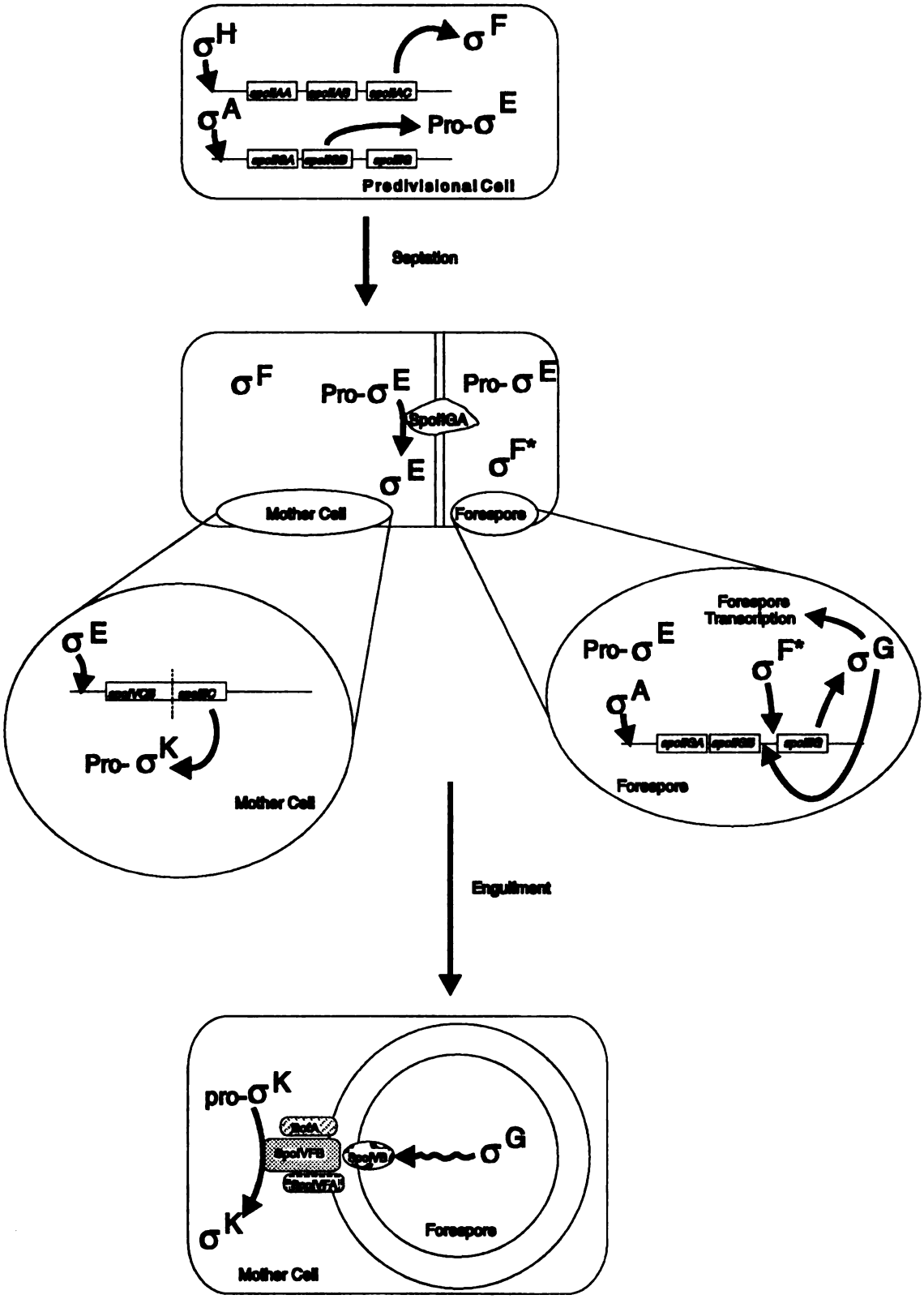
B. subtilis

The ordered expression of genes required for each stage of development in *B. subtilis* is controlled by the activities of a series of sigma factors (reviewed by Stragier and Losick, 1990). Sigma factors are involved in the recognition of specific promoter sequences in DNA and associate with the core RNA polymerase to initiate transcription (McClure, 1985). Sigma factors usually contain both a conserved region that interacts with RNA polymerase and a unique region within the DNA-binding domain. Because of their conserved region, it has been possible to identify many sigma factors by hybridization with heterologous sigma-factor DNA probes. The ordered appearance of activity of the various sigma factors in *B. subtilis* is controlled transcriptionally and by other regulatory mechanisms, including genomic rearrangement and post-translational modification. The activation of each sigma factor relies, at least in part, on the activity of the preceding sigma factor. As mentioned in the previous section,"Processes involved in the initiation of differentiation. *B. subtilis*", the increased activity of the initiating sigma factor, σ^H, involves a stabilization of the corresponding mRNA and of the protein itself (Healy et al., 1991); the resulting increase in the level of σ^H leads to an induction of genes whose promoters are recognized by σ^H. σ^H directs the enhanced transcription of the *ftsAZ* operon (Beal et al., 1988), which is required for septum formation

(Beal and Lutkenhaus, 1991), and of the *spoIIA* operon (Figure 1.5), which contains the *spoIIAC* gene that encodes σ^F (Figure 1.5; Wu et al., 1989). σ^F is required for the completion of septation. Recent results suggest that active σ^F (shown as σ^{F*} in Figure 1.5) is restricted to the forespore even though the *spoIIA* operon is transcribed in the predivisional cell (Gholamhoseinian and Piggot, 1989; Losick and Stragier, 1992) and the σ^F protein is also present in the mother cell. The regulation of σ^F is thought to be carried out by the promoter-proximal products of the *spoIIA* operon, SpoIIAA and SpoIIAB (Partridge et al., 1991; Schmidt et al., 1990). Genetic experiments indicate that SpoIIAB inhibits σ^F activity and that SpoIIAA relieves the inhibition in a regulatable manner. Precisely how σ^F is regulated is unknown.

Transcription of the *spoIIG* operon, in which *spoIIGB* encodes σ^E (Figure 1.5; Stragier et al., 1984; Trempy et al., 1985), is initiated by the vegetative sigma factor, σ^A, and also occurs in the predivisional cell (Gholamhoseinian and Piggot, 1989). The σ^E protein is initially found as an inactive precursor called Pro-σ^E (LaBell et al., 1987). Processing of Pro-σ^E appears to require the product of the first gene in the *spoIIG* operon, *spoIIGA*, that encodes a protease (Jonas et al., 1988; Stragier et al., 1988). The activity of SpoIIGA, and consequently σ^E, has been proposed to require the completion of septation, and activation of σ^E is restricted to the mother cell compartment (Stragier et al., 1988; Trempy et al., 1985). Such a regulatory network makes the temporal and spatial regulation of σ^F- and σ^E-dependent genes reliant upon the establishment of septation.

Figure 1.5 Diagram showing the sequential expression and activation of the various sigmas factors, and their transcriptional dependencies, required for sporulation in *Bacillus subtilis*. The arrows indicate either the action or the synthesis of sigma factors. Active σ^F is denoted as σ^{F*}. Adapted from Stragier and Losick (1990) and Kaiser and Losick (1993).


σH
σF
spoIIA
spoIIAB
spoIIAC
σA
Pro-σE
spoIIGA
spoIIGB
spoIIR
Predivisional Cell
Septation
σF
Pro-σE
Pro-σE
SpoIIGA
σE
σF*
Mother Cell
Forespore
σE
spoIVCB
spoIID
Pro-σK
Mother Cell
Forespore
Transcription
Pro-σE
σF*
σG
σA
spoIIGA
spoIIGB
spoIIR
Forespore
Engulfment
pro-σK
SpoIVA
SpoIVFB
SpoIVB
σG
BofA
σK
Forespore
Mother Cell

The expression of *spoIIID* is directed by σ^E; for that reason, SpoIIID activity is limited to the mother cell compartment (Kroos et al., 1989; Kunkel et al., 1989; Stevens and Errington, 1990). SpoIIID is a DNA-binding protein that regulates the expression of many late genes that are active in the mother cell, including the mother cell specific sigma factor, σ^K. σ^K is encoded by *spoIVCB* and *spoIIIC*, which are separated by a 42-kb DNA element called 'skin'. The 'skin' element is excised exclusively from the mother cell chromosome and forms a circular, extrachromosomal element (Kunkel et al., 1990; Stragier et al., 1989). The excision is carried out by a site-specific recombinase encoded within 'skin' (Kunkel et al., 1990; Sato et al., 1990) and requires SpoIIID. However, the genomic rearrangement appears to be a redundant regulatory scheme: strains that lack the 'skin' insertion show proper regulation of σ^K expression (Kunkel et al., 1990). SpoIIID is also required for the σ^E-directed transcription of the spliced *spoIVCB/spoIIIC* gene (Kunkel et al., 1989). Like σ^E, σ^K is produced as an inactive precursor, Pro-σ^K, which is activated by processing (Kroos et al., 1989; Lu et al., 1990). Processing of Pro-σ^K requires the activity of SpoIVFB, which is negatively affected by BofA and SpoIVFA; all of these proteins are present within the mother cell or outer forespore membrane (Figure 1.5; Cutting et al., 1990, 1991b; Ricca et al., 1992). The activation of σ^K is stimulated by the protein encoded by the *spoIVB* gene, which is expressed only within the forespore. Transcription of *spoIVB* is limited to the forespore because its transcription requires the forespore-specific sigma factor, σ^G (Cutting et al., 1991a). SpoIVB is made in the forespore and then acts,

directly or indirectly, across the forespore membrane to activate the product of the *spoIVFB* gene. The activated SpoIVFB product then, directly or indirectly, controls the processing of Pro-σ^K to an active form (Figure 1.5; Cutting et al., 1990, 1991a; Lu et al., 1990). Once activated, σ^K can direct the initiation of its own transcription, thereby amplifying its expression within the mother cell. σ^K also directs the transcription of *gerE*, which encodes a DNA-binding protein that acts in conjunction with σ^K to activate transcription of late mother cell-specific genes (Kroos et al., 1989; Zheng and Losick, 1990).

The mechanism that leads to the initiation of σ^G transcription in the forespore is still not clear, but once σ^G is activated it is capable of directing its own transcription to amplify its presence within the forespore (Karmazyn-Campelli et al., 1989). The promoter for the σ^G gene is recognized by active σ^F, so regulation of σ^F activity by the SpoIIAA/SpoIIAB system may control the time at which σ^G is expressed (Partridge et al., 1991; Schmidt et al., 1990; Sun et al., 1991), while the activity of σ^G may also be held in check by the SpoIIAA/SpoIIAB system that also regulates σ^F activity (Figure 1.5; Rather et al., 1990; Schmidt et al., 1990; Stragier, 1991). The mechanisms that regulate the activity of σ^G and σ^K coordinate forespore and mother cell development.

The development of competence in *Bacillus* is not well understood. As mentioned in the previous section, the expression of the surfactin synthetase operon appears to be the sole or major requirement for competence development (Hahn and Dubnau, 1991; Nakano and Zuber, 1991). Surfactin is composed of seven amino acids and a ß-hydroxy fatty acid moiety (Vater,

1989). The constituent amino acids are activated by adenylylation to form acyl adenylate intermediates. Each aminoacyl adenylate is covalently bound to the enzyme complex via a carboxyl thioester linkage to a cofactor, apparently 4'-phosphopantetheine (Kleinkauf et al., 1970; Kleinkauf and von Döhren, 1990; Lipmann, 1980). Each functional domain that adds a single constituent amino acid contains within a highly conserved region a serine residue to which the cofactor is covalently attached (D'Sousa et al., 1993). The attached cofactors act as arms that shuttle the growing peptide chain to the various enzymatic domains that carry out the sequential condensations of the constituent amino acids. The synthesis is similar to syntheses carried out by multifunctional (Type I) fatty acid and polyketide synthases that shuttle growing acyl chains through multiple domains that contain 4'-phosphopantetheine attachment sites and carry out sequential specific chain elongations and modifications (Hopwood and Sherman, 1990).

The surfactin synthetase operon, *srfA*, is composed of four genes. The first three genes in the *srfA* operon encode multifunctional proteins, each of which contains separate, linearly arranged domains; each domain incorporates a single constituent amino acid. The fourth gene encodes a thioesterase-like protein that has been proposed to release the completed lipopeptide product (Marahiel et al., 1993). The *srfAA* gene encodes the protein that initiates surfactin biosynthesis with a glutamate residue, to which the fatty acyl moiety is added (Vater, 1989). The other domains of SrfAA catalyse the incorporation of L-leucine and D-leucine. SrfAB catalyses the addition of valine, asparagine and D-

leucine, and SrfAC completes surfactin synthesis by adding a final L-leucine residue. Converting the cofactor-binding serine residue to alanine in any one of the domains blocks the further synthesis of surfactin. However, none of the serine to alanine conversions blocks competence development (D'Sousa et al., 1993). Thus, despite the requirement for expression of the surfactin synthetase operon, surfactin itself is not required for cells to gain competence. However, other evidence suggests that some activity associated with the valine-activating domain of *srfAB* is required for competence development (van Sinderen et al., 1993). What the activity is and how it induces competence development is still unknown.

Streptomyces

Spatial control of gene expression and pattern formation in the *Streptomyces* has not received a great deal of attention. All of the studies mentioned in this section were performed on *S. coelicolor* which, because it is the species of *Streptomyces* most amenable to genetic manipulation, has been the favored organism for developmental studies. Promoters that are turned on in a temporally and spatially controlled manner were found by cloning random fragments of chromosomal DNA upstream from the luciferase genes, *luxAB* (Schauer et al., 1988; Schauer, 1988; Schauer and Im, 1988). However, these promoter fragments have not been further characterized. The *sapA* gene was identified using reverse genetics on the spore associated proteins (Saps) that are abundantly produced in aerial mycelia and are associated with the spore

coat. *sapA-luxAB* fusions show that the *sapA* gene is expressed only in the aerial mycelia, demonstrating that spatially regulated gene expression does occur during sporulation in *Streptomyces* (Guijarro et al., 1988). An 18-residue peptide, called SapB, has also been identified (Willey et al., 1991). SapB, however, appears to be produced by a non-ribosomal mechanism (Willey et al., 1993), probably using a thiotemplate mechanism similar to that used by surfactin synthetase and other peptide antibiotic synthetases. Extracellular addition of SapB to many *bld* mutants rescued their ability to produce aerial mycelia. In fact, it was found that coculture of pairs of different *bld* mutants, which individually are incapable of producing SapB, rescued SapB production and the formation of aerial mycelia in one member of the pair (Willey et al., 1993). Only one member of the pair was rescued, and the aerial mycelia formed by the rescued cells extended toward the donor-cell colony. In addition, cells that acted as a donor in one pairing could become a responder in a different pairing. The complementation analysis led to the proposal that at least four separate signal molecules are involved in the extracellular complementation and regulation of SapB expression. It has been proposed that SapB acts as a morphogenic component that reduces surface tension, thereby allowing aerial growth (Willey et al., 1991, 1993).

A unique regulatory phenomenon seen in the *Streptomyces* was discovered by the analysis of *bldA* mutants. The gene affected in these mutants was found to encode a tRNA for the rare UUA leucine codon (Lawlor et al., 1987). A majority of the genes found to contain a UUA codon are directly involved in

regulating antibiotic production and sporulation (Chater, 1989; Wright and Bibb, 1992). It was recently shown that the low levels of the UUA-tRNA in vegetative cells are a result of inefficient processing of the 5′ end of the primary transcript and not of transcriptional regulation (Leskiw et al., 1993). However, other recent work suggests that the *bldA* gene does not play a central role in regulating antibiotic production (Gramajo et al., 1993), and the fact that the *bldA* mutant phenotype is only expressed in certain media suggests that *bldA* has only a limited role in regulating sporulation.

The *whi* mutations block spore formation and the production of a grey pigment encoded by the *whiE* locus (Davis and Chater, 1990). The *whi* designation was given to describe what were therefore white colonies. The strong effects associated with the overproduction of the WhiG protein (see above under "Processes involved in the initiation of differentiation. *Streptomyces*") suggest that the localization of sigma-WhiG expression may be the primary contribution of earlier regulatory factors, and is required for spatially regulated expression of sporulation-associated genes (Chater et al., 1989). The role of sigma-WhiG in controlling differentiation may be different than that expressed by the cascade of sigma factors associated with sporulation in *B. subtilis*. Early expression of late sigma factors in *B. subtilis* does not usually cause sporulation to occur in vegetative cells, nor does it lead to defective spatial regulation. Other sigma factors have been identified in the *Streptomyces* both biochemically and by heterologous hybridization (Buttner, 1989; Tanaka et al., 1988; Westpheling et al., 1985), and promoter switching has been found

for a number of genes during development (Buttner et al., 1990). Nonetheless, no developmental role has been found for these other sigma factors.

M. xanthus

The production and exchange of extracellular factors is required for each stage of morphological differentiation of *Myxococcus xanthus* (Hagen et al., 1978). However, the cells must also be motile in order to aggregate, form fruiting bodies, and sporulate (Kim and Kaiser, 1990b,c; Kroos et al., 1988; Sager and Kaiser, 1993). There are two types of motility systems, called A (for adventurous) and S (for social); each has unique genetic loci. Additional loci, denoted *mgl* (for mutual gliding), are required for all motility (Hodgkin and Kaiser, 1977, 1979). Most mutations within the S system adversely affect development, while mutations in the A system were initially shown to have little effect on development. However, strains that carry mutations within two different A system loci -- loci that are induced by dissimilar signals -- fail to sporulate (Kalos and Zissler, 1990). Therefore, the A motility system appears to be essential for differentiation, and may have multiple pathways for stimulation.

The best characterized signal in *M. xanthus* is the C-signal, which shows the clearest relationship between cell motility and development. The C factor is a 17-kD protein, encoded by the *csgA* gene, that is present on the cell surface (Hagen and Shimkets, 1990; Kim and Kaiser, 1990a; Shimkets and Rafiee, 1990). Immunogold labeling showed that between 1000 and 2000 molecules

of C factor are present on the surface of a single cell (Shimkets and Rafiee, 1990). *csgA* mutants fail to form fruiting bodies or sporulate, a phenotype that is similar to the phenotype of non-motile (*mgl*) mutants (Figure 1.4; Kroos et al., 1988). Furthermore, *csgA* mutants and non-motile strains both fail to induce developmental genes that are normally expressed after six hours into development. These data suggested that motility is required for C factor to function properly. Subsequent experiments showed that motility is required for both donation and response to C factor; that is, non-motile cells (which produce C factor) cannot induce sporulation in *csgA* mutants (which are motile), and wild-type cells cannot induce sporulation in non-motile cells. Surprisingly, purified C factor is able to induce sporulation in non-motile cells even though non-motile cells already produce C factor (Kim and Kaiser, 1990b). Non-motile mutants that are artificially aligned also regain the ability to sporulate (Kim and Kaiser, 1990c), which shows that motility is normally required to achieve a crucial spatial alignment between cells that allows for C factor signalling. It is not known whether end to end or side by side is the crucial cellular arrangement.

C factor has been shown to influence two distinct morphological and transcriptional responses in *csgA* cells in a concentration-dependent manner (Kim and Kaiser, 1991). Addition of low levels of purified C factor to *csgA* mutants restores transcription of early C-dependent genes (those induced at six hours of development) and aggregation of cells. Adding higher levels of C factor restores the induction of late C-dependent genes (those induced at ca. 12 hours

of development) and complete sporulation. Additionally, it was found that *csgA* itself was induced by low levels of C factor. C factor is therefore required for aggregation and then, through auto-induction, increases its cellular concentration to induce sporulation genes. The relationship between cell motility, alignment, and C-dependent gene expression suggests that induction of sporulation genes requires tight packing of cells (i.e., a fruiting body). A dense population of germinating spores may be ecologically advantageous.

Anabaena

In *Anabaena* sp. strain PCC 7120 the transcription of genes that are involved in various aspects of growth and metabolism has been shown to be restricted to specific cells. For instance, the transcription of genes encoding ribulose bisphosphate carboxylase, required for CO_2 fixation, is primarily limited to vegetative cells while transcription of the *nif* genes, encoding nitrogenase, and *hepA*, required for heterocyst envelope formation (Holland and Wolk, 1990), is limited to the heterocysts (Elhai and Wolk, 1990; Wolk et al., 1993). In many species of *Anabaena* and *Nostoc*, the induction of nitrogenase within heterocysts has been found to require the excision of intervening DNA from the chromosome of developing heterocysts (Golden et al., 1985, 1988; Matveyev et al., 1993). However, loss of the intervening DNA from vegetative cells does not affect heterocyst differentiation, just as loss of the 'skin' element from vegetative cells of *B. subtilis* does not affect sporulation. Therefore, genomic rearrangements appear not to be required as regulatory mechanisms except,

perhaps, for preventing expression in vegetative cells. In *Anabaena* sp. strain PCC 7120, unlike in most other diazotrophs, the induction of the *nif* genes has been found to be under developmental control and not simply to be inducible in an environment devoid of fixed nitrogen with low oxygen tension (Elhai and Wolk, 1990). The developmental program is required in order to produce nitrogen-fixing cells.

The questions raised by heterocyst differentiation are i) what form of regulation causes genes to be differentially transcribed in the different cells and ii) what aspects of transcriptional regulation are involved in the patterned formation of heterocysts? Two models have been proposed that could account for the non-random distribution of heterocysts (Wolk, 1989). One model postulates that the first cells to sense nitrogen deprivation start to differentiate into heterocysts. Differentiating cells produce an inhibitor that prevents nearby cells from differentiating and can even reverse early stages of differentiation of neighboring cells. Because localization of differentiation precedes nitrogen fixation, the inhibitor cannot be a direct product of nitrogen fixation (Neilson et al., 1971). According to a second model, cells that sense nitrogen deprivation induce uptake systems by which they scavenge nitrogenous compounds from neighboring cells. The last cells to sense nitrogen deprivation would be subjected to extreme deprivation that would induce heterocyst differentiation. The first model provided an interpretation for early experiments that showed that differentiating cells are able to prevent or reverse differentiation in neighboring cells (Wilcox et al., 1973a; Wolk, 1967). It was suggested that

'zones of inhibition' (Mitchison and Wilcox, 1976) are generated by the most advanced differentiated cells. The second model is primarily based on the fact that other procaryotes induce uptake systems upon nutrient deprivation, and that high-affinity amino acid transport systems in cyanobacteria have been described (Flores and Muro-Pastor, 1988; Herrero and Flores, 1990). A distinguishing aspect of the two models is whether the first or last cell to sense nitrogen deprivation will be the first to differentiate. The second model would appear to be supported by the fact that *hetR*, which clearly plays a central role in development, is not induced until 2 hours following nitrogen deprivation, whereas cells are capable of sensing and responding to nitrogen deprivation within 20 minutes (e.g., by activation of the nitrate reductase operon; Wolk et al., 1991; Y. Cai, personal communication). However, the cellular localization of *hetR* expression, using *luxAB* fusions, shows that small foci of cells emit enhanced levels of light 3.5 hours after the removal of nitrate, and that by ca. six hours the light emission is mostly localized to single cells. Following 24 hours of nitrogen deprivation, light is emitted most intensely from within cells that lie approximately midway between the positions of two existing heterocysts, i.e., at the position at which a heterocyst would be expected to form. These data indicate that mechanisms that act following the induction of *hetR* can limit the positions at which the induced expression of *hetR* is maintained. The production of a diffusible inhibitor within the most advanced differentiated cells could limit the positions of *hetR* expression. However, the induction of uptake systems could also control *hetR* expression, with the last

cell to induce an uptake response continuing to transcribe *hetR*. How *hetR* is regulated may be of central importance in understanding how heterocyst differentiation is initiated and perhaps how pattern formation occurs.

Characterization of the N10 mutant and of the genes that surround the site of insertion of the transposon in N10, presented in chapter 3 of this dissertation, leads me to suggest that a secondary metabolite, perhaps a polyketide, may be involved in regulating the spacing of heterocyst differentiation. As discussed in chapter 3, the gene that is interrupted in N10, *hetN*, and the genes that surround *hetN* may be required for the production of a diffusible compound that inhibits heterocyst differentiation. Because the presence of *hetN* on a replicating plasmid suffices to suppress heterocyst differentiation, *hetN* appears to play a central role in the process of suppression. A gene, called *hetI*, that lies adjacent to *hetN* may regulate the production of the metabolite because the presence of *hetI* on the same plasmid as *hetN* relieves the inhibition of heterocyst differentiation. *hetI* also appears to be essential for vegetative growth because, thus far, I have been unable to inactivate *hetI*. The information obtained from my work on the N10 locus lends support to the inhibitor model of pattern formation.

On the basis of what was known, and what has been shown in this dissertation, about the genes involved in the patterned differentiation of heterocysts, I propose to specify the functions within a mathematical model that has been developed to interpret one-dimensional pattern formation (Meinhardt, 1982). The basis of the model is that two components are essential

for the de novo production of pattern. The first component is an inhibitor that can rapidly diffuse or be transported along the filament. The second component is an activator that cannot readily diffuse from the site of activation. The activator must induce its own expression, thereby ensuring that activation by the activator exceeds inhibition by the inhibitor. In order to produce a stable gradient of inhibitor that can prevent the differentiation of neighboring cells, production of the inhibitor should also be induced within differentiating cells. During vegetative growth, the system is maintained in a homeostasis that prevents the differentiation of heterocysts. Following nitrogen stepdown, the system may be perturbed by fluctuations that either decrease the amount of the inhibitor or increase the amount of the activator. Even if the sites of initial perturbations are relatively random, the gradient of the inhibitor produced by the differentiating cells would lead to a patterned spacing of the final sites of differentiation (Meinhardt, 1982).

The molecule proposed to be produced by *hetN* and the surrounding genes could fulfill the role of the inhibitor. My preliminary experiments have suggested that *hetN* is induced within 2 to 3 hours following nitrogen stepdown, which (if *hetN* is involved in production of an inhibitor) is consistent with the possibility that enhanced levels of inhibitor are produced following nitrogen stepdown. The auto-activation of *hetR* and the dependence of differentiation upon *hetR*, as well as the ability of *hetR* to induce differentiation when present in multiple copies, shows that HetR can fit the role of an auto-inducible activator.

What perturbation of the system would lead to the initiation of differentiation? A sensory transduction pathway that responds to environmental or internal cues (or perhaps an integration of signals from both environmental and internal stimuli) could act to disrupt the balance of inhibitor and activator. Perhaps the sensory transduction pathway acts through PatA, an apparent response regulator protein. Although PatA has been proposed to be a transcription factor (Liang et al., 1992), it is possible that PatA is required to activate the protein(s) that can inactivate or act counter to the inhibitory molecule produced by differentiating cells. In fact, in a *patA* mutation that was constructed in our laboratory, many filaments failed to form heterocysts (Cai and Wolk, unpublished observations). If *patA* strains cannot inactivate or counteract the inhibitor, they may be self-inhibited. If the putative inhibitor were able to diffuse slowly out of cells, then the additional surface area of the terminal cells might permit enough diffusion of the inhibitor to allow differentiation sometimes to proceed within terminal cells. Therefore, the formation of terminal heterocysts, which is the phenotype reported for *patA* mutants (Liang et al., 1992), may result from physical differences in terminal cells rather than from a specific regulatory role for PatA. Hence, the sensory response to nitrogen stepdown may act through PatA to decrease the levels (or perhaps the activity) of the inhibitor, thereby allowing initiation of differentiation to take place. Because the perturbation could occur randomly within filaments, the reversal of heterocyst differentiation, perhaps due to the production of an inhibitor by nearby differentiating cells, may be required for

the semiregular spacing of the final sites of differentiation.

If the production and movement of an inhibitory molecule plays a direct role in pattern formation, then an important question remains: how does the inhibitor move between cells? Because no extracellular control over the spacing of heterocysts is known, the presumptive inhibitory molecule may be polar and therefore remain within the filaments rather than being secreted into the medium. To move from cell to cell, the inhibitor would have to be transported or there would have to be channels for intercellular diffusion. Electron microscopy of thin sectioned (Lang and Fay, 1971; Wilden and Mercer, 1963) and freeze fractured material (Giddings and Staehlin, 1978, 1981) suggests that microplasmodesmata may provide aqueous channels between cells. However, proof of the existence of such channels has not been obtained. In other filamentous cyanobacteria, in which presumed microplasmodesmata have also been visualized, there is evidence that low-resistance electrical coupling exists between cells (Häder, 1987; Severina et al., 1988). Although microplasmodesmata would provide low resistance coupling, the exact nature of the electrical continuity is not known. It is also possible that specific transport complexes could facilitate movement of the inhibitor. It has been shown that, in *E. coli*, flagellar proteins are specifically targeted to cell poles (Maddock and Shapiro, 1993); perhaps a distinct feature of the membrane that is made during septation can direct proteins to the poles of the cells. If a similar feature exists in *Anabaena*, then specific transport systems could be localized at the cell poles to provide intercellular transport. It is also possible that

receptor proteins can be specifically positioned at the cell poles. This may be the case in *M. xanthus*, where cells may require an end-to-end alignment in order to transmit and receive the C factor signal. Because *Anabaena* cells are already aligned end to end within the filament, a signal peptide or some other factor produced and exported through the cell pole, or incorporated into the outer membrane, could transmit signals between cells.

Whether promoter switching or use of alternative, development-specific sigma factors is required for heterocyst differentiation is still unclear. There are examples of developmentally regulated genes, such as *hetR* (Buikema and Haselkorn, 1992), *glnA* (Tumer et al., 1983) and *sigA* (the major vegetative sigma factor; Brahamsha and Haselkorn, 1991), that use alternative transcriptional start sites. Two genes encoding alternative sigma factors, *sigB* and *sigC*, have been identified by heterologous hybridization. The levels of mRNA for both genes increase following the inception of nitrogen deprivation; however, inactivation of either or both of the genes does not affect heterocyst differentiation (Brahamsha and Haselkorn, 1992). Therefore, temporal and spatial control of the various stages of differentiation by a succession of sigma factors, which appears to be the primary means of developmental regulation in *B. subtilis*, may not be of central importance for the differentiation of heterocysts.

Chapter 2

SPATIAL EXPRESSION AND AUTOREGULATION OF *hetR*, A GENE INVOLVED IN THE CONTROL OF HETEROCYST DEVELOPMENT IN *Anabaena*[1]

SUMMARY

The spatially patterned differentiation of heterocysts in the filamentous cyanobacterium *Anabaena* requires a functional *hetR* gene. Transcriptional fusions to *luxAB* show that *hetR* is transcribed at a low level throughout the filament when *Anabaena* is grown with combined nitrogen, and that induction of the gene begins within 2 hours following nitrogen deprivation. By 3.5 hours, induction is localized to spaced foci. By 6 hours, there is an overall induction of at least three fold in whole cultures, reflecting at least a 20-fold increase within spatially separated cells. The induction requires the presence of a functional *hetR* locus, indicating that *hetR* is autoregulatory. Full induction of a heterocyst structural gene, *hepA*, also requires a functional *hetR* locus.

[1] The essential content of this chapter has been published in Molecular Microbiology (Black, Cai, and Wolk, 1993).

INTRODUCTION

Study of *nifH-luxAB* fusions and of mutants that fail to differentiate have shown that in *Anabaena* sp. strain PCC 7120, nitrogenase is expressed only in heterocysts and that its transcriptional induction is developmentally regulated (Elhai and Wolk, 1990; Ernst et al., 1992). The induction of *nifH* at about 16 hours after the removal of nitrate (Elhai and Wolk, 1990) is a late event in heterocyst development. *hepA* (see Ernst et al., 1992), a gene that is required for normal elaboration of the heterocyst envelope (Wolk et al., 1988), is induced within 7 hours of nitrogen deprivation (Holland and Wolk, 1990), before morphological changes in the vegetative cells can be visualized by bright-field light microscopy. Luminescence from *hepA-lux* fusions is confined to semi-regularly spaced cells that (once morphological differentiation is discernible) are seen to be differentiating into heterocysts (Wolk et al., 1993).

Buikema and Haselkorn (1991b) identified a gene, *hetR*, that is essential for heterocyst differentiation. Its presence on a replicating plasmid leads to the formation of heterocysts in the presence of fixed nitrogen and to the formation of multiple, contiguous heterocysts following nitrogen deprivation. The level of *hetR* mRNA increases within 6 hours of nitrogen stepdown (idem). Our laboratory has reported (Ernst et al., 1992) that many but not all of the transposon-generated mutations that arrest heterocyst development prior to morphological differentiation lie within *hetR.* Although it is apparent that *hetR* plays a regulatory role in heterocyst differentiation, HetR has shown no sequence similarities to regulatory (or other) proteins. Because of the

importance of *hetR* in differentiation, I have examined its temporal and spatial expression in differentiating filaments and its effect on the spatially and developmentally regulated gene, *hepA*.

MATERIALS AND METHODS

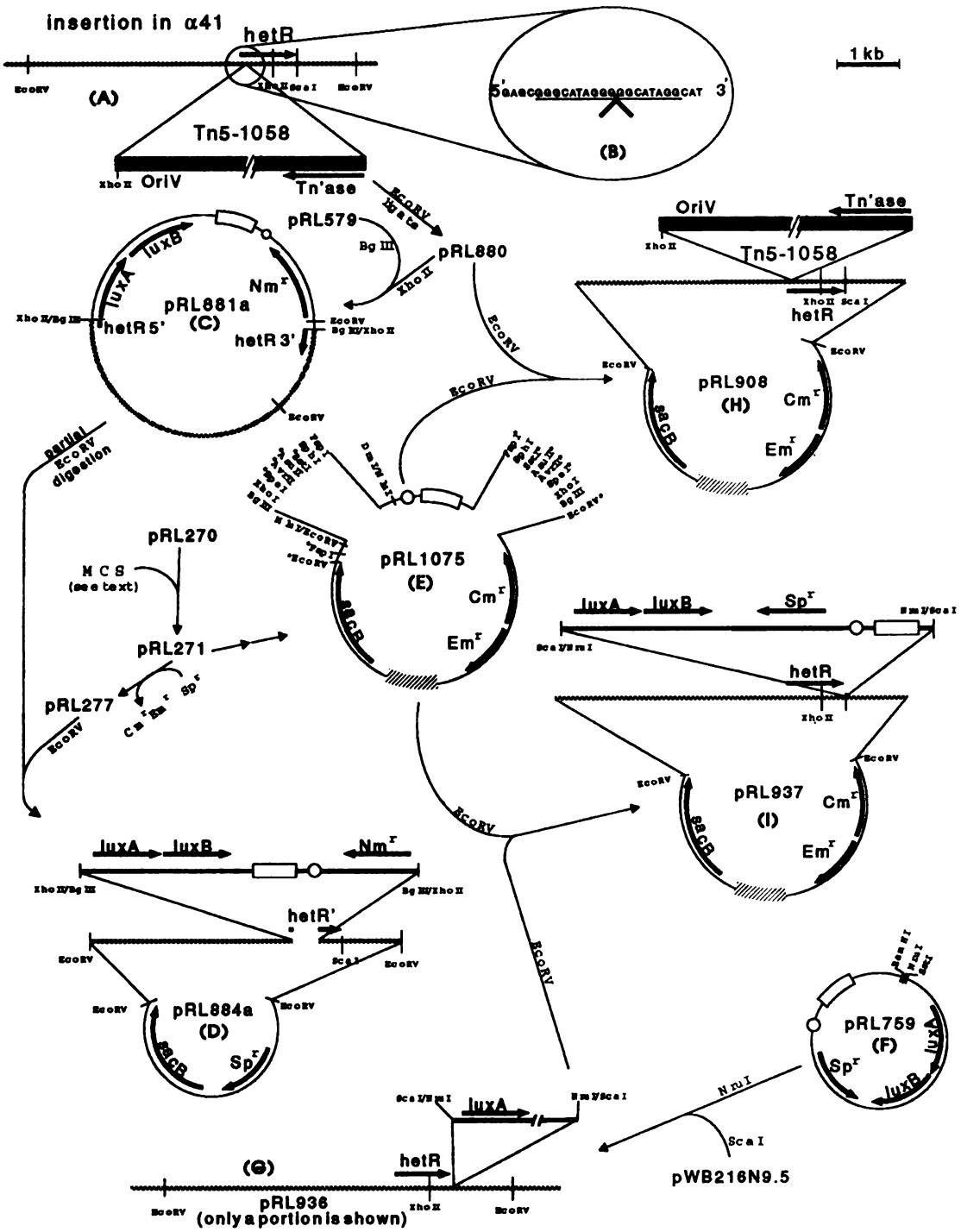
Plasmids and strains. Strains of *Anabaena* were grown at 30° with continuous illumination (ca. 3500 erg cm^{-2} s^{-1}) on a rotary shaker in 125-ml Erlenmeyer flasks containing 50 ml of AA/8 nitrate (Hu et al., 1982). Transposons and plasmids containing *hetR* constructions were introduced by conjugation (Wolk et al., 1984, 1991), and double recombinants were selected as described by Cai and Wolk (1990). Suspension cultures of DR884a and of strains containing transposon insertions were supplemented with 50 μg neomycin sulfate (Nm) ml^{-1}, while suspension cultures of SR884a and of DR908-DR1069 were supplemented with 50 μg Nm ml^{-1} + 5 μg spectinomycin (Sp) ml^{-1}. DR937 and DR1069 were grown in the presence of 5 μg Sp ml^{-1}.

Several plasmids that were designed to facilitate the *sacB*-mediated positive selection for double recombinants (Cai and Wolk, 1990) were used in this study. Plasmid pRL271 was derived from pRL270 (Maldener et al., 1991) by (1) cutting *dam*-methylated plasmid with *Xba*I, treating with Klenow fragment, and religating; and (2) inserting between the *Xho*I and *Sst*I sites of the pIC20R polylinker (Marsh et al., 1984; erroneously given as the pIC20H polylinker by Maldener et al., 1991) a 527-bp *Xho*I-*Sst*I polylinker derived from bp 1943 to 1572 and bp 1443 to 1289 of plasmid pJRD184 (Heusterspreute et al., 1985;

bp 1571 to 1444 were deleted by treatment with T4 DNA polymerase of the *Bgl*I and *Pst*I ends, and subsequent ligation). The polylinker region of pRL271 contains, from Cm^r to *oriV*, unique sites for *Eco*RV, *Xba*I (methylated in *dam*$^+$ strains), *Bgl*I, *Xho*I, *Nae*I, *Bss*HII, *Bst*EII, EcoNI, *Spe*I, *Sph*I, *Xba*I (not methylated in *dam*$^+$ strains), *Avr*II, *Afl*II, *Asu*II, *Sst*I, *Nru*I, *Sph*I, *Pst*I, *Ase*I, *Bgl*I, Eam1105I, and *Bsp*HI. The *Xmn*I and *Sca*I sites (both blunt) in the 3′ portion of the Em^r and Cm^r genes, respectively, permit convenient replacement of antibiotic resistance cassettes to generate derivatives of pRL271. A Sm^{r}Sp^r derivative, pRL277, and a Km^{r}Nm^r derivative, pRL278, were made by ligation of the larger *Xmn*I-*Sca*I fragment of pRL271 with the *aadA*-containing *Dra*I fragment from the Ω interposon (Prentki et al., 1991), and with the *nptII*-containing cassette C.K3 (Elhai and Wolk, 1988a; *Xba*I ends made blunt by filling-in reactions), respectively.

Plasmid pRL1075 (Figure 2.1E), based on pRL271 but bearing an additional *oriT*, was constructed by inserting most of the polylinker region, from *EcoRV* to *Dra*I (between *Eam*1105I and *Bsp*HI), in inverted orientation into the (blunted) *Mlu*I site of pRL271, and then adding the *oriT* region of RK2 from pAT187 (Trieu-Cuot et al., 1987) to the unique *Sal*I site of the resulting plasmid. The *Pst*I ends of the RK2 *oriT* fragment were converted to *Sal*I ends by passage through pRL500 (Elhai and Wolk, 1988a). Plasmid pRL1075 is useful for reconstructing mutations generated by transposons Tn*5*-1058 and Tn*5*-1063 (Wolk et al., 1991). These transposons are conveniently excised from the chromosome of *Anabaena* with *Cla*I, compatible with *Asu*II;

Figure 2.1 Several DNA constructs used in this work. See text for details. (A) The *hetR* region of the genome of mutant *a*41, interrupted by Tn*5*-1058 which generates 9-bp direct repeats (B), was excised with *Eco*RV, and religated, forming pRL880. To introduce *luxAB* as a reporter, the transposon was replaced with pRL579 (Elhai and Wolk, 1990), yielding plasmid pRL881a (C); *sacB* was then added by ligation to a part of pRL277, producing plasmid pRL884a (D). Plasmid pRL271, derived from pRL270 (Maldener et al., 1991) by addition of a multiple cloning site (MCS; see text), served as a precursor of pRL1075 (E) and pRL277. Plasmid pRL759 (F), cassette BLOS2 with its ends joined by an extended polylinker (see Materials and methods), was fused by *Sca*I-*Nru*I junctions with pWB216N9.5 (Buikema and Haselkorn, 1991a) to produce plasmid pRL936 (G), of which only a portion is shown and in which *luxAB* serves as reporter. pRL880 and the *Eco*RV fragment of pRL936 that is shown were inserted separately between *Eco*RV sites of pRL1075 to form, respectively, pRL908 (H) and pRL937 (I). O, *oriT* of pMBI; open rectangle, *oriV* of pMBI; thick hatched arc, *oriT* region of RK2; solid bar, Tn*5*-1058 (Wolk et al., 1991) including *oriV* of p15A; thin cross-hatched line or curve, *Anabaena* DNA; Tn'ase, Tn*5* transposase gene; Cm^r, Em^r, Nm^r, Sp^r, genes conferring resistance to chloramphenicol, erythromycin, neomycin (and kanamycin), and spectinomycin (and streptomycin), respectively; *, sites useful for adding *sacB*, Cm^rEm^r and *oriT* (RK2) to transposon-bearing fragments of the *Anabaena* chromosome (see Materials and methods). The bar at the upper right shows the length of 1 kb of DNA, valid for horizontal (linear) and circular portions of the plasmids illustrated.


insertion in α41
hetR
(A)
Tn5-1058
OriV
Tn'ase
(B)
1 kb
pRL579
pRL880
pRL881a
(C)
luxA
luxB
Nm^r
hetR 5'
hetR 3'
pRL908
(H)
Cm^r
Em^r
pRL1075
(E)
pRL270
MCS
(see text)
pRL271
pRL277
Sp^r
pRL937
(I)
pRL884a
(D)
hetR'
pRL759
(F)
(G)
pRL936
(only a portion is shown)
pWB216N9.5
Partial EcoRV digestion
EcoRV
Xho II
Bg III
Sca I
Nru I

*Spe*I, compatible with *Avr*II and *Spe*I; and *Dra*I, *Eco*RV (see above), *Sca*I and *Sna*BI, compatible with *Fsp*I, *Ecl*136II (a neoisoschizomer of *Sst*I), and *Eco*RV (however, ligation to that site somewhat impairs the activity of *sacB*).

A set of *lux* fusion cassettes, collectively called BLOS, was designed to generate *lux* fusions in recovered *Anabaena* DNA fragments. Cassette BLOS1 was derived from a portion of pRL1050 (Wolk et al., 1993): bp 1 to 2567 are *Sst*I (5′ from *luxAB*) to *Dra*I (destroyed; 3′ from the T7 terminator); bp 2568 to 5391 are *Stu*I (within cassette C.S4, destroyed; a description of C.S4 was given by Bancroft and Wolk, 1989, and amended by Wolk et al., 1993) to *Alw*NI. To this fragment was ligated *Alw*NI, bp 2884, to bp 3230 (*Dra*I, destroyed) from pBR322, and an 8-bp linker *Sma*I (destroyed)-*Bam*HI, completing 5666-bp cassette BLOS1. An *Eco*RV site that had been generated 5′ from C.S4 in pRL1050 by an S1 nuclease digestion and subsequent ligation was eliminated by cutting with *Eco*RV and *Eco*47III, and religating, thus deleting 54 bp and forming BLOS2. The ends of cassette BLOS1 are connected by different polylinkers to form plasmids pRL739, pRL739B, and pRL739S, and from BLOS2 to form pRL759 (Figure 2.1F) and pRL759B. The polylinker in pRL739 and pRL759 is (from the *luxAB*-distal end) *Bam*HI-*Xba*I-*Sal*I-*Pst*I-*Sph*I-*Hin*dIII-*Nru*I-*Sst*I-*Xho*I-*Bgl*II-[*Xba*I]-G-*Kpn*I-*Sst*I (*Bam*HI to [*Xba*I] from pIC20R [Marsh et al., 1984], the rest from pUC [Vieira and Messing, 1982]); in pRL739B and pRL759B is *Bam*HI-*Sma*I-*Kpn*I-*Sst*I; and in pRL739S is an *Sst*I deletion of the polylinker in pRL739. The *Xba*I site in brackets is methylated by *dam*$^+$ strains of *E. coli*. The *Bam*HI site 3′ from *luxAB* in BLOS2 in pRL759

was eliminated by partial digestion with *Bam*HI followed by a filling-in reaction using T4 DNA polymerase, producing plasmid pRL759D and cassette BLOS3.

Plasmid pRL58 consists of the large *Sal*I fragment of pRL139 (Elhai and Wolk, 1988a) to which was ligated the following 4379-bp *Sal*I fragment that can be excised with *Sal*I or *Sma*I, and that lacks *Eco*RV sites: *Vibrio harveyi luxAB* (as reporter) from the *Sal*I site 137 bp 5′ from *luxA* (Cohn et al., 1985) through to the *Pvu*II (destroyed) 195 bp 3′ from *luxB* (Johnston et al., 1986), followed by bases GG, a *Bam*HI site, andthen polylinker *Xba*I-*Eco*RI-*Xba*I from L.HEH2 (Elhai and Wolk, 1988a) with C.S4 (Bancroft and Wolk, 1989; corrected in Wolk et al., 1993; antiparallel to *luxAB*) inserted in the *Eco*RI, site followed by *Sal*I. That *lux*-bearing cassette, excised with *Sma*I, was inserted into the *Nru*I site of *hep*A (Holland and Wolk, 1990; renamed by Ernst et al., 1992) in the 2.1-kb *Eco*RV fragment shown in Figure 5 of Holland and Wolk (1990), and was oriented to have *luxAB* report on transcription from P_{hepA}. The entire resulting 6.5-kb *Eco*RV fragment was then transferred to the unique *Nru*I site of pRL271, yielding plasmid pRL1069.

The genomic *Eco*RV fragment bearing the transposon in *a*41 was cloned as plasmid pRL880 as described (Wolk et al., 1991), and was maintained in *E.coli* HB101. DNA contiguous with the transposon was subjected to automated sequencing (Applied Biosystems Inc., Foster City, CA) outwards from primers (CTCAAGAAGATCATC and CACATGGAATATCAG) corresponding to unique sequences near the left and right ends of transposon Tn*5*-1058, respectively. Plasmids pRL881a and pRL881b were constructed from pRL880 by excising,

with *Xho*II, all of Tn*5*-1058 but the terminal 60 bp at the left end through to bp 607 3′ from the translational start of *hetR* (Figure 2.1A) and by inserting, in its place, the *lux*-fusion vector pRL579 (Elhai and Wolk, 1990) cut with *Bgl*II. Insertion of *lux* fusion cassette BL0S2 (*Nru*I-cut pRL759) into the *Sca*I site 21 bp downstream from the stop codon of *hetR* in pWB216N9.5 (Buikema and Haselkorn, 1991b) produced plasmid pRL936 (Figure 2.1G). To permit selection for double recombinants (Cai and Wolk, 1990) of pRL881a and pRL881b in *Anabaena*, these plasmids were partially digested with *Eco*RV and ligated with the larger, *sacB*-containing *Eco*RV fragment of pRL277 yielding pRL884a (Figure 2.1D) and pRL884b, respectively. For the same reason as well as to permit mobilization, *Eco*RV-cut pRL880 and pRL936 were each ligated with the larger *Eco*RV fragment of pRL1075 (Figure 2.1E) that contains the Cm^{r}Em^r-*oriT*(RK2)-*sacB* cassette, yielding plasmids pRL908 (Figure 2.1H) and pRL937 (Figure 2.1I), respectively. The latter two plasmids were recombined with *hepA*::*luxAB* strain DR1069 and with wild type *Anabaena* sp. strain PCC 7120, respectively, to produce double recombinants DR908-DR1069 and DR937.

Measurement of luminescence. Three-microliter portions of lightly greened liquid cultures were spotted onto filters atop solidified AA medium containing 10 mM nitrate and lacking antibiotics (Allen and Arnon, 1955). Following four days of growth on the solid medium, individual spots were moved to solidified AA medium at various times. Following the final time point, whole plates were exposed to vapor of n-decanal for 2 min and then immediately assayed for

luminescence using a Hamamatsu Photonics System, model C1966-20 (Wolk et al., 1991).

Microscopy and assays of luminescence of suspension cultures were performed as described by Elhai and Wolk (1990). For induction experiments, cultures grown with AA/8 nitrate plus appropriate antibiotics were washed three times with AA/8, and were resuspended in AA/8 without antibiotics. The cultures were maintained under growth conditions throughout the time course. Images of luminescence are 20-min integrals. Concentrations of chlorophyll were measured in methanolic extracts (Mackinney, 1941).

Results

Transposon insertions in *hetR*. Transposon-generated mutants *a*41, *a*63, T34, T47, and YC49 (Ernst et al., 1992) showed little sign of differentiation after 24 to 48 hours of nitrogen-deprivation, although some cells within the filaments displayed a less granular, more homogeneous appearance suggestive of a very early stage of differentiation. Southern analysis (Maniatis et al., 1982) of genomic DNA (data not shown) disclosed that, as expected for insertions within the *hetR* open reading frame, the *Cla*I, *Eco*RI, *Eco*RV, and *Spe*I fragments bearing the transposons were the same size for each of the mutants. Plasmid pRL880, containing the transposon-bearing *Eco*RV fragment from *a*41 (see Materials and methods), hybridized to the appropriate 5-kb *Eco*RV fragment of *hetR* clone pWB216N9.5 (Buikema and Haselkorn, 1991a; data not shown). The transposon in *a*41 was found to have inserted 101 bp 3′ from the

translational start site of *hetR*, generating a 9-bp direct repeat as is characteristic of Tn*5* (Figure 2.1A,B).

Transcriptional control of *hetR-luxAB* fusions following removal of fixed nitrogen. To measure transcription of *hetR*, Tn*5*-1058 (Wolk et al., 1991) in pRL880 was replaced (see Materials and methods) with linearized plasmid pRL579, which bears the *Vibrio fischeri* bioluminescence genes, *luxAB*, as a transcriptional reporter (Elhai and Wolk, 1990). Plasmids were obtained with the reporter inserted in two orientations: pRL881a (Figure 2.1C) reports transcription initiated 5′ from the site of insertion of the transposon in *hetR* and pRL881b reports possible transcription initiated 3′ from that site (Figure 2.1C). The larger *Eco*RV fragment of the positive selection vector pRL277 containing the *Bacillus subtilis* levansucrase gene (*sacB*) and conferring resistance to streptomycin (Sm) and spectinomycin (Sp) was placed into the *Eco*RV site used for the genomic cloning, yielding pRL884a,b (Figure 2.1D). These plasmids were transferred by conjugation to *Anabaena* sp. strain PCC 7120. Single recombinant exconjugants (SR884a,b; shown by our unpublished data to have recombined at the *hetR* locus) were identified as $Sm^rSp^rNm^rSuc^s$ and had a Fox^+ Het^+ phenotype (terminology of Ernst et al., 1992; Fox refers to the ability to grow in an oxygenated environment on media lacking a source of fixed nitrogen). Plating the single recombinants on sucrose-containing medium (Cai and Wolk, 1990) gave rise to $Sm^sSp^sNm^rSuc^r$ *d*ouble *r*ecombinants (DR884a,b) at the *hetR* locus that were Fox^- Het^-.

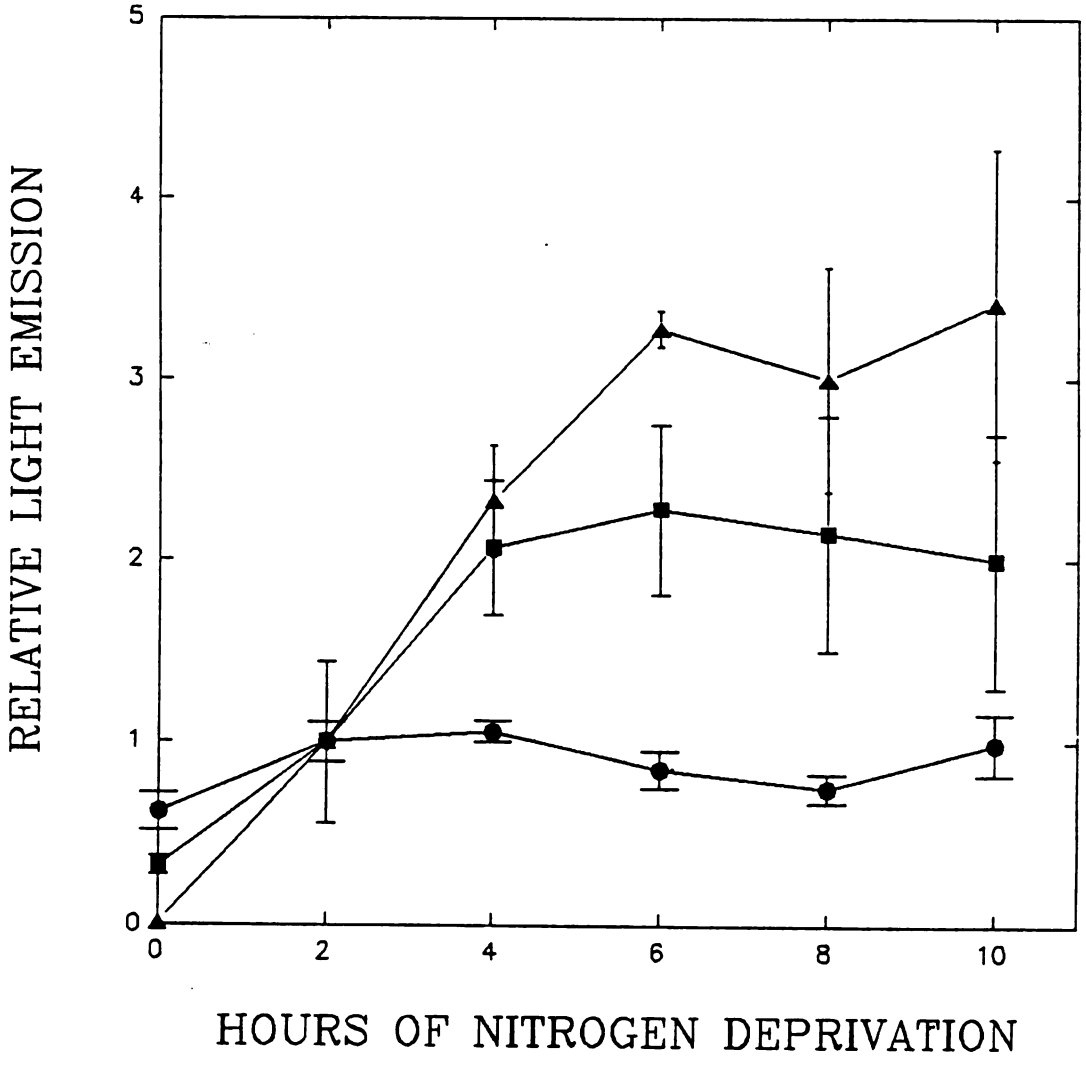
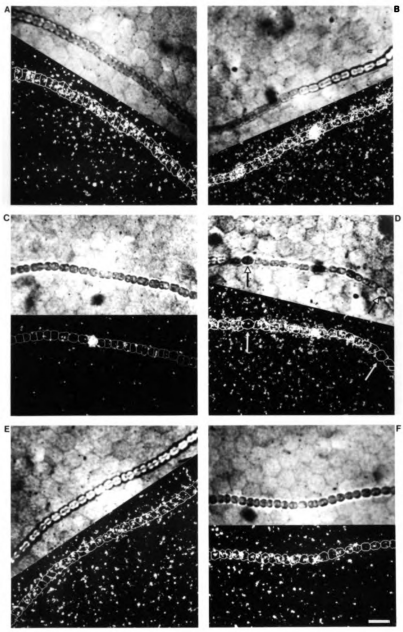


Figure 2.2 Relative light emission from suspensions of *hetR-luxAB* constructs SR884a (■), DR884a (●) and DR937 (▲) at various intervals after removal of nitrate from the medium. The results graphed are averages derived from three, three and two independent experiments, respectively. Values of luminescence during successive measurements on a given suspension were, for the sake of clarity of the graph, normalized to the corresponding mean values at 2 hours, and results for different suspensions then averaged. The mean values of light emission ($\times 10^{-7}$) at 2 hours for SR884a, DR884a, and DR937 were 278.4 ± 121.3, 126 ± 13.8 and 16.1 ± 0.54 quanta (μg Chl)$^{-1}$ min^{-1}, respectively.

Luminescence from spots of filaments (see Materials and methods) of SR884b and DR884b, in which *luxAB* was oriented opposite to the direction of transcription of *hetR*, was very low, and constitutive (data not shown). Luminescence from spots of filaments of SR884a was initially low, but increased within 2 hours following removal of nitrate, and within 8 hours reached a level 3-4 fold greater than that seen on nitrate (data not shown). Nitrogen-deprived suspension cultures of SR884a showed a rapid increase in luminescence (Figure 2.2). In contrast, the luminescence from spots of filaments (data not shown) and suspension cultures of DR884a, whose level of expression at 0 hours was similar to that of SR884a, showed no significant increase (Figure 2.2). A *luxAB* fusion generated 21 bp downstream from the *hetR* termination codon (pRL937; see Figure 2.1i) was introduced as a double recombinant (DR937) into the wild type strain. Heterocyst differentiation proceeded normally, and the luminescence of this recombinant, like that of SR884a (Figure 2.2), increased rapidly after removal of nitrate, although absolute values of luminescence were much lower.

Localization of expression of *hetR-luxAB* during differentiation. At various times after nitrogen stepdown of suspension cultures of SR884a and DR884a, small aliquots were removed from the cultures, a suspension of n-decanal was added, and luminescence was collected from filaments through an oil immersion lens (Elhai and Wolk, 1990). SR884a initially displayed a uniform distribution of luminescence throughout the filament (Figure 2.3A). After 3.5 hours without nitrate, single cells or in some cases clusters of 2 or 3 cells luminesced

Figure 2.3 Images of luminescence (20-min integrals corrected for charge spreading: see Elhai and Wolk, 1990) from individual filaments of SR884a at (A) 0, (B) 3.5, (C) 6, and (D) 24 hours, and of DR884a at (E) 0 and (F) 24 hours, following nitrogen stepdown. Adjacent analog images of the filaments have not been manipulated to eliminate an instrumentally generated honeycomb pattern (compare Elhai and Wolk, 1990; Wolk et al., 1993). In (D), the arrows point to mature heterocysts that are non-luminescent due either to a lack of O_2, a substrate of luciferase, within the heterocyst, or possibly to diminished transcription of *hetR* in mature heterocysts. Values of light flux density are presented as means ± standard deviations of the mean for areas the size of cells, after correcting for charge spreading. In (A), (E) and (F), the light flux density averaged over the filament was 0.38 ± 0.03, 0.32 ± 0.06, and 0.29 ± 0.03, and outside of the filaments was 0.06 ± 0.01, 0.04 ± 0.006, and 0.05 ± 0.01 cpm μm^{-2}, respectively. In (B), the light flux densities of the two bright cells toward the left, the single bright cell near the center, the remainder of the filament, and the background were 1.69, 2.08, 2.93, 0.72 ± 0.05, and 0.18 ± 0.02 cpm μm^{-2}, respectively. In (C), the single bright cell, the remainder of the filament and the background had respective values of light flux density of 2.50, 0.09 ± 0.02, and 0.012 ± 0.003 cpm μm^{-2}. In (D), the central bright cell, the left-hand and right-hand heterocysts (arrows), the other cells, and the background had values of light flux density of 3.26, 0.13, 0.19, 0.75 ± 0.07 and 0.12 ± 0.01 cpm μm^{-2}, respectively. The bar represents 10 μm.


A
B
C
D
E
F

much more brightly than did the remainder of the cells of the filament. Noncontiguous, especially luminescent cells, when observed, were separated by about 8-9 less luminescent cells (Figure 2.3B). As of 6 hours, expression was particularly clearly localized (Figure 2.3C). After 24 or more hours of incubation of the filaments in the absence of fixed nitrogen, the positions of especially luminescent cells approximately bisected the intervals between observed heterocysts (Figure 2.3D). Luminescence of DR884a was weak and constitutive in all cells in the presence or absence of nitrate (Figure 2.3E,F).

Effect of *hetR* on expression of *hepA*. The expression of luciferase from fusions to *hepA* has also been found to occur in a patterned array prior to morphological differentiation of heterocysts (Wolk et al., 1993). The induction of *hepA* fusions, however, does not occur until 5-7 hours after the removal of fixed nitrogen, much later than the induction of *hetR*. To determine whether a mutation in *hetR* would affect the expression of *hepA*, we made use of Sm^{r}Sp^r strain DR1069, which bears a *hepA-luxAB* fusion. Plasmid pRL908 (Figure 2.1H), bearing an insertion within *hetR*, was transferred by conjugation to strain DR1069. There, it recombined into the genome at the *hetR* locus (data not shown). Sucrose-resistant double recombinants (DR908-DR1069) displayed a typical Het$^-$ phenotype, characteristic of a *hetR* mutant. Twenty-four hours after nitrogen stepdown (and averaged over three pairs of independent experiments), DR1069 showed a 334-fold increase in luminescence to 1635 $\pm$ 90 x 10^7 quanta (μg Chl)$^{-1}$ min^{-1} while DR908-DR1069 showed only a 19-fold increase to 6.5 $\pm$ 1.0 x 10^7 quanta (μg Chl)$^{-1}$ min^{-1}.

DISCUSSION

Buikema and Haselkorn (1991b) showed both that *hetR* is required for differentiation of heterocysts and that it leads to formation of supernumerary heterocysts when present in multiple copies. Those authors therefore suggested that *hetR* is a positive regulator of heterocyst differentiation. Our results confirm their suggestion and show, further, that *hetR* is also involved in regulation of its own expression. Transcriptional fusions to *luxAB* within the coding region of *hetR* block induction of the reporter following nitrogen deprivation unless a wild type copy of *hetR* is maintained. Transcripts that range in size from 1.4 to 2.4 kilobases have been reported for *hetR*, whose open reading frame spans 0.9 kb (Buikema and Haselkorn, 1991b). It was therefore possible that the regulatory effect that we saw in *hetR*⁻ strains was due to a polar effect of the insertion on a cotranscribed factor lying downstream from *hetR*. To examine this possibility, a *lux* fusion was made downstream from the *hetR* coding region. Although the absolute values of luminescence obtained from this fusion were much lower than from fusions within *hetR*, induction of *hetR* was found to proceed normally as reported by (i) increased luminescence measured following removal of fixed nitrogen, and (ii) unimpaired differentiation. The lower levels of luminescence seen in DR937 can potentially be accounted for in many ways but is probably based, in part, on the use of a different reporter construct. The very low level of luminescence from DR937 at zero hours apparently cannot have resulted from the presence of only one *hetR* promoter region compared with two promoter regions in the

single recombinant strain because the levels of luminescence of SR884a and DR884a are very similar at zero hours. However, the greater relative increase in luminescence seen in DR937 compared with SR884a could result from the extra *hetR* promoter in SR884a, although there are also numerous other possible explanations for this difference. Nonetheless, it is apparent that HetR, the product of *hetR*, is required for enhanced expression of *hetR*.

The positive feedback seen with *hetR* is similar to that seen with the *B. subtilis* sporulation factors σ^K (Kroos et al., 1989) and σ^G (Sun et al., 1989; Karmazyn-Campelli et al., 1989). Once these sigma factors are synthesized, they activate their own transcription from specific promoters to assure their enhanced expression in the appropriate compartment and at the appropriate stage in development (Stragier and Losick, 1990). The deduced sequence of the HetR protein, however, shows no similarities to the sequences of known sigma factors. The expression of σ^K requires the presence of an auxiliary DNA-binding protein (Kroos et al., 1989), while σ^G may require an effector encoded by the *spoIIIA* locus (Stragier and Losick, 1990) to overcome repression by SpoIIAB (Rather et al., 1990). Although HetR contains no known DNA-binding motifs (Buikema and Haselkorn, 1991b), it could interact with a development-specific sigma factor or another DNA-binding protein and thereby enhance the expression of *hetR* as well as of other heterocyst-specific genes.

Full induction of *hepA* depends upon the presence of a functional *hetR* gene, but may depend only indirectly on the *hetR* product because *hepA* is first activated several hours after *hetR* is induced. The time course of induction of

a *hepA-lux* fusion has been published (Wolk et al., 1993). The DR1069 *hepA-lux* fusion has a similar increase in light emission between 4 and 20 hours after nitrogen stepdown. In the presence of the DR908 mutation, an increase in light emission is also seen, but the much lower values of light emission preclude accurate quantitation at early times following nitrogen stepdown. The presence of DR908 reduces the measured expression of DR1069 (quanta min^{-1} [μg chl]$^{-1}$) by typically >99%.

The spatially constitutive luminescence seen in nitrate-grown filaments, the results of Northern blot analysis (Buikema and Haselkorn, 1991b), and the higher levels of luminescence seen in cultures of DR884a than in cultures of DR884b (both, in the presence of nitrate) indicate that *hetR* is transcribed at a low level in all vegetative cells. Upon nitrogen stepdown, induction of *hetR* begins within 2 hours; and within 3.5 hours, enhanced transcription of *hetR* can be visualized within specific cells. Because expression of *hetR* in differentiating filaments is restricted to no more than 10% of the cells, and because, later, luminescence from *hetR-luxAB* fusions is seen in the cells that lie midway between two pre-existing heterocysts, i.e., at the sites at which new heterocysts would be anticipated to form, it appears that transcription of *hetR* is enhanced only in cells that have initiated differentiation into heterocysts. If the overall induction of at least 3 fold by 6 hours is attributed to the increase in at most 10% of the cells, then within those cells, *hetR* must have been induced at least 20-fold. Induction of *hetR* thus appears to occur within a pattern of cells and may, through positive autoregulation, contribute

to the stabilization of the pattern of heterocyst spacing; that it contributes to the initial establishment of that pattern is not excluded.

Wilcox et al. (1973a,b) observed that incompletely differentiated cells, called proheterocysts, often arise in clusters with all but one cell in a cluster eventually reverting to a state of vegetative growth. Those authors considered the theory that a differentiation-inhibitory substance generated by a proheterocyst increases in concentration until it represses differentiation of contiguous cells, even if those cells have also initiated differentiation. According to this theory, the differentiating cell must be immune to the effects of the inhibitor that it generates. If the *hetR* product were a factor that confers immunity to (or, more generally, acts counter to) such an inhibitor, and if the level of inhibitor were low in nitrate-grown cells, differentiation of nitrate-grown cells in the presence of multiple copies of *hetR* (Buikema and Haselkorn, 1991b, and my confirmatory observations) might be explained. Likewise, the appearance of multiple heterocysts in N_2-grown filaments can be interpreted as implying that there is no change in the specification of the foci of differentiation, but that the balance between inhibitory and counteracting factors is shifted in favor of differentiation when *hetR* is present in higher copy number than in the wild type strain. Strains lacking HetR would be unable to overcome self-repression, and therefore unable to differentiate. According to this interpretation, *hetR* would be involved not in the generation of the primary pattern, but in the resolution of clusters and stabilization of the differentiation process. Such a conclusion would be consistent with our observations and

that of Buikema and Haselkorn (1991a) that in a *hetR* mutant, "non-granular cells are visible and may represent cells that have degraded their internal stores of nitrogen more fully than other cells." Nonetheless, as has been pointed out (Wolk, 1982), several substances that are known to elicit the formation of clusters of heterocysts also lead to decreased spacing of the clusters, suggesting that mechanisms underlying the resolution of clusters, and spacing, may well be related.

I presume that cellular factors help to determine the non-uniform expression of *hetR* along a filament: the identification of these factors is crucial for elucidation of the involvement of *hetR* in heterocyst differentiation. Other genes are also required for all, or all but the earliest, stages of heterocyst differentiation (Ernst et al., 1992); it will be of great interest to determine whether they or their products interact with HetR or affect transcription of *hetR*.

Chapter 3

ANALYSIS OF N10, A Het$^-$ MUTANT IN *Anabaena* SP., PROVIDES EVIDENCE THAT A SECONDARY METABOLITE MAY REGULATE HETEROCYST SPACING

SUMMARY

Transposon-generated mutant N10 has a Het$^-$ phenotype (Ernst et al., 1992). Reconstruction of the mutation has reproduced a Het$^-$ phenotype, but other insertions at the position of the transposon have produced strains that form multiple contiguous heterocysts. Sequence analysis around the site of insertion of the transposon showed that the insertion lies within the 5′ end of an 861-bp *orf* (*hetN*). The product of translation of *hetN* shows extensive similarity to NAD(P)H-dependent oxido-reductases that are involved in biosyntheses of fatty acids, poly-ß-hydroxybutyrate, *nod* factor and polyketides. A second, 1518-bp *orf* (5′ *orf*) that ends 557 bp 5′ from the start of *hetN* appears to encode a protein that has at least two functional domains: its amino-terminus is similar to an acyl-carrier protein (ACP), while its central portion is similar to domains of proteins that perform reductive reactions. A third 711-bp *orf* (*hetI*) encoded on the opposite strand ends 42 bp away from the 3′ end of *hetN*. The

translated product of *hetI* shows similarity to the *B. subtilis* protein Sfp that regulates biosynthesis of the cyclic peptide, surfactin. Clones from a λ-EMBL3 library that contain the wild-type *hetN* gene do not complement the N10 mutant. The presence of *hetN*, as the only *orf*, on a replicating plasmid suppresses heterocyst formation in wild-type cells, while the additional presence of *hetI* alleviates this effect.

INTRODUCTION

Study of heterocyst differentiation and pattern formation has been greatly stimulated by the development of methods for genetic analysis of *Anabaena* sp. (Wolk, 1991; Wolk et al., 1984). Despite facile transposon mutagenesis of *Anabaena* sp. with derivatives of transposon Tn*5* (Ernst et al., 1992; Wolk et al., 1991), only one mutation has been reported that clearly alters pattern formation: the PAT-1 mutant causes heterocysts to form almost exclusively at the ends of filaments (Liang et al., 1992). Several mutations have been found that prevent heterocyst differentiation. Inactivation of *hetR* by a point mutation that converts a serine residue to an asparagine (Buikema and Haselkorn, 1991a,b) or by insertion of a transposon (Black et al., 1993) prevents evident differentiation of heterocysts. Supernumerary copies of *hetR* lead to the formation of heterocysts when filaments are grown on media containing combined nitrogen, and to the formation of multiple contiguous heterocysts in the absence of fixed nitrogen (Buikema and Haselkorn, 1991b; and unpublished observations). Like mutations of *hetR*, an insertion in *hetP* prevents heterocyst differentiation (Ernst et al., 1992) while extra copies of *hetP* lead to the

production of multiple contiguous heterocysts during growth on N_2 (F. Fernández Piñas and C. P. Wolk, unpublished observations). Another mutant, N10, was reported to be similar to *hetR* in that heterocyst differentiation was blocked at an early stage; however, this mutant has a tendency to revert to a form that generates multiple contiguous heterocysts (strain N16) when inoculated on media lacking fixed nitrogen (Ernst et al., 1992). The site of transposition in the N10 mutant has been mapped near the arbitrary origin of the physical map (Kuritz et al., 1993). I report a characterization of the sequence of the DNA surrounding the site of transposition in mutant N10 and a genetic analysis of the function of that DNA in heterocyst differentiation. My data lead me to suggest that the gene inactivated by the transposon in N10 may be involved in the production of a secondary metabolite that can inhibit heterocyst differentiation.

MATERIALS AND METHODS

Growth and manipulations of *Anabaena* sp. Wild-type *Anabaena* sp. strain PCC 7120 and its various mutant derivatives were grown at 30°C in the light (ca. 3,500 ergs $cm^{-2}s^{-1}$) on a rotary shaker in 50 ml of AA/8 medium (Hu et al., 1982) supplemented with 5 mM nitrate in 125-ml Erlenmeyer flasks. Plasmids containing the various insertions were introduced by conjugation (Elhai and Wolk, 1988b; Wolk et al., 1984), and double recombinants were selected as described by Cai and Wolk (1990). Mutant strains were grown in the presence of appropriate antibiotics (Table 3.1). To induce heterocyst formation, portions

of rapidly growing cultures (ca. 1 to 2 μg of chlorophyll *a* ml^{-1}) were washed twice with AA/8, suspended in an equal volume of AA/8 without antibiotics, and incubated under growth conditions. Filaments were examined by microscopy 48 to 72 hours following nitrogen stepdown.

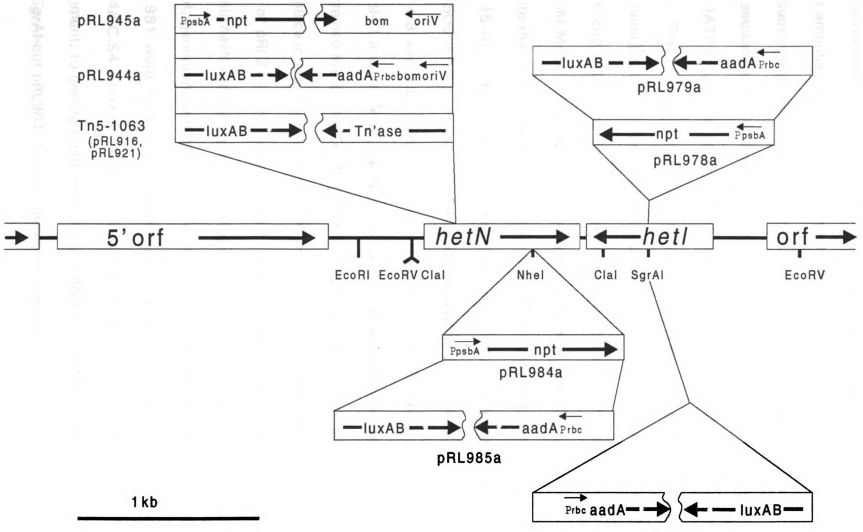
Recovery of DNA contiguous with the transposon in mutant N10. Ca. 1 μg of DNA isolated from mutant N10 was cut with 10 u of *Eco*RV. The reaction mixture was then heated to 65°C for 15 min, diluted 20-fold, subjected to self-ligation for four hours at 25°C, and the DNA sedimented. Half of the DNA was used for electroporation into *E. coli* HB101. Colonies that grew on plates of L-agar containing 50 μg kanamycin-sulfate (Km) ml^{-1} were analyzed for the presence of the transposon, Tn*5*-1063a (Wolk et al., 1991). Plasmid pRL916, containing 2.1 kb of *Anabaena* DNA, was isolated from a positive colony. Larger regions of *Anabaena* DNA, ca. 3.5, 2.1 and 13 kb, respectively, flanking the left end of the transposon were obtained by recovering transposon-containing fragments after digestion of total N10 DNA with *Eco47*III, *Mlu*I and *Mun*I. pRL921 was made by ligating *Eco*RV-cut pRL916 to an *Eco*RV fragment from plasmid pRL1075 (Black et al., 1993) that contains the genes conferring resistance to chloramphenicol (Cm) and erythromycin (Em), and sensitivity to sucrose (*sacB*), and the *oriT* region of RK2.

To replace the transposon, pRL921 was cut with *Alw*NI, the ends blunted with the Klenow fragment, and then cut with *Bam*HI. This fragment containing *Anabaena* DNA was ligated with the BLOS2 vectors (Black et al., 1993)

pRL759 (cut with *Bgl*II + *Nru*I), generating pRL944a, and pRL759D (cut with *Bam*HI + *Nru*I), generating pRL944b. The BLOS2 insert in pRL944b was removed by cutting with *Bam*HI + *Bgl*II and was replaced with the S.K3 vector, pRL498 (Elhai and Wolk, 1988a), that had been cut with *Bam*HI, producing pRL945a (Figure 3.1) and pRL945b. To generate insertions within *hetI*, plasmid pRL945a was cut with *Nco*I + *Nhe*I and the fragment containing *hetI* was isolated and ligated with the positive selection vector pRL271 (Black et al., 1993) producing pRL975. pRL975 was then cut with *Sgr*AI, and the C.K3 cassette, excised from pRL648 [pUC/L.EHEI/(*Xba*I)/C.K3: nomenclature of Elhai and Wolk, 1988a] with *Ava*I, was inserted producing pRL978a and pRL978b. The C.K3 insertion in pRL978a was then removed by cutting with *Bam*HI and replaced with the *luxAB*-C.S4 cassette (Black et al., 1993) present in pRL65 with *Bgl*II-ends, generating pRL979a and pRL979b.

Construction of a λ-EMBL3 library. Chromosomal DNA from wild-type *Anabaena* sp. strain PCC 7120 was partially digested with *Sau*3AI. The digestion mixture was separated on a 10-40% continuous sucrose gradient. Fractions were collected and examined on 0.2% agarose gels. Fractions that contained fragments between ca. 15 and 25 kb in length were pooled, concentrated and ligated with λ-EMBL3 arms that had been cut with *BamHI* and treated with calf intestinal alkaline phosphatase (purchased from Promega, Madison, WI). The ligated DNA was packaged in vitro and plated on *E. coli* KW251 (*mcrA*$^-$*mcrB*$^-$) and LE392 (*mcrA*$^-$ *mcrB*$^+$). The packaged mixture formed ca. 1.5 x 10^6 pfu ml^{-1} on KW251 but gave ca. 1 x 10^4 pfu ml^{-1} on LE392, while

Figure 3.1 The region of the *Anabaena* genome that contains the 5' *orf*, *hetN*, and *hetI*. Insertions shown above the genomic map were made using plasmids derived from mutant N10. Reconstructions shown below the genetic map were made using a wild-type clone of the region obtained from a λ-EMBL3 library. With the exception of pRL989, each insert was obtained in both orientations, although only the "a" orientation is shown.


pRL945a
PpsbA
npt
bom
oriV
pRL944a
luxAB
aadA
Prbc
bom
oriV
Tn5-1063
(pRL916,
pRL921)
luxAB
Tn'ase
luxAB
aadA
Prbc
pRL979a
npt
PpsbA
pRL978a
5' orf
hetN
hetI
orf
EcoRI
EcoRV
ClaI
NheI
ClaI
SgrAI
EcoRV
PpsbA
npt
pRL984a
luxAB
aadA
Prbc
pRL985a
1 kb
Prbc
aadA
luxAB
pRL989

a control insertion gave equal plating efficiency on the two strains. The library was amplified on KW251; the eluted lysate contained ca. 10^9 pfu ml^{-1}.

Subcloning the wild-type N10 DNA from the λ-EMBL3 library, and subsequent plasmid constructions. Plasmid pRL916 was labeled with [α-^{32}P]dATP by using a random primer labeling kit from GIBCO BRL (Gaithersburg, MD). The pRL916 probe was used to screen two sets of duplicate filters containing a total of ca. 1200 plaques. A single positive plaque was amplified on *E. coli* LE392 and the phage were eluted with 3 ml of 10 mM Tris, pH 7.5, 10mM $MgSO_4$. The plate-amplified lysate was used to produce 10 ml of liquid lysate that was used for isolation of phage DNA (Maniatis et al., 1982). The ca. 15-kb insert, cut from the phage DNA with *Sal*I, was ligated with *Sal*I-cut pRL500 (Elhai and Wolk, 1988a), yielding plasmid pRL946. *Eco*RI fragments ca. 4.8 and 4.5 kb in length were subcloned into *Eco*RI-cut pRL500 giving pRL947 and pRL948, respectively. Restriction analysis showed that pRL948 contained the 2.1-kb *Eco*RV genomic fragment that had been disrupted in N10. To place interposons within this fragment, it was first sub-cloned into the *Nru*I site of pRL271, producing pRL981. pRL981 was cut at the unique *Nhe*I site within *hetN* and then ligated with the C.K3 cassette, excised from pRL648 with *Xba*I, generating pRL984a and pRL984b. Alternatively, the ends of *Nhe*I-cut pRL981 were filled in with the Klenow fragment and then ligated with the *luxAB*-C.S4 cassette, removed from pRL58 (Black et al., 1993) as a *Sma*I fragment, to generate pRL985a and pRL985b. Transfer of the same cassette to *Sgr*AI-cut pRL981, filled in, generated pRL989.

Plasmids pRL959 and pRL982, containing wild-type N10 DNA in a (pDU1-based) plasmid that can replicate in *Anabaena* sp. were made by inserting the 4.5-kb *Eco*RI fragment and the 1.3-kb *Cla*I fragment, respectively, into the unique *Eco*RI and *Cla*I sites of pRL1049, the *lux*-less immediate precursor of pRL1050 (Wolk et al., 1993). The same single orientation was obtained for both insertions with *hetN* oriented parallel to the *aadA* gene. Deletions pRL982BN and pRL982RN were generated from pRL982 by cutting pRL982 with *Bam*HI and *Nhe*I or *Eco*RI and *Nhe*I, respectively, blunting with the Klenow fragment, and self-ligating. Cassette C.A1 (Elhai and Wolk, 1988a), excised from pRL494 (S.K4/L.XSX1(*Eco*RI)/C.A1; nomenclature of Elhai and Wolk, 1988a) with *Xba*I, was inserted into the *Nhe*I site of pRL982 to generate pRL982C.A1.

Plasmids pRL967a and pRL967b that bear the *hetR* gene and can replicate in *Anabaena* sp. were made as follows. The 5.3-kb, *hetR*-containing, *Eco*RV fragment from pWB216N9.5 (Buikema and Haselkorn, 1991a), provided with *Bam*HI ends by transfer to *Hinc*II-cut pRL500, was ligated into the *Bam*HI site of pRL1049. Two orientations of the insert were obtained; pRL967b has the *hetR* gene oriented parallel to the *aad*A gene.

Plasmids pRL1450a and pRL1450b (F. Fernández-Piñas and C. P. Wolk, unpublished results), that contain the wild-type DNA surrounding the site of the transposon insertion in mutant P6, were made as follows. First, pRL1404 was made by adding a portion of the polylinker L.HEH1 and the C.C1 cassette, excised from pRL171 (Elhai and Wolk, 1988a) with a partial *Bam*HI digestion,

to the *Bam*HI site of pRL1049. Next, pRL1404ΔCN was made by cutting pRL1404 with *Cla*I and *Not*I, blunting the ends with the Klenow fragment, and then self-ligating to remove an additional *Eco*RI site and a portion of pDU1 that is not required for replication in *Anabaena*. Finally, a 4.3-kb *Eco*RV fragment from a λ-EMBL3 clone was ligated between the *Ecl*136II sites of pRL1404CN.

Southern analysis. Double-recombinant insertions were confirmed by Southern analysis of chromosomal DNA that was isolated from the mutants and digested with *Eco*RV. Probes were made with the 2.1-kb *Eco*RV fragment from pRL948 that was labeled with [α-^{32}P]dATP or digoxigenin-11-dUTP using kits purchased from GIBCO BRL or Boehringer Mannheim (Indianapolis, IN).

Sequencing. Automated sequencing (Applied Biosystems Inc., Foster City, CA) was performed using universal primers on a variety of fragments that had been subcloned from pRL916 and pRL948 into vectors pUC18, pUC19, pUC118, and pUC119 (Vieira and Messing, 1982; 1987). Database comparisons and alignments of the translated sequences were performed, using the default settings of the algorithm developed by Altschul *et al.* (1990), at the National Center for Biotechnology Information (NCBI) using the BLAST network service. Single-sequence alignments obtained from NCBI were combined to give the multiple-sequence alignments. In some cases, sequence in addition to that returned from BLAST was added.

RESULTS

Isolation, recovery and reconstruction of the N10 mutation. Whether the Het$^-$ phenotype of mutant N10 (Ernst et al., 1992) was a result of the transposon insertion was tested by regenerating the mutation in the wild-type strain as described by Black *et al.* (1993). The transposon and flanking *Anabaena* DNA, cloned from the N10 chromosome as pRL916, were combined with the *sac*B-containing portion of pRL1075, and the resulting plasmid (pRL921) was transferred by conjugation into wild-type *Anabaena*. The locus of insertion was confirmed by Southern analysis (data not shown). The resulting Nm^{r}Suc^r double recombinants (DR921) displayed the same Het$^-$ phenotype as N10. However, when the transposon in pRL921 was replaced with a variety of other insertions, the most frequently derived phenotype of the double recombinants was the production of multiple contiguous heterocysts (Mch; Table 3.1). The double recombinants (DRs) from at least three independent single recombinant strains were examined in each case, and all isolated DRs had the same phenotype (for DR984a, the same combination of phenotypes) for each particular construction. Two to three rounds of growth, fragmentation and selection on plates containing 5% sucrose were used in order to obtain fully segregated double recombinants with plasmids pRL944a, pRL944b, pRL945a, pRL945b, and pRL984a, whereas double recombinants were more readily obtained with pRL944a and pRL944b in the *hetR* strain, DR908 (Black et al., 1993; data not shown).

TABLE 3.1 Construction, antibiotic resistances and phenotypes of the strains.

Strain	Source of insert	Antibiotic resistance[†]	Phenotype[‡]
Insertions in *hetN*			
N10	Tn*5*-1063a	BmNmSm	Het$^-$
DR921	pRL921	BmNmSm	Het$^-$
DR944(a,b)	pRL944(a,b)	Sm/Sp	Mch
DR945(a,b)	pRL945(a,b)	Nm	Mch
DR984a	pRL984a	Nm	wild type and Het$^-$
DR984b	pRL984b	Nm	Mch
DR985(a,b)	pRL985(a,b)	Sm/Sp	Mch
Insertions in *hetI*[•]			
DR978a	pRL978a	Nm	wild type and Mch
DR978b	pRL978b	Nm	wild type and Het$^-$
DR979(a,b)	pRL979(a,b)	Sm/Sp	wild type
DR989	pRL989	Sm/Sp	wild type but fragmented

[†]Bm = bleomycin^r, Nm = neomycin^r, Sm = streptomycin^r, and Sp = spectinomycin^r.

Concentrations used in liquid media were 50 μg Nm ml^{-1} or a combination of 1 μg Sm ml^{-1} + 5 μg Sp ml^{-1}.

Concentrations used in solid media were 200 μg Nm ml^{-1} or a combination of 1 μg Sm ml^{-1} + 10 μg Sp ml^{-1}.

[•] In no instance were doubly recombinant chromosomes completely segregated.

[‡] Observed 48-72 h after the removal of nitrate. Mch = multiple contiguous heterocysts. In cases where two phenotypes are listed, clonal cultures had filaments that displayed different phenotypes.

Cloning and attempted complementation of the N10 mutants. Plasmid pRL916 was used to probe a λ-EMBL3 library. The wild-type DNA for the region interrupted by the transposon was subcloned from the library as a 4.5-kb *Eco*RI fragment into pRL500 (Elhai and Wolk, 1988a), producing pRL948. The wild-type *Eco*RI clone contained 400 bp upstream and ca. 3.3 kb downstream from the *orf* that had been interrupted by the transposon in N10 (*hetN*). This clone, in replicating vector pRL959, was unable to complement the N10 or DR921 mutants. This result was surprising because sequence analysis confirmed that the wild-type clone was identical in sequence to the DNA surrounding the transposon (see below). Plasmid pRL959 also produced no effect in wild-type *Anabaena* sp. strain PCC 7120. However, a 1.3-kb *Cla*I subclone, containing 48 bp upstream and 371 bp downstream from the *hetN* and no other complete *orf*, inhibited heterocyst formation by wild-type cells when in replicating vector pRL982. Like pRL959, pRL982 was unable to complement N10 or DR921. Heterocyst differentiation in wild-type cells was not suppressed by plasmid pRL982BN, which has the region 3′ from the *Nhe*I site in *hetN* deleted; pRL982RN, which has the region 5′ from the *Nhe*I site in *hetN* deleted; or pRL982C.A1, which contains an insertion within the *Nhe*I site in *hetN*.

To test whether the Het⁻ phenotype of N10 and DR921 was due to a second-site mutation in a gene known to inhibit heterocyst formation, *hetR* and the cloned wild-type DNA containing *hetP* were introduced into the N10 and DR921 strains on replicating vectors (pRL967 for *hetR*, and pRL1450a and pRL1450b for *hetP*). None of these plasmids complemented the Het⁻

phenotype of N10 or DR921.

It was possible that the phenotypic differences seen in the various reconstructions were due to a second mutation contained within the DNA near the transposon. To examine this possibility, plasmids (pRL984a, pRL984b, pRL985a, and pRL985b) constructed from the λ-EMBL3 clone were used to generate insertion mutations. The phenotypes of the resulting double-recombinant strains DR984a, DR984b, DR985a and DR985b, are shown in Table 3.1. Except for DR984a, these disruptions of *hetN* consistently yielded a Mch phenotype.

Sequence analysis of DNA near the transposon insertion in N10. Sequence analysis (sequence is available from Genbank under accession number L22883; Figure 3.2) showed that the transposon in N10 had inserted 179 bp from the 5' end of an 861-bp *orf*, called *hetN*, and that other *orf*s were nearby (Figures 3.1 and 3.2). A 2.3-kb *Eco*RV fragment from the λ-EMBL3 clone containing *hetN* was also sequenced, and was found to be identical in sequence to the clone recovered with the transposon. The predicted amino acid sequence from each reading frame was compared to those contained in the NCBI database using the blastp program. A large number of highly similar proteins was found for the protein sequence encoded by *hetN*. Alignments with some of the most similar proteins are shown in Figure 3.3. This class of proteins can be grouped as a superfamily of NAD(P)H-dependent oxido-reductases. The HetN protein aligns with known oxido-reductases for approximately 190 amino acid residues from the amino terminus, before diverging. The protein sequences that show

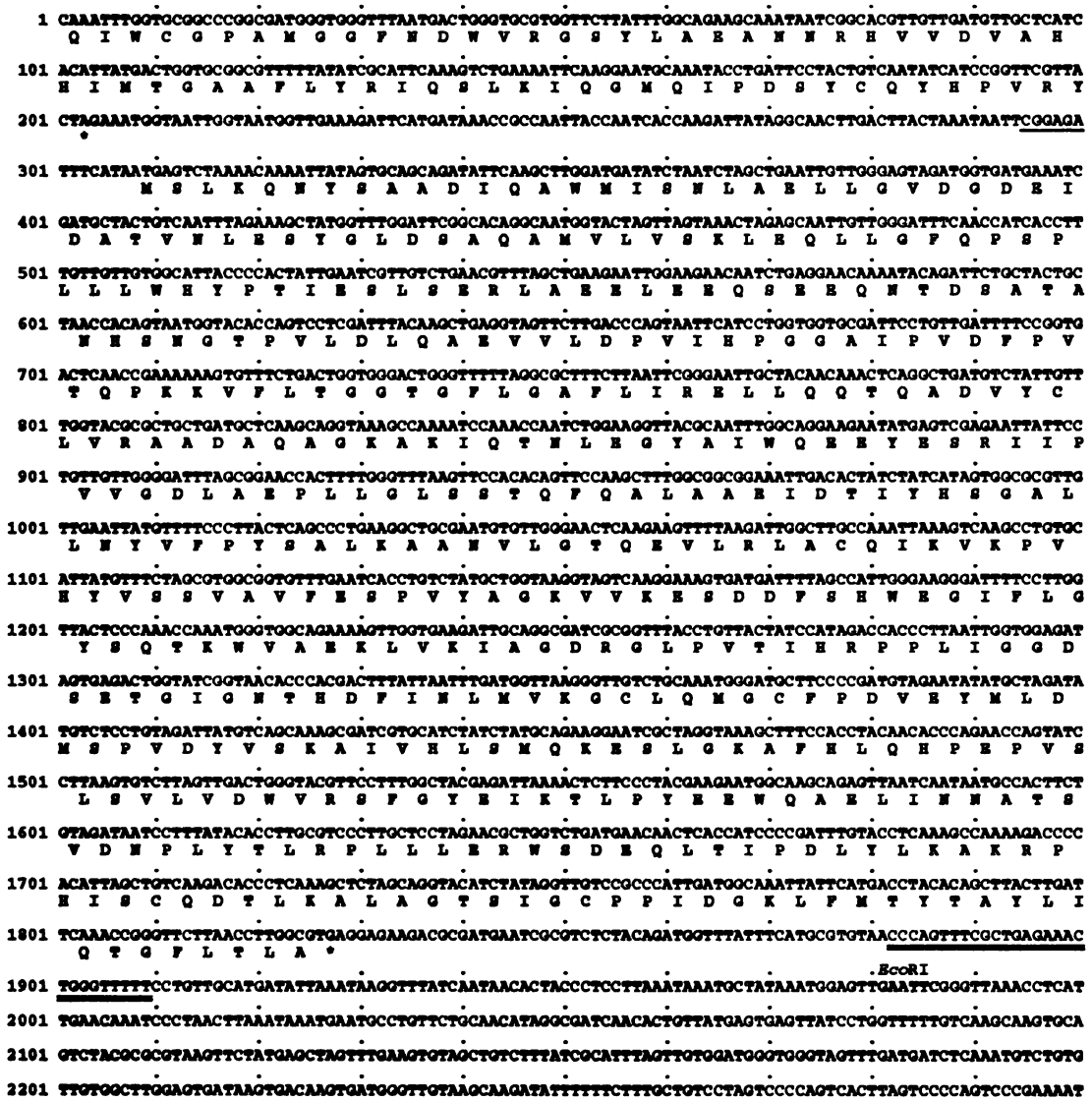


Figure 3.2 Nucleotide sequence of the 4.7-kb fragment shown in Figure 3.1. The underlined sequences indicate putative ribosome-binding sites. The double underline shows the position of a possible factor-independent transcriptional terminator following the 5′ *orf*. The overlined sequence shows the 9 bp that were duplicated during transposition. Restriction sites mentioned in the text are shown in bold type.

Figure 3.2 (cont'd)

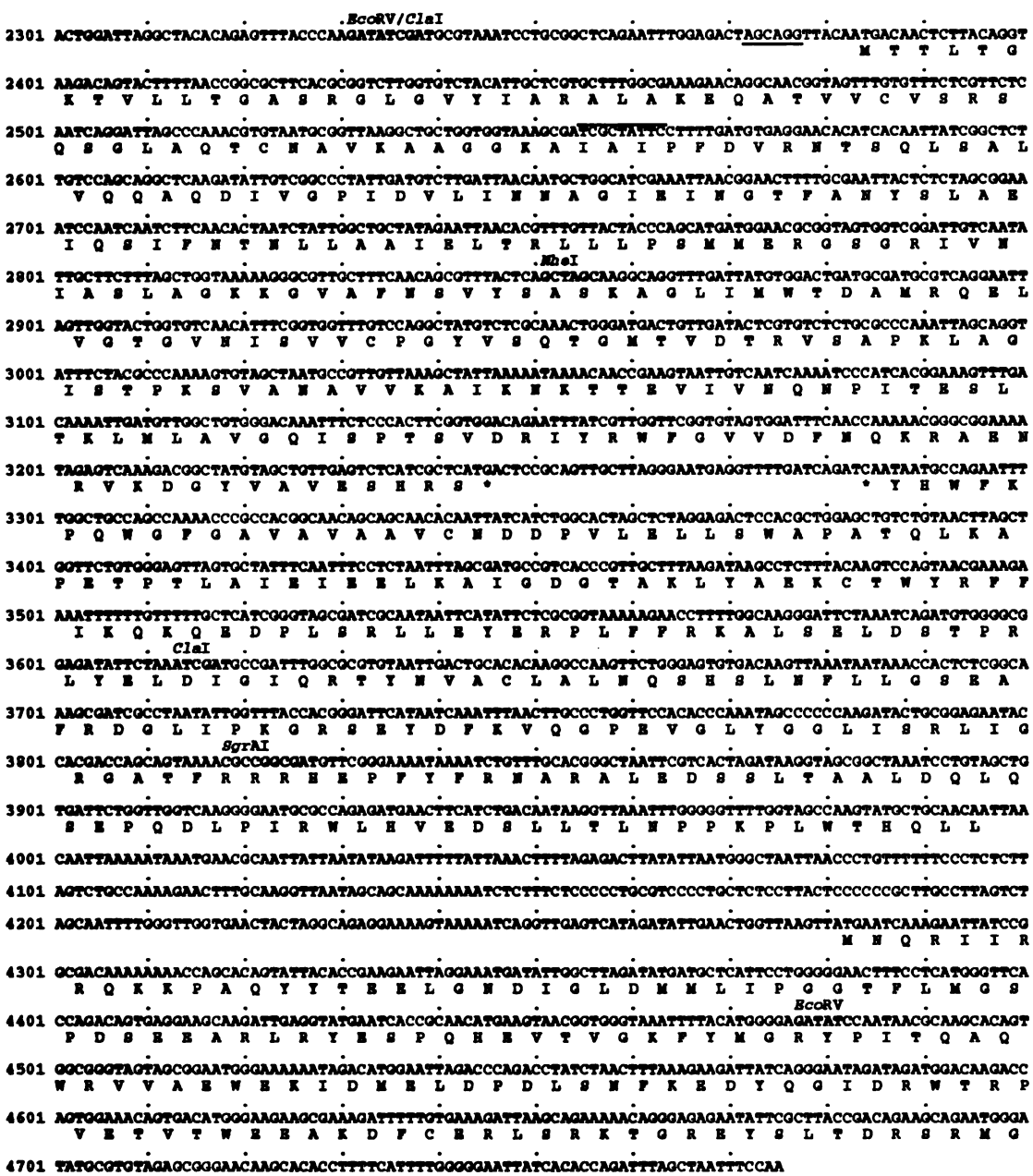


Figure 3.3 Alignment of the protein encoded by *hetN* with other proteins: Orf4 from *S. cinnamonensis* (S.cin [Arrowsmith et al., 1992]), ActIII from *S. coelicolor* (S.coe [Hallam et al., 1988]), NodG from *R. meliloti* (R.mel [Debellè and Sharma, 1986]), FabG from *E. coli* (E.col [Rawlings and Cronan, 1992]), 3-oxoacyl-ACP-reductase from *Arabidopsis thaliana* (A.tha [Slabas et al., 1992]), acetoacetyl-CoA-reductase from *Alcaligenes eutrophus* (A.eut [Peoples et al., 1989]) and the estradiol 17ß-dehydrogenase from *Homo sapiens* (H.sap [Peltoketo et al., 1988]). The residues showing the greatest conservation are shaded.

```
HetN :     1 MTTLTGKTVLLTGASRGLGVYIARALAKEQATVVCVSRSQSGLAQTCNAV.KAAGGKAIAI 60
S.cin:     1 MTQSTSRVALVTGATSGIGLATARLLAAQGHLVFLGARTESDVIATVKAL.RNDGLEAEGQ 60
S.coe:     1 MATQDSEVALVTGATSGIGLEIARRLGKEGLRVFVCARGEECLRTTLKEL.REAGVEADGR 60
R.mel:     1 MFELTGRKALVTGASGAIGGAIARVLHAQGAIVGLHGTQIEKLETLATEL....GDRVKLF 57
E.col:     1 .MNFEGKIALVTGASRGIGRAIAETLAARGGKVIGTATSENGAQAISDYL....GANGKGL 56
A.tha:    71 VQKVESPVVVITGASRGIGKAIALALGKAGCKVLVNYARSAKEAEEVAKQIEEYGGQAITF 131
A.eut:     1    MTQRIAYVTGGMGGIGTAICQRLAKDGFRVVAGCGPNSPRREKWLEQQKALGFDFIAS 57
H.sap      1    MARTVVLITGCSSGIGLHLAVRLASDPSQSFKVYATLRDLKTQGRLWEAARALACPPG 57

HetN :    61 PFDVRNTSQLSALVQQAQDIVGPIDVLINNAGIEINGTFANYSLAEIQSIFNTNLLAAIE 120
S.cin:    61 VLDVRDGASVTAFVQAAVDRYGRIDVLVNNAGRSGGGVTADLTDELWDDVIDTNLNSVFR 120
S.coe:    61 TCDVRSVPEIEALVAAVVERYGPVDVLVNNAGRPGGGATAELADELWLDVVETNLTGVFR 120
R.mel:    58 PANLANRDEVKALGQRAEADLEGVDILVNNAGITKDGLFLHMADPDWDIVLEVNLTAMFR 117
E.col:    57 MLNVTDPASIESVLEKIRAEFGEVDILVNNAGITRDNLLMRMKDEEWNDIIETNLSSVFR 116
A.tha:   132 GGDVSKATDVDAMMKTALDKWGTIDVVVNNAGITRDTLLIRMKQSQWDEVIALNLTGVFL 191
A.eut:    58 EGNVADWDSTKTAFDKVKSEVGEVDVLINNAGITRDVVFRKMTRADWDAVIDTNLTSLFN 120
H.sap:    62 QLDVRDSKSVAAARERV..TEGRVDVLVCNAGLGLLGPLEALGEDAVASVLDVNVVGTV  120

HetN :   121 LTRLLL..PSMMERGSGRIVNIASLAGKKGVAFNSVYSASKAGLIMWTDAMRQELVGTGVNI 180
S.cin:   121 MTRAVLTTXGMRTRERGRIINVASTAGKQGVVLGAPYSASKHGVVGFTKALGNELAPTGITV 182
S.coe:   121 VTKQVLKAGGMLERGTGRIVNIASTGGKQGVVHAAPYSASKHGVVGFTKALGLELARTGITV 182
R.mel:   118 LTREITQ..QMIRRRNGRIINVTSVAGAIGNPGQTNYCASKAGMIGFSKSLAQEIATRNITV 177
E.col:   117 LSKAVMRA..MMKKRHGRIITIGSVVGTMGNGGQANYAAAKAGLIGFSKSLAREVASRGITV 176
A.tha:   192 CTQAAVKI..MMKKKRGRIINISSVVGLIGNIGQANYTATKGGVISFSETPAREGASRNINV 251
A.eut:   121 ..VTKQVIDGMADRGWGRIVNISSVNGQKGQFGQTNYSTAKAGLHGFTMALAQEVATKGVTV 161
H.sap:   121  RMLQAFLPDMKRRGSGRVLVTGSVGGLMGLPFNDVYCASKFALEGLCESLAVLLLPFGVHL 181

HetN :   181 SVVCPGYVS 187
S.cin:   183 NAVCPGYV  190
S.coe:   183 NAVCPGFV  190
R.mel:   178 NCVAPGFI  185
E.col:   177 NVVAPGFI  184
A.tha:   252 NVVCPGFIA 260
A.eut:   179 NTVSPGYIA 187
H.sap:   182 SLIECGPVH 190
```

the greatest similarity, as much as 40% identity through the first 190 amino acid residues, are 3-ketoacyl-(acyl carrier protein [ACP]) reductases involved in the biosyntheses of polyketides (PKs), poly-ß-hydroxybutyrate (PHB) and fatty acids (FAs). The 100 carboxy-terminal amino acid residues of HetN do not show significant sequence similarity to other protein sequences contained in the databases.

A 711-bp *orf*, *hetI*, lies 3' from *hetN* and is encoded on the opposite strand of DNA. This *orf* ends 42 bp from the 3' end of *hetN*. *hetI* lacks an apparent ribosome binding site and an initiator AUG codon but has two adjacent UUG codons that could initiate translation. The sequence of the protein encoded by *hetI* shows similarity (28% identity) to portions of the proteins encoded by the *sfp* (Nakano et al., 1992) and *orfX* (Krätzschmar et al., 1989) genes from *Bacillus subtilis* and *B. brevis*, respectively (Figure 3.4). During the late-growth phase of *B. subtilis*, Sfp regulates the production of surfactin, a cyclic lipopeptide; affects genetic competence and sporulation; and may regulate various genes that are induced in the presence of alternative nitrogen sources (Marahiel et al., 1993). *orfX*, only partially sequenced, lies adjacent to the 5' end of the operon that encodes the proteins involved in the biosynthesis of the cyclic lipopeptide, gramicidin.

A third complete reading frame, the 5' *orf*, lies 5' from *hetN* and is encoded on the same strand of DNA as *hetN*. The 5' *orf* is 1518 bp in length, ends 557 bp from the start of *hetN,* and appears to be followed by a transcriptional terminator (Figure 3.2; Carafa et al., 1990). The protein sequence encoded by

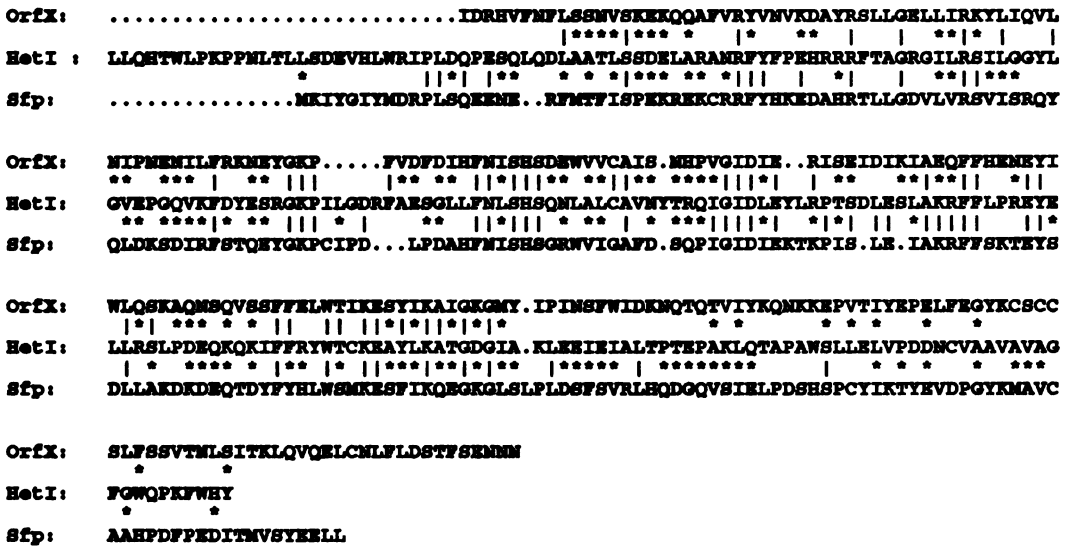


Figure 3.4 Alignment of the protein encoded by the *hetI* with Sfp (Nakano et al., 1992) and OrfX (Krätzschmar et al., 1989) from *B. subtilis* and *B. brevis*, respectively. Identical residues are marked with an (|) and similar residues with an (*).

the 5′ *orf* shows great similarity (26% identity) over its entire length to the carboxyl-terminal half of the yeast LYS2 protein (Morris and Jinks-Robinson, 1991; Figures 3.5 and 3.6), and a small region of both proteins, beginning at ca. residue 27 in the protein encoded by the 5′ *orf*, is similar to the 4′-phosphopantetheine-binding domains of putative and confirmed ACPs and ACP domains (Figure 3.6A). The ACP domains from the multifunctional *eryA* gene products of *Saccharopolyspora erythraea* (Cortes et al., 1990; Donadio et al., 1991; Donadio and Katz, 1992) which produce the polyketide-derived macrolide antibiotic, erythromycin, show particularly strong similarities to the ACP domain in the 5′ *orf*. Beginning at amino acid residue 136 in the protein encoded by the 5′ *orf*, and in LYS2, lies a consensus NAD(P)H binding site that is also found in EryA and other polyketide synthases (PKSs) and fatty acid synthases ([FASs] Figures 3.5 and 3.6B). The remainder of the protein encoded by the 5′ *orf* has a region of similarity to the acyl transferase domains that are weakly conserved in PKSs (Figure 3.6C).

Another long *orf*, only partially sequenced (Figures 3.1 and 3.2), lies just upstream from, and is transcribed in the same direction as, the 5′ *orf*. Preliminary alignments with the translated protein from this *orf* also show similarity to PKSs (data not shown). Another apparent *orf*, also only partially sequenced, is found to extend beyond the 3′ end of the fragment shown in Figures 3.1 and 3.2.

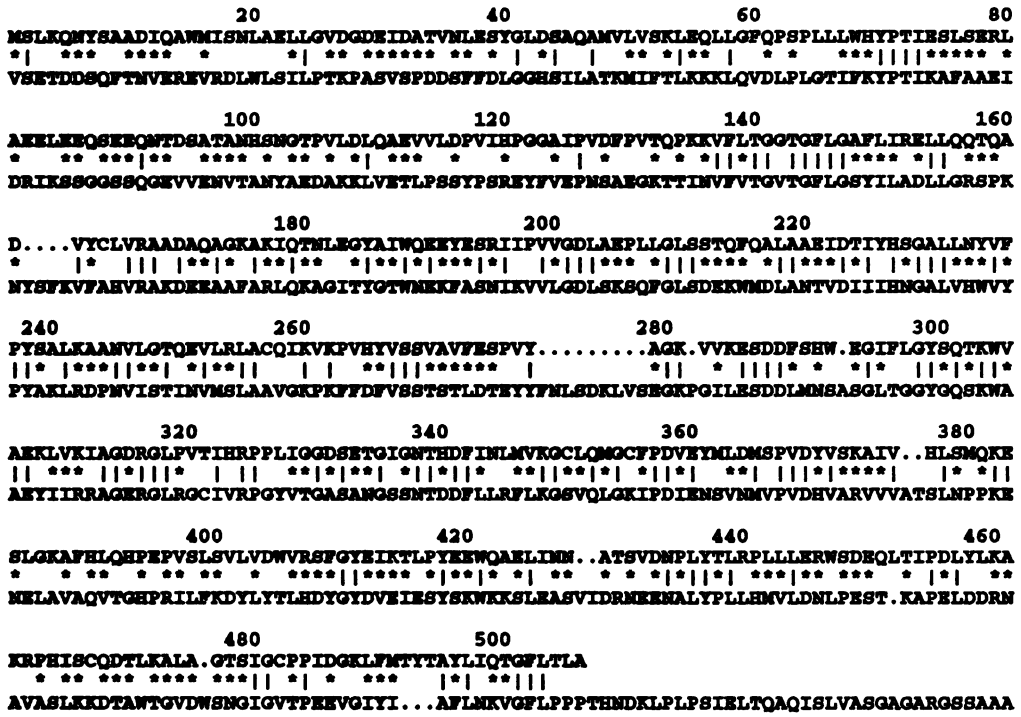
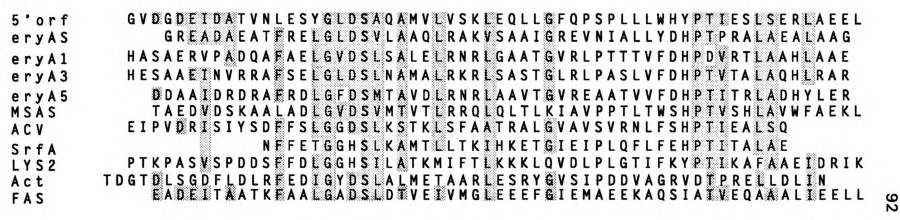


Figure 3.5 Alignment of the entire protein encoded by the 5′ *orf* (shown on top) with the carboxy-terminal half of the LYS2 protein from *S. cerevisiae* (Morris and Jinks-Robinson, 1991), starting at amino acid residue 837. Identical residues are marked with an (|) and similar residues with an (*). The numbers shown above the sequence refer to the position within the translated 5′ *orf*.

Figure 3.6 Alignments of domains within the 5′ *orf*-encoded protein and (A) acyl carrier protein, (B) ketoreductase and (C) acyl-transferase domains from other proteins. eryA(S,1-5) represent the subunits within EryAI,-II and -III from *S. erythraea* (Cortes et al., 1990; Donadio et al., 1991; Donadio and Katz, 1992). MSAS represents domains from the 6-methylsalicylic acid synthase of *Penicillium patulum* (Beck et al., 1990), ACV is the α-aminoadipyl-cysteinyl-valine synthetase from *Acremonium chrysogenum* (a.k.a. *Cephalosporium acremonium* [Gutierrez et al., 1991]), SrfA is a domain from the surfactin synthetase from *B. subtilis* (Fuma et al., 1993), LYS2 is from *S. cerevisiae* (Morris and Jinks-Robinson, 1991), Act is the ACP from the actinorhodin PKS of *S.coelicolor* (Hallam et al., 1988), and FAS is the FAS-ACP domain from *A.thaliana* (Lampa and Jacks, 1991) in (A) and the 2-oxoacyl reductase from the chicken FAS (Chirala et al., 1989) in (A). The serine residue shown in bold type in (A) represents the putative 4′-phosphopantetheine attachment site. Residues showing the greatest conservation are shaded.


A
5'orf GVDGDEIDATVNLESYGLDSAQAMVLVSKLEQLLGFQPSPLLLWHYPTIESLSERLAEEL
eryAS GREADAEATFRELGLDSVLAAQLRAKVSAAIGREVNIALLYDHPTPRALAEALAAG
eryA1 HASAERVPADQAFAELGVDSLSALELRNRLGAATGVRLPTTTVFDHPDVRTLAAHLAAE
eryA3 HESAAEINVRRAFSELGLDSLNAMALRKRLSASTGLRLPASLVFDHPTVTALAQHLRAR
eryA5 DDAAIDRDRAFRDLGFDSMTAVDLRNRLAAVTGVREAATVVFDHPTITRLADHYLER
MSAS TAEDVDSKAALADLGVDSVMTVTLRRQLQLTLKIAVPPTLTWSHPTVSHLAVWFAEKL
ACV EIPVDRISIYSDFFSLGGDSLKSTKLSFAATRALGVAVSVRNLFSHPTIEALSQ
SrfA NFFETGGHSLKAMTLLTKIHKETGIEIPLQFLFEHPTITALAE
LYS2 PTKPASVSPDDSFFDLGGHSILATKMIFTLKKKLQVDLPLGTIFKYPTIKAFAAEIDRIK
Act TDGTDLSGDFLDLRFEDIGYDSLALMETAARLESRYGVSIPDDVAGRVDTPRELLDLIN
FAS EADEITAATKFAALGADSLDTVEIVMGLEEEFGIEMAEEKAQSIATVEQAAALIEELL
B
5'orf: PVTQPKKVFLTGGTGFLGAFLIRELLQQTQADV
eryA2: YAVPGGTILVTGGTAGLGAEVARWL.AGRGAEH
eryA3: SWTPHGTVLVTGAASPVGDQLVRWL.ADRGAER
eryA4: PVDPDGTVLITGGTGTLGRLLARHLVTEHGVRH
MSAS: LPRPEGTYLITGGLGVLGLEVADFLVEKGARRLL
FAS: SCPPTKSYIITGGLGGFGLELAQWLIERGAQKLV
LYS2: EGKTTINVFVTGVTGFLGSYILADLLGRSPKNY
C
5'orf: TIHRPPLIGGDSETGIGNTHDFINLMVKGCLQM
LYS2: CIVRPGYVTGASANGSSNTDDFLLRFLKGSVQL
eryAS: FGVTPDAVVGHSIGELAAAHVCGAAGAADAARA
MSAS: NGITPQAVIGHSVGEIAASVVAGALSPAEGALIVT

Preliminary alignments with the translated protein from this *orf* also show similarity to PKSs (data not shown). Another apparent *orf*, also only partially sequenced, is found to extend beyond the 3′ end of the fragment shown in Figures 3.1 and 3.2.

Attempts to inactivate the *hetI*. The *hetN* gene alone, but not together with *hetI*, on a replicating plasmid, inhibited heterocyst differentiation. To examine the possibility that *hetI*, or its product, regulates *hetN* or its product, I attempted to inactivate *hetI*. None of the insertion mutations attempted yielded fully segregated double recombinants despite multiple rounds of growth and selection for sucrose-resistant colonies.

DISCUSSION

Testing that a transposon insertion is the cause of an observed phenotype is normally essential when working with *Anabaena* sp. strain PCC 7120 (see Liang et al., 1992; 1993; and personal observations). Confirmation that the original Het$^-$ phenotype of mutant N10 was due to the Tn*5*-1063a insertion was apparently achieved when the reconstructed mutant (DR921) also proved to be Het$^-$. However, when the transposon was replaced with a variety of other insertions, the predominant phenotype was the production of multiple (i.e., two or more) contiguous heterocysts, a result that contrasts with the original phenotype. Moreover, the presence of the wild-type *hetN* gene on a replicating vector failed to complement the (original) Het$^-$ N10 mutation or its reconstructed version, DR921. In fact, the wild-type *hetN* gene inhibited the

formation of heterocysts in wild-type cells when it was the only *Anabaena* DNA present in pRL982. Partial deletions of, or an insertion within, *hetN* alleviated the suppression of heterocyst formation in wild-type cells, indicating that suppression required the product encoded by *hetN*.

Several explanations could account for these results. (i) Perhaps a second-site mutation that occurred during the original transposon mutagenesis led to the Het$^-$ phenotype of N10. In that case, however, the same (or a phenotypically identical) mutation must have arisen during the reconstruction of the mutation in DR921, a result that seems improbable. However, the inhibition of heterocyst formation seen when *hetN* is carried on a plasmid suggests that *hetN* may be involved in repressing heterocyst differentiation and may be essential for preventing all vegetative cells from differentiating. There may, therefore, be very strong selective pressure for second-site Het$^-$ mutations in N10 and DR921 if the transposon-mutated cells are to survive and grow. Indeed, double recombinant insertions within *hetN* were more readily obtained in a *hetR* strain, which was already Het$^-$. Why the other insertion mutations that were generated did not also lead to a Het$^-$ phenotype is unclear. Perhaps *hetN* retains partial function when differently interrupted. Although *hetR* and the gene that complements the P6 mutation were unable to rescue the Het$^-$ phenotype of N10, the occurrence of a differentiation-blocking mutation at an as yet unidentified locus remains possible.

(ii) The recovered clone of the N10 region may have contained an additional mutation. As mentioned above, the N10 mutant had a tendency to revert to

a strain that produced multiple contiguous heterocysts. Perhaps the DNA recovered from N10 contained such an additional mutation. The phenotypes of the subsequent double recombinant strains would have then been dependent upon the sites of recombination. This interpretation appears unlikely because the sequence of the DNA recovered from N10 matches, base for base, that of a wild-type clone that includes *hetN* and *hetI*.

(iii) Perhaps the transposon and the various other insertions had unique, *cis*-dominant effects that gave rise to the variety of phenotypes, e.g., by altering the expression of *hetI*. This explanation is tempting in view of the arrangement and proximity of *hetN* and *hetI*. My inability to inactivate *hetI* also suggests that it may play an essential role in maintaining vegetative growth: if *cis*-dominant effects of the interposons generated the Het$^-$ and Mch phenotypes, this could also explain why the wild-type clone was unable to complement the Het$^-$ mutations. I am tempted to attribute to *cis* interactions between *hetN* and *hetI*, the observation that plasmid pRL959, which contains those two *orf*s, did not inhibit heterocyst formation in wild-type cells whereas plasmid pRL982, which lacks *hetI*, did. However, the differing effects of pRL959 and pRL982 might also be attributed to the additional absence from pRL982 of a 352-bp sequence, upstream from *hetN*, that may contain regulatory sites. A different pDU1-based vector that contained both *hetI* and *hetN*, but stops 48 bp upstream from *hetN* at the *Cla*I site that terminates the sequence in pRL982, also failed to inhibit heterocyst differentiation (J. Golden, personal communication). This observation appears to strengthen the interpretation that

it is the presence of *hetI* on pRL959, and not the presence of bp -48 to -352 upstream from *hetN*, that is responsible for the loss of heterocyst suppression.

The protein encoded by *hetN* is similar to a large number of oxido-reductases. Most similar are synthases of PKs, PHB and FAs, including the FA moiety found on some NOD factors that are required for host-specific recognition of symbiotic bacteria by legumes. These proteins also contain regions that are highly conserved in the steroid hormone-modifying dehydrogenases (Baker, 1991). However, other proteins involved in dehydrogenase-like reactions also contain these highly conserved domains, including the proposed NAD(P)H binding site. Thus, sequence comparisons do not narrowly delimit the possible activities of the protein encoded by *hetN*.

The protein encoded by the nearby 5′ *orf* shows similarities to multifunctional PKS and FAS proteins, and particularly to domains within the EryA proteins from *S. erythraea*. It is of potential evolutionary interest that the putative transposase of insertion sequence IS*1136* that is found between the first and second subunits of the EryA PKS gene cluster (Donadio and Staver, 1993) is similar in sequence to that encoded by insertion sequence IS*891* of *Anabaena* (Bancroft and Wolk, 1989). The similarity of the 5′ *orf* to the LYS2 protein of *Saccharomyces cerevisiae* may reflect the similarity of the latter to PKSs and FASs, such as the presence of a possible 4′-phosphopantetheine attachment site and a domain that carries out an NADPH-dependent reduction of the bound substrate. The strong similarity between the 5′ *orf* and LYS2 may reflect similarities in the substrates that are bound to the enzymes. It is known

that PKSs can initiate with, or add, a variety of molecules to the growing acyl chain, which increases the diversity of the side chains that provide unique properties to the metabolites that are generated (Hopwood and Sherman, 1990).

The consortium of LYS2 and LYS5, referred to as α-aminoadipate reductase, carries out the NADPH-dependent reduction of α-aminoadipate to α-aminoadipate-δ-semialdehyde, a biosynthetic precursor of lysine in *S. cerevisiae* (Bhattacharjee, 1985). The similarity between LYS2 and the protein encoded by the 5′ *orf* begins at a putative 4′-phosphopantetheine attachment site (Figures 3.5 and 3.6A). The amino-terminal half of LYS2 is homologous to the synthetases that produce cyclic peptides, such as surfactin and gramicidin, and to δ-(L-α-aminoadipyl)-L-cysteine-D-valine (ACV)-synthetases (ACV is a precursor in the biosynthesis of penicillin). These enzymes appear to use a thiotemplate mechanism in which acyladenylated substrates are covalently attached to a 4′-phosphopantetheine cofactor; the cofactor supports the condensation and translocation of the growing peptide chain (Lipmann, 1980). The thiotemplate mechanism is analogous to the mechanism used by FASs and PKSs in which the 4′-phosphopantetheine of the ACP or ACP domain supports the condensation and translocation of growing acyl chains (Hopwood and Sherman, 1990). Consequently, the putative cofactor attachment sites are conserved among the cyclic peptide synthetases, FASs and PKSs, particularly around the highly conserved serine residue that provides the ester linkage to the cofactor (D'Sousa et al., 1993; Hopwood and Sherman, 1990; Lipmann,

1980). In fact, PKS ACPs can be functionally replaced by FAS ACPs (Khosla et al., 1992).The possible attachment site in the protein encoded by the 5' *orf* shows greater similarity to ACPs and ACP domains found in PKSs and FASs than it does to LYS2 and cyclic peptide synthetases (Figure 3.6A).

Because the protein encoded by the 5' *orf* may contain at least two functional domains, an ACP domain and a ketoreductase domain, it may be a type-I PKS. Furthermore, if the LYS2 protein maintains a covalently attached cofactor that has a thioester linkage to the substrate, then it may also possess thioesterase or acyltransferase functions that release the reduced substrate, since free α-aminoadipate is produced in vitro by α-aminoadipate reductase. Although the LYS5 protein could carry out this function, a domain within LYS2, and the 5' *orf* protein, shows similarity to the proposed acyltransferase domains from other PKSs. Thus, the protein encoded by the 5' *orf* may contain a third functional domain.

The protein encoded by *hetI* shows less similarity to other entries in the databases, but it is intriguing that it is most similar to a gene (*sfp*) that is proximal to, and involved in the regulation of, genes that encode structural proteins for the biosynthesis of a secondary metabolite, surfactin (Nakano et al., 1992). The late growth phase expression of genes whose regulation is dependent on the source of nitrogen may also be influenced by *sfp* (Marahiel et al., 1993). Interestingly, it appears that *sfp* strains can be complemented by an extracellular factor produced by sfp^+ strains (Marahiel et al., 1993). Another similar protein is encoded by *orfX* from (*B. brevis* [Krätzschmar et al.,

1989]), a presumptive gene that lies adjacent to, and may possibly regulate, the operon that encodes the proteins required for the biosynthesis of gramicidin (Krätzschmar et al., 1989; Turgay et al. 1992).

The sequence of *hetN* and of the DNA surrounding it raises new possibilities concerning the control of pattern formation in *Anabaena*. I conjecture that *hetN* and the 5′ *orf* encode, and *hetI* regulates, functions that are required for the synthesis of a PK, FA, or modified amino acid. Because most insertions within *hetN* result in the production of multiple contiguous heterocysts, while supernumerary copies of *hetN* suppress heterocyst formation, a substance produced or modified by the product of *hetN* may inhibit heterocyst differentiation. Such a substance may even mediate intercellular interactions that regulate development. Therefore, an endeavor should be undertaken to identify a substance whose synthesis is affected by the integrity, and presence on plasmids, of the *orf*s that I have studied. Reduced synthesis of the substance might lead to massive initiation of differentiation, which could in turn prevent vegetative growth, and thus be effectively lethal. My inability, to date, to inactivate *hetI* suggests that it is essential for maintaining vegetative growth. Perhaps *hetI* acts, via *hetN*, to inhibit differentiation. There is no indication, in *Anabaena*, that cell density affects the differentiation of heterocysts, other than in terms of growth-rate effects on spacing (Antarikanonda and Lorenzen, 1982; Fogg, 1949). A putative inhibitory metabolite may, therefore, remain within the filament.

Procaryotic secondary metabolites, some that have highly specific receptors,

have been found to control and coordinate cellular differentiation within populations of cells (Beppu, 1992). Such compounds are considered to be the evolutionary precursors to the hormones that play a vital role in coordinating eucaryotic metabolism, growth and differentiation. It will be of great interest to determine whether (and if so, how) a secondary metabolite acts intercellularly to control pattern formation within ancient, heterocyst-forming procaryotes.

Chapter 4

A STUDY OF INTERCELLULAR COMMUNICATION IN THE FILAMENTOUS CYANOBACTERIUM *Anabaena* SP. STRAIN PCC 7120: IMMUNOLOGICAL EVIDENCE FOR A GAP JUNCTION-RELATED PEPTIDE IN A PROCARYOTE

SUMMARY

The existence of intercellular channels between cells of *Anabaena* sp. has been examined by physical and immunological techniques. Fluorescence redistribution assays on filaments labeled with a variety of dyes were employed to assess intercellular movement of the dyes; however, no dye movement was visualized. Antibodies raised against the α-peptide of the 26-kD rat liver gap junction protein were used to probe protein extracts from *Anabaena*. Primarily a 52-kD protein was labeled by the antibodies. The 52-kD protein was localized to sedimented fractions of crude extracts, and appeared to be enriched within isolated plasma membrane fractions.

INTRODUCTION

Intercellular interactions are clearly required for pattern formation in *Anabaena* (Adams, 1992; Wilcox et al., 1973a,b; Wolk, 1967; Wolk and Quine, 1975). Nonetheless, the means by which cells communicate is essentially unknown. Speculation on the existence of cytoplasmic continuity between cells in filamentous cyanobacteria began years ago when the dyes methylene blue (Brand, 1901; Schmid, 1923) and ruthenium red (Metzner, 1955) were reported to move through filaments via the cell septa.

Drews (1959) reported that filaments of *Phormidium uncinatum*, a motile, non-heterocyst-forming organism, reversed their direction of movement when the front 10% of the cells in the filament were shaded. Shading the rear cells had no effect. The generation of an electrochemical potential, induced by light and mediated by fluxes of protons and of calcium ions, and propagated along the filaments through low resistance channels (Häder, 1978,1981; Häder and Poff, 1982), appears to be responsible for controlling the direction of movement. Similarly, illumination of a portion of the filament was found to generate a membrane potential that was rapidly propagated along the entire filament (Severina et al., 1988). Low resistance electrical coupling, shown thus to occur in cyanobacteria, is mediated by gap junctions in animal cells and by plasmodesmata in plants (Goodwin, 1976).

More evidence for the existence of structures mediating intercellular communication in *Anabaena* was obtained from thin section electron micrographs (Lang and Fay, 1971; Wildon and Mercer, 1963). These

micrographs showed intercellular connections that were 5 - 20 nm in diameter and ≥ 40 nm long. These connecting elements, called microplasmodesmata after their presumed plant counterparts (Clowes and Juniper, 1968), may provide aqueous channels that mediate cytoplasmic continuity between cells. Freeze fracture electron micrographs display pits on the protoplasmic fracture face and protrusions on the exoplasmic fracture face of plasma membranes (Giddings and Staehlin, 1978), characteristics that are consistent with the premise that protein complexes traverse the cytoplasmic membrane and cell wall.

During a survey of a number of filamentous species (Giddings and Staehlin, 1981), it was noted that heterocyst-forming species tend to form a greater number of microplasmodesmata between vegetative cells (100 to 250 per cell) than do non-heterocyst-forming species (30 to 40 per cell). This finding was interpreted as meaning that heterocyst-forming species require a greater degree of intercellular communication, and that the increased presence of microplasmodesmata may fulfill this requirement. The same study showed that there are 80% fewer microplasmodesmata between vegetative cells and heterocysts than between two vegetative cells, an observation that is consistent with the fact that the area of contact at the septa between heterocysts and vegetative cells is approximately one-tenth of that between neighboring vegetative cells. The decreased contact area and fewer microplasmodesmata may prevent excessive entry, into the heterocyst, of oxygen that could decrease the nitrogenase activity within the heterocyst.

Although the observations presented are consistent with the idea that microplasmodesmata exist and confer cytoplasmic continuity, the actual existence of microplasmodesmata remains in doubt. Furthermore, little additional work has been performed to characterize presumptive microplasmodesmata despite their potential importance in regulating cell-cell interactions in *Anabaena*. Gap junctions and plasmodesmata are structures that provide cytoplasmic continuity between animal and plant cells, respectively (Robards, 1976; Unwin and Zampighi, 1980). The passage of regulatory molecules and metabolites through these channels allows cells to interact and function in a synchronized manner. My study of microplasmodesmata was inspired by investigations that demonstrated antigenic relatedness of the proteins that constitute gap junctions and the proteins that are thought to constitute plasmodesmata (Meiners and Schindler, 1987, 1989; Yahalom et al., 1991). Transport via plasmodesmata and gap junctions is also regulated by similar factors, such as calcium ions and calmodulin-dependent protein kinases, pH, and phorbol esters (Baron-Epel et al., 1988; Rink et al., 1980; Wade et al., 1986), providing further evidence of their similarity. Therefore, the evolution of intercellular channels may have an early phylogenetic history. Consequently, I inspected *Anabaena* cells for a protein immunologically related to a gap junction protein, and thereby to the putative plasmodesmatal protein, and also looked for intercellular movement of fluorescent dyes.

MATERIALS AND METHODS

Growth of strains. The laboratory strain *Anabaena* sp. strain PCC 7120 was maintained in liquid culture with Allen and Arnon medium diluted eight-fold (AA/8 [Hu et al., 1981]). 5 mM nitrate (2.5 mM $NaNO_3$ + 2.5 mM KNO_3) was added to repress heterocyst formation and maintain vegetative growth of filaments. Cells were grown at 30°C with gentle shaking under continuous illumination. To obtain heterocyst-containing filaments, nitrate-grown cells were washed once and then resuspended in AA/8 to give a final concentration of 0.3 μg of chlorophyll *a* (Chl *a*) ml^{-1}. The cells were allowed to grow under normal conditions for 24-28 hours. Mature heterocysts were present after 20 hours.

Protein isolation. Cells grown in AA/8 + NO_3^- were washed and then concentrated 10-fold in lysis buffer (50 mM Tris [pH 7.5], 0.1 M NaCl, 0.5 mM $CaCl_2$). Cells were lysed by cavitation, and cell wall material and unlysed cells were removed by sedimentation at 3000 X g for 10 min. The supernatant solution was used as the crude extract. For further fractionation, the crude extract was centrifuged at 100,000 X g for one hour; the supernatant solution was retained and the pellet was resuspended in the lysis buffer. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE; 12% acrylamide) under reducing conditions (Laemmli, 1970). For western blot analysis, proteins were transferred to nitrocellulose using a Gelman Bio Trans semidry electrophoretic transfer unit (Gelman, Ann Arbor, MI) as described by the manufacturer. After transfer, the nitrocellulose was washed in TBS (20 mM Tris [pH 7.5], 0.5M NaCl) and then blocked for 2 hours in TBS

+ 5% BSA. The filter was washed in TBS + 0.05% TWEEN 20 and then rinsed in distilled water. Antibody raised against a 27-amino acid peptide (α-peptide) from the rat liver gap junction 26-kD polypeptide (connexin32; Meiners et al., 1991; provided by Dr. Melvin Schindler, Dept. of Biochemistry, Michigan State University, East Lansing) was diluted 1:50 (final concentration: 10 μg/ml) in TBS + 1% BSA and incubated with the filter overnight at 4°C. The filter was washed in TBS + 0.05% TWEEN 20, incubated with alkaline phosphatase-linked goat anti-rabbit antibody (purchased from Sigma Chemical Company, St. Louis, MO), and developed according to the manufacturer's instructions.

Isolation of plasma membrane fractions. Plasma membranes were isolated as described by Murata and Omata (1988). Approximately 3 grams of cells (wet weight) were washed with 60 ml of 10 mM TES (pH 7.0) and resuspended in 30 ml of spheroplast buffer (10 mM TES [pH 7.0] + 600 mM sucrose + 2 mM EDTA), and 0.05% (w/v) of lysozyme then added. Digestion with lysozyme was carried out under growth conditions for two hours; the cells were collected by centrifugation at 5000 X g for 10 min, and then washed twice with extraction buffer (20 mM TES [pH 7.0] + 600 mM sucrose). The final pellet was resuspended in 30 mls of ice cold extraction buffer and passed through a French press at 5800 psi. The cell extract obtained from the French press was incubated 15 min with DNaseI by adding 0.1 ml of 3% PMSF and 0.05 ml of DNase buffer (10 mM NaOAc [pH 5.6] + 1 mM $MgCl_2$ + 0.1% [w/v] DNaseI). Cell debris was removed by centrifugation for 10 min at 5000 X g. The supernatant solution was adjusted to 50% (w/v) sucrose by adding

0.74 volumes of a 90% (w/v) sucrose solution containing 24 mM TES [pH 7.0] + 12 mM EDTA + 24 mM NaCl. Fifteen-ml portions of the cell extract, adjusted to 50% sucrose, were placed into 35-ml centrifuge tubes and discontinuous sucrose gradients were made by overlaying with 8 ml of 39%, 3 ml of 30%, and 7 ml of 10% sucrose solutions (w/v), each containing 10 mM TES (pH 7.0) + 5 mM EDTA + 10 mM NaCl + 1 mM PMSF. The sucrose gradients were centrifuged in a swinging bucket rotor at 130,000 X g for 16 hours at 4°C. A yellow-orange band (plasma membrane fraction) that formed within the 30% sucrose layer, and a green band (thylakoid membrane fraction) at the interface of the 39% and 50% sucrose layers were collected using a syringe, and pooled with the equivalent fractions collected from other tubes. The pooled material was diluted with 3 volumes of 10 mM TES (pH 7.0) + 10 mM NaCl, placed into a centrifuge tube, and sedimented for 1 hour at 300,000 X g. The pellets were resuspended in 10 mM TES (pH 7.0) + 10 mM NaCl. The purity of the fractions was established by measuring the absorption spectra of diluted portions of the resuspended membranes.

Labeling the filaments with fluorescent dyes. One-ml portions of rapidly growing filaments were washed and concentrated 10-fold in either AA/8 or AA/8 + NO_3^- media buffered at pH 7.3 with 5 mM HEPES (final cell concentration: 3 μg Chl *a* ml^{-1}). Fluorescent dyes were then added as described below. All the dyes, unless otherwise indicated, were provided by Dr. Melvin Schindler. The cells were incubated with the dyes under growth conditions. After incubation with the dyes, cells were washed 3 times with fresh media as

follows: sedimentation for one minute in a microcentrifuge was followed by rapid decanting and resuspension in one ml of fresh medium. The cells were kept at ca. 25°C both during and after the washings. Approximately 10 μl of each cell suspension was placed onto a microscope slide and covered with a coverslip. The edges of the coverslip were sealed with paraffin wax. Each slide was examined within five minutes.

Use of polar cytosolic dyes to measure cytoplasmic continuity. Carboxyfluorescein or fluorescein, each in the non-fluorescent, membrane-permeable diacetate form, was added to the cells at a final concentration of 10 μg ml^{-1}. Once inside the cells, the acetoxymethyl esters were cleaved by internal esterases to give the fluorescent products, carboxyfluorescein or fluorescein. When carboxyfluorescein was used to label developing heterocysts, vegetatively growing filaments were washed once in AA/8 and concentrated 10-fold in AA/8. Ten ug ml^{-1} of carboxyfluorescein was added, and filaments were placed under normal conditions of growth for 24 hours. 4-Methylumbelliferyl ß-D-galactopyranoside (Sigma) and 3-carboxyumbelliferyl ß-D-galactopyranoside (Molecular Probes, Eugene, OR) were added to filaments at a final concentration of 0.1 mg ml^{-1}. The carboxyl derivative was also added in media with pH's of 4.5, 5, and 6 (adjusted with 5 mM MES). The two umbelliferyl galactosides are non-fluorescent until cleaved by ß-galactosidase. To ensure the presence of high levels of ß-galactosidase, cells were used that contained the plasmid pRL544. pRL544 has the *lacZ* gene fused to the *tac* promoter which, although constitutively very active, can be induced 3-4 fold

by the addition of 10 μM IPTG (J. Elhai, personal communication, and my unpublished observations). Filaments were labeled with the dyes ca. five hours after IPTG had been added and fluorescence was semi-quantitatively analyzed in photographs by comparing ultraviolet light-induced fluorescence from spots of labeled cells and control spots of solution containing known concentrations of free umbelliferone.

Use of lipophilic membrane dyes to measure membrane continuity. N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-phosphatidylcholine (NBD-PC),4-(N,N-dihexadecyl)amino-7-nitrobenz-2-oxa-1,3-diazole (NBD-diC16amine) and 5(N-dodecanoyl)aminofluorescein were all added to filaments at a final concentration of 50 μg ml^{-1}. The mixtures of dye and filaments were shaken vigorously and examined.

Examination of intercellular movement of dye. A Meridian Instruments ACAS 470 workstation was used to measure fluorescence and the cell to cell movement of the dyes. This instrument contains a 5-watt argon laser that generates narrow band excitation beams to induce fluorescence of the dyes. To examine a field, the stage moved at a speed of 2 cm s^{-1} with a step size of 0.25 μm. At each step, the emitted light is detected by a photomultiplier tube that generates quantitative electronic signals. The signals are accumulated by a microprocessor that generates a false color image of the labeled cells indicating relative fluorescence. A 530/540 nm narrow band-pass filter was placed in front of the photomultiplier tube to block autofluorescence. Control experiments (data not shown) showed only much lower levels of fluorescence

from unlabeled filaments.

To examine the movement of the dyes, the fluorescence redistribution after photobleaching (FRAP [Baron-Epel et al., 1988]) technique was utilized. In this technique, a single cell within a filament is bleached by a high intensity laser beam. The recovery of fluorescence within the bleached cell is then followed by a series of scans with a lower intensity beam. The timed scans, in my case, 1 to 2 minutes between scans, reveal any changes in intensity of fluorescence of the cells as a function of time. Recovery of fluorescence in a bleached cell indicates that dye is diffusing into the cell from adjacent cells. Therefore, a decrease in fluorescence from within the adjacent cells is expected. At least 50 individual filaments labeled with carboxyfluorescein, 20 filaments labeled with fluorescein, and 20 filaments labeled with NBD-PC were examined.

RESULTS

Immunological analysis of *Anabaena* proteins. To determine whether *Anabaena* filaments contain a polypeptide similar to a gap junction polypeptide, antibodies raised against, and purified with, the α-peptide from rat liver gap junction connexin32 were used to probe crude extracts from vegetative filaments of *Anabaena* sp. strain PCC 7120. Figure 4.1 depicts *Anabaena* proteins and rat liver gap junction proteins separated by electrophoresis on a 12% SDS PAGE gel and probed with antibodies against the α-peptide. A number of protein bands are seen: rat liver gap junction has bands at 27, 50, 70, and 100 kD, and *Anabaena* has bands at 25, 48, 68, 92, and 100 kD.

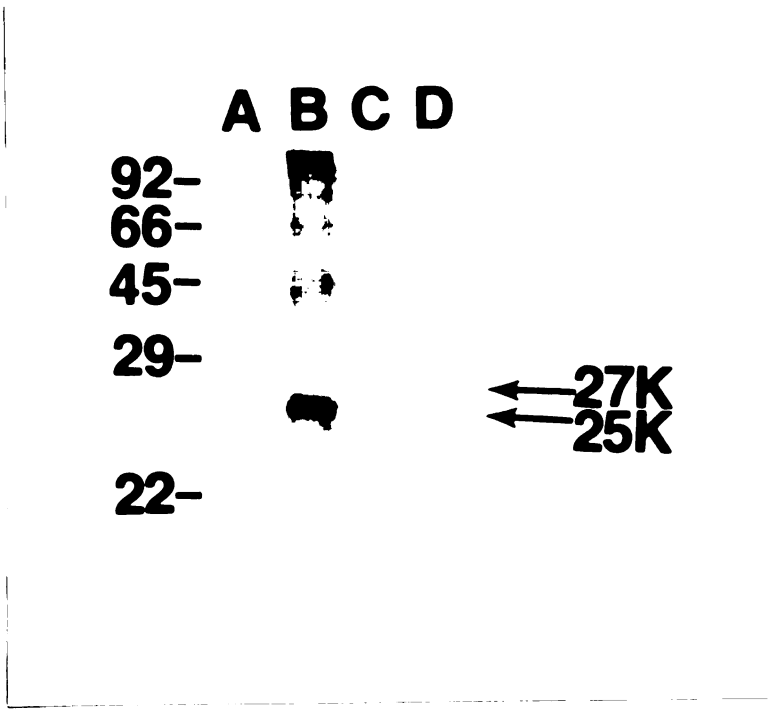


Figure 4.1 Proteins resolved on an SDS-PAGE gel, transferred to nitrocellulose, and decorated with antibodies raised against the α-peptide of the rat liver gap junction (10 μg ml^{-1}) in lanes A and B or preimmune serum (25 μg ml^{-1}) in lanes C and D. Lanes A and C contain fractions with rat liver gap junction (15 μg each) and lanes B and D contain crude extracts of *Anabaena* (60 μg each). The positions of molecular weight standards are shown to the left. The rat liver gap junction fraction was provided, and the western blot was performed, by Sally Meiners, Dept. of Biochemistry, Michigan State University.

The similarity in the pattern of the aggregates formed is striking. Immunological cross-reactivity with the anti-rat liver gap junction antibody and aggregate formation has also been seen for proteins from a variety of higher plant extracts (Meiners and Schindler, 1987, 1989). However, I was never able to obtain a similar result showing the pattern of aggregate formation. In my experiments, a 52-kD protein band was consistently found in crude extracts of *Anabaena*, often along with a 45-kD band and occasionally another band at 50 kD. The 52- and 45-kD bands were found to be present in the pellet of crude extracts that had been subjected to high speed centrifugation and virtually absent from the soluble fraction of the same extract (Figure 4.2). The same protein bands along with a 27-kD protein were enriched in plasma membrane fractions, and absent from the thylakoid membrane fractions (Figure 4.3). The purity of the membrane fractions was ascertained by measuring the absorption spectra of the fractions. The spectra (Figure 4.4) are nearly identical to those reported by Murata and Omata (1988).

Examination of fluorescent labeling and FRAP. Vegetative filaments labeled with carboxyfluorescein are shown in Figure 4.5A. The false color image displays two filaments that cross each other, as well as a single cell. The filaments demonstrate a high degree of labeling, which shows that carboxyfluorescein is able to enter the cells and is maintained within them for up to 3 hours. Figure 4.5B shows the same filaments after photobleaching. The two cells that were bleached show a significant decrease in fluorescence, while the remainder of the cells appear unchanged. Figure 4.5C shows the filaments

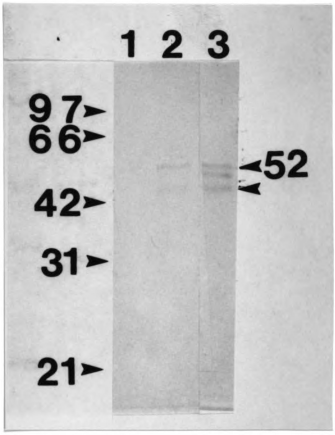


Figure 4.2 Proteins from *Anabaena* resolved on an SDS-PAGE gel, transferred to nitrocellulose, and decorated with antibodies raised against the α-peptide of the rat liver gap junction (10 μg ml^{-1}). Each lane was loaded with 30 μg of protein. Lane 1 contains the soluble fraction of proteins from crude extract that was subjected to high speed centrifugation while lane 2 contains the sedimented proteins from the same sample. Lane 3 contains crude extract that was not fractionated further. The arrows on the left indicate the positions of molecular weight standards. The arrows on the right indicate the positions of the protein bands typically labeled by the antibody.

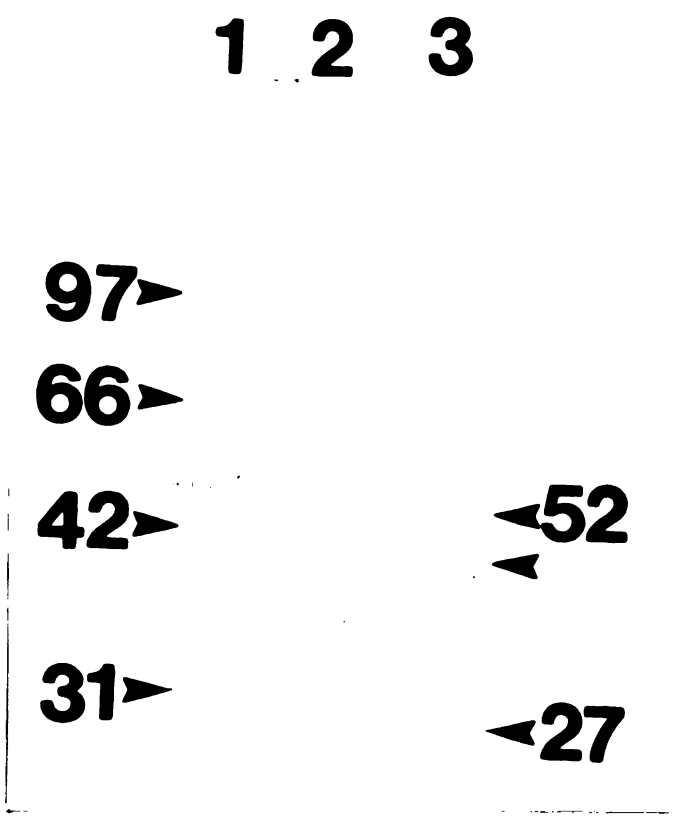


Figure 4.3 Proteins from: 1) crude extract, 2) thylakoid membrane fraction, and 3) plasma membrane fraction of *Anabaena* resolved on an SDS-PAGE gel, transferred to nitrocellulose, and decorated with antibodies raised against the α-peptide of the rat liver gap junction (10 μg ml^{-1}). Each lane was loaded with 30 μg of protein. The positions of molecular weight standards are shown on the left. Arrows indicating the positions of labeled protein bands found in the plasma membrane fraction are shown on the right.

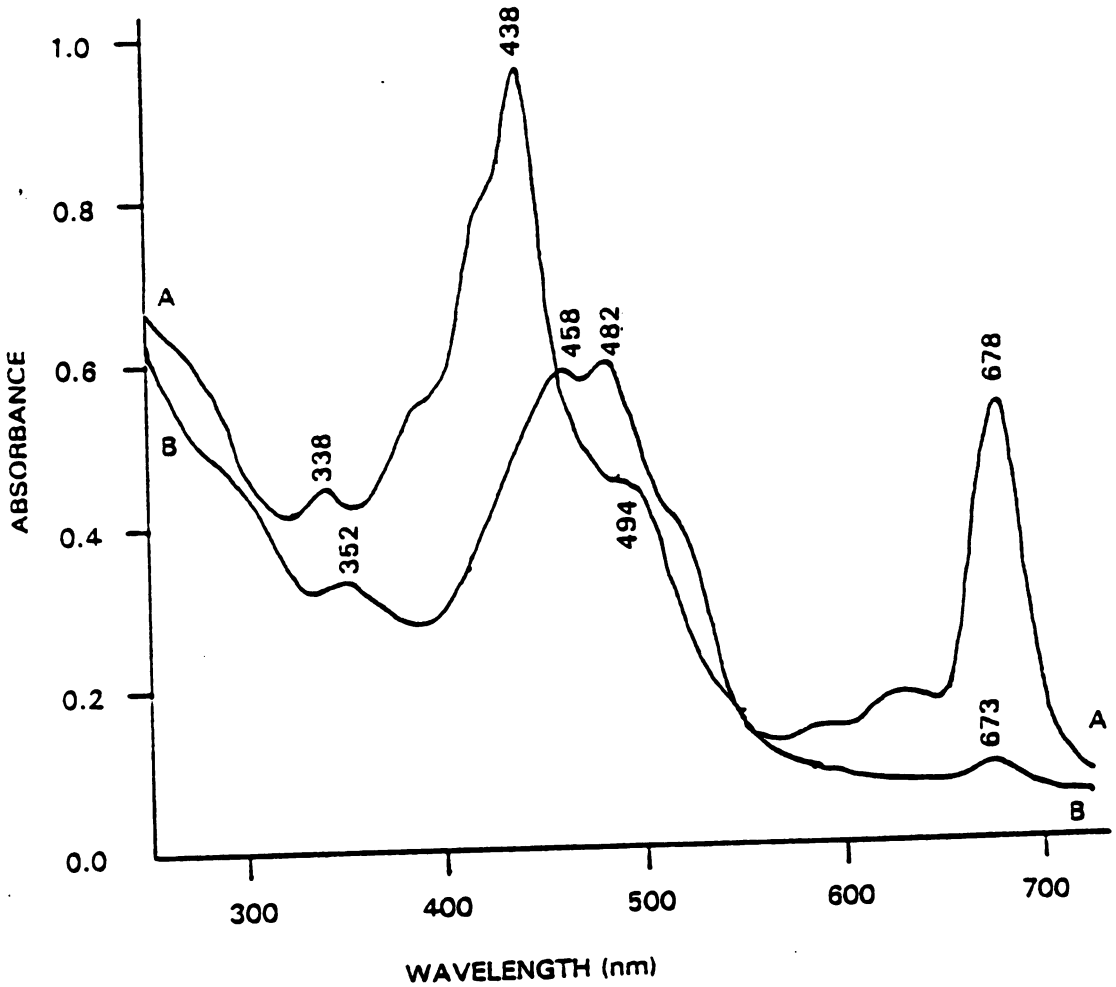


Figure 4.4 Absorption spectra of (A) thylakoid and (B) plasma membrane fractions from *Anabaena* sp. strain PCC 7120. The spectra were measured at room temperature (ca. 28°C).

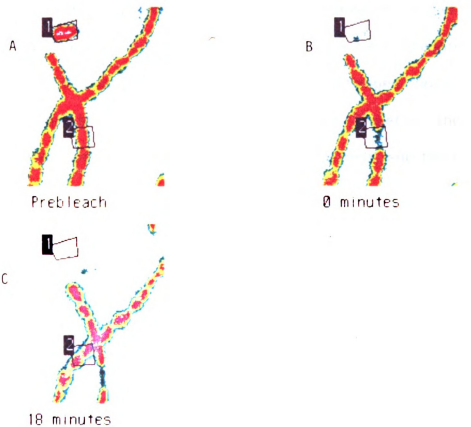


Figure 4.5 Vegetative filaments of *Anabaena* sp. strain PCC 7120 labeled with carboxyfluorescein diacetate as presented by the ACAS 470 workstation. Two separate, crossing filaments as well as a single cell (box 1) are shown (A). The fluorescence from the same filaments is shown immediately after photobleaching the cells in boxes 1 and 2 (B). The photobleached cells show a significant decrease in fluorescence. Fluorescence of the same filaments 18 minutes later (C) shows no recovery of fluorescence within the photobleached cells. Note that the filaments have moved slightly and the photobleached cells are no longer contained within the boxes. (Magnification = ca. 1500x)

after 18 minutes of recovery. It is apparent that no recovery of fluorescence has occurred within the bleached cells.It should also be noted here that neither of the bleached cells loses any additional fluorescence after bleaching. Therefore, the bleaching process does not appear to disrupt the membranes, a consequence that would allow dye to leak out of the cells. The intensity of fluorescence of the unbleached cells also remains unchanged, indicating that the cells are not bleached by the scanning process. Therefore, the overall procedure does not appear to be detrimental to the cells, and the dyes are sufficiently photostable for use in the assay.

Carboxyfluorescein labeling of a filament containing a heterocyst is shown in Figure 4.6. The vegetative cells are highly labeled, whereas the heterocyst displays a low level of fluorescence. Even filaments that are incubated with the dye for up to five hours have only weakly labeled heterocysts. Therefore, carboxyfluorescein does not appear to enter heterocysts either from the medium or from the adjacent vegetative cells. The assay of photobleaching of heterocysts or vegetative cells in these filaments gave the same result as with vegetative filaments: no movement of the dye was observed.

In an attempt to obtain labeled heterocysts, vegetative filaments were induced to form heterocysts in the presence of carboxyfluorescein diacetate. Although normal heterocysts were formed, no label was seen within them. Hence, the metabolism of the cells and their ability to differentiate is not affected by the dye.

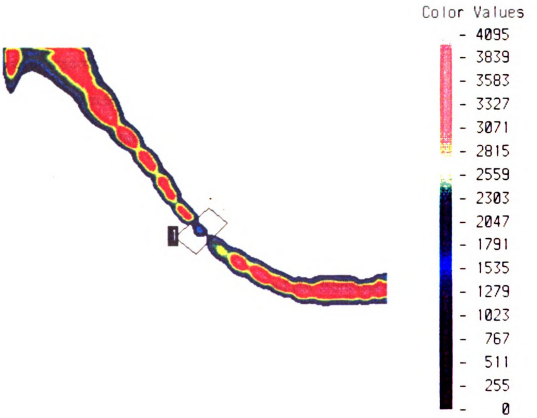


Figure 4.6 A single filament of *Anabaena* sp. strain PCC 7120, grown in nitrogen-free medium and containing a heterocyst (box 1), which was then labeled with carboxyfluorescein diacetate. The heterocyst is very weakly labeled in comparison to the vegetative cells within the filament. The color bar at the right displays relative values of fluorescence. The increasing size of the vegetative cells at the ends of the filament is due to over- and under-focusing, the result of the narrow depth of focus at high magnifications. (Magnification = ca. 3000x)

Because the negative charge on the carboxyl group of the carboxyfluorescein molecule may have prevented movement of the dye between cells, the FRAP assay was attempted using fluorescein diacetate. The experiment was more difficult to perform because fluorescein diffuses out of the cells with a half-time of 10-15 min. Nonetheless, the results of experiments with fluorescein (data not shown) did not differ from those obtained with carboxyfluorescein.

The other cytosolic dyes utilized, methylumbelliferone and carboxyumbelliferone were not helpful in measuring cell-cell communication in *Anabaena*. The methylumbelliferone diffused out from the cells into the medium much too rapidly ($t_{1/2} \leq 5$ min) to allow measurements to be made, whereas the carboxyumbelliferyl ß-D-galactopyranoside was never hydrolyzed to its fluorescent form and may not have entered the cells. The pKa of the carboxyl group is very low, so that the dye may have been too highly charged to penetrate the cell membrane.

Figure 4.7A shows vegetative filaments labeled with the lipophilic probe NBD-PC. The borders of the cells are intensely labeled, especially at the cell septa, suggesting that the membranes are labeled. However, it is also possible that the outer membrane or cell wall may have absorbed the dye. The lower level of intensity of fluorescence in the interior of the cells suggests that NBD-PC does not enter the cells and label the thylakoid membranes. After photobleaching and scanning, the dye is rapidly lost into the medium (Figures 4.7B and 4.7C). Dispersion of the dye, perhaps a result of hyperpolarization of the membranes due to the intensity of the scanning light, probably makes NBD-

PC and similar dyes useless for further studies of *Anabaena*. Although the NBD-PC should be isolated from the labeled cells to ensure that the dye is intact in these cells and not metabolized to another form. Likewise, labeled cells should be washed with salt solutions or other solvents to ensure that the dye is, in fact, labeling the cytoplasmic membrane of the cells. Figure 4.8 shows a heterocyst-containing filament that was labeled with NBD-PC. The heterocyst does not show any preferential degree of labeling despite the presence of a thickened glycolipid layer.

DISCUSSION

Intercellular communication in plant and animal cells is mediated by protein channels called plasmodesmata and gap junctions, respectively (Robards, 1976; Unwin and Zampighi, 1980). Structural and regulatory similarities between the two types of channels have been demonstrated (Baron-Epel et al., 1988; Rink et al., 1980; Robards, 1976; Unwin and Zampighi, 1908; Wade et al., 1986). Anti-gap junction antibodies have also shown that various plants contain an immunologically related peptide (Meiners and Schindler, 1987,1989; Yahalom et al., 1991). Although it has not been proven that the cross-reactive protein is associated with the

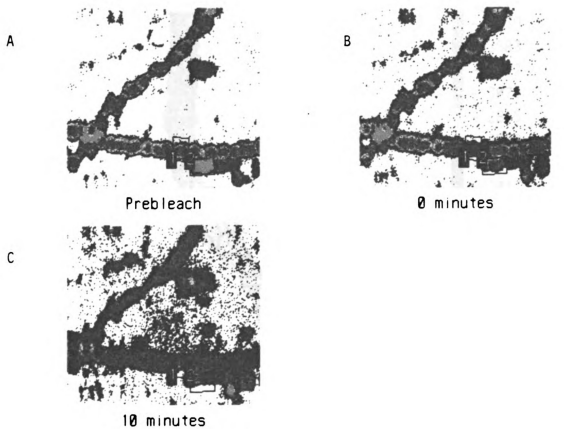


Figure 4.7 Vegetative filaments of *Anabaena* sp. strain PCC 7120 labeled with the lipophilic dye NBD-PC (A). Two filaments that cross as well as a three-celled filament that lies below and adjacent to the horizontal filament are shown. The fluorescence from the same filaments is shown immediately after photobleaching the cells in boxes 1 and 2 (B). The photobleached cells show a significant decrease in fluorescence. Fluorescence of the same filaments 10 minutes later (C) shows no recovery of fluorescence within the photobleached cells and shows that NBD-PC is lost to the medium. Note that the filaments have moved slightly and the photobleached cells are no longer contained within the boxes. (Magnification = ca. 1500x)

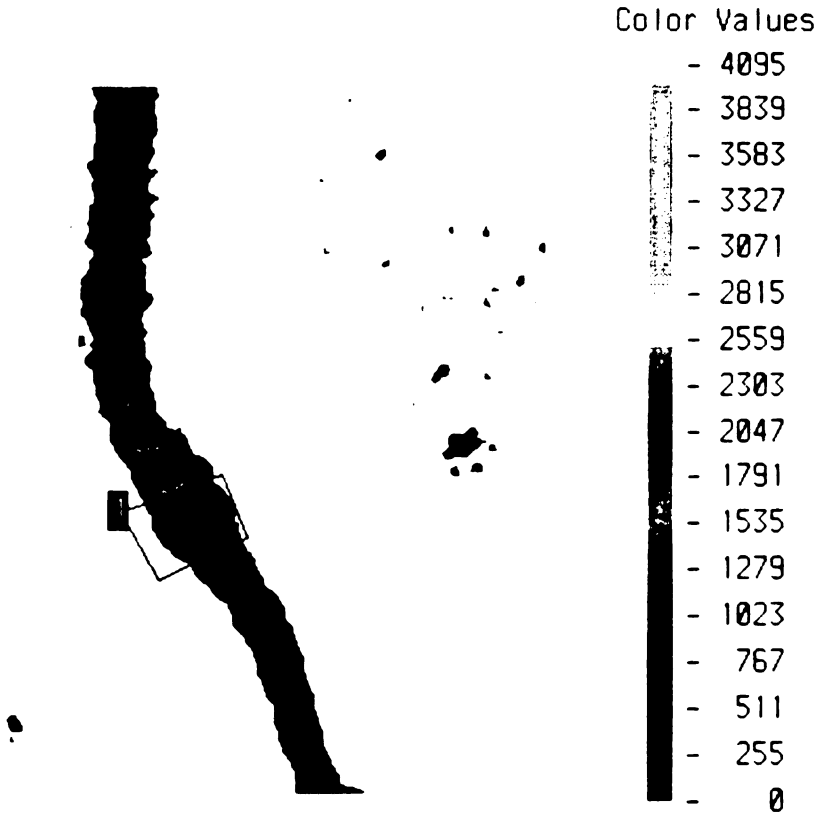


Figure 4.8 A single filament of *Anabaena* sp. strain PCC 7120, grown in nitrogen-free medium and containing a heterocyst (box 1), which was then labeled with the lipophilic dye NBD-PC. The slightly more intense areas of labeling correspond to the cell septa. (Magnification = ca. 3000x)

plasmodesmata, immunolocalization and cell fractionation strongly suggest that such is the case. Results from the current study on microplasmodesmata show that *Anabaena* also contains a protein with a peptide that is immunologically related to gap junction and plasmodesmatal polypeptides. The western blot performed by Sally Meiners (Figure 4.1) shows a similar behavior of aggregate formation by rat liver gap junction and the cross-reactive protein in *Anabaena*. The formation of aggregates by rat liver gap junction connexin32 has been well documented. The way in which samples are handled prior to loading on gels greatly influences the pattern of aggregation. Typically, extensive boiling of connexin32 in gel loading buffer leads to the formation of aggregates. Storage of frozen protein extracts has also been found to increase the presence of aggregates. It might also be that differences in the age of the reducing component of the sample buffer could result in different degrees of protein denaturation and of subsequent formation of aggregates. My results indicate that a 52-kD protein is consistently labeled and is present in cytoplasmic membrane fractions. It is possible that the 52-kD band represents a dimer of the 25-kD band seen in Figure 4.1 or of the 27-kD band seen in Figure 4.3. Whether the protein that gives rise to the 52-kD band is localized at the cell septa remains to be determined. The presence of a protein that is antigenically related to a gap junction protein poses the possibility that channel-forming proteins may provide conduits between cells in filaments of *Anabaena*. The α-peptide forms an alpha helix that is positioned within the cytoplasmic portion of the rat liver gap junction protein (Meiners et al., 1991). Therefore, the α-

peptide portion of connexin32 may be involved in interactions between the homohexamers that comprise a gap junction (Hertzberg and Johnson, 1988), or could be involved in regulating the channel. Either of these roles, and therefore the peptide sequence and structure, may have been conserved during evolution or obtained through convergent evolution. In more recent studies, Yahalom et al. (1991) found that antibodies raised against connexin32 decorated a 27-kD protein band (PAP27) that was present in both cell wall and membrane fractions from maize. The western blots for PAP27, like my own blots with extracts from *Anabaena*, did not show the formation of aggregates that is characteristic of connexin32, but there was an apparent 54-kD dimer band present in their blots. Immunolocalization of PAP27 showed that the neck region (cytoplasmic portion) of plasmodesmata was most highly labeled, suggesting that the antibodies recognize a cytoplasmic loop of PAP27 just as anti-α antibodies recognize a cytoplasmic domain of connexin32.

That plasmodesmata are aqueous channels that provide intercellular cytoplasmic continuity has been confirmed by a number of studies in which the intercellular movement of fluorescent probes was examined (Baron-Epel et al., 1988; Terry and Robards, 1987). Carboxyfluorescein as well as larger fluorescein derivatives (up to MW = 1000) have been shown to permeate plasmodesmata freely. The analogous experiment, using the FRAP assay, in filaments of *Anabaena* has failed to demonstrate any intercellular movement of these dyes. FRAP assays on plant cells showed that there were two rate components associated with recovery of fluorescence (Baron-Epel et al., 1988).

A short phase of recovery that occurred immediately following photobleaching which lasted for ca. 30 seconds was attributed to intracellular redistribution of unbleached dye. The second component was slower than the first, but still gave a measureable rate of recovery of fluorescence within one minute after photobleaching. By comparison, the length of time in which the filaments of *Anabaena* were examined (18 minutes in Figure 4.5) should have been more that sufficient for visualizing movement of the dye after photobleaching.

The external dimensions of plasmodesmata (diameter = 60 nm; Robards, 1976) are much larger than the dimensions of microplasmodesmata (diameter = 5 to 20 nm). However, the morphology of the plasmodesmata is quite complex, and a single plasmodesma may contain six to nine small pores (pore diameter = 1 to 2 nm; Baron-Epel et al., 1988; Terry and Robards, 1989) rather than one large single pore. Therefore, the actual pore size may not vary much between a plasmodesma and a microplasmodesma, especially if each microplasmodesma contains a single pore. Consequently, it is somewhat surprising that carboxyfluorescein does not move between the cyanobacterial cells if microplasmodesmata are in fact aqueous channels between cells.

The largest molecule that has been proposed to move between cells is a disaccharide from vegetative cells (Wolk, 1982), possibly sucrose (Schilling and Ehrnsperger, 1985), which may provide the heterocyst with a source of reductant required for nitrogen fixation. The molecular weight of sucrose (MW = 342; atomic radius = 0.44 nm) is just slightly less than that of carboxyfluorescein (MW = 378; atomic radius = 0.63 nm), although fluorescein

is a rigid molecule and has different charge characteristics. Work by Terry and Robards (1987) has shown that the hydrodynamic radius of a molecule is the sole factor affecting its ability to permeate plasmodesmata. It may be that sucrose is near the size exclusion limit of the microplasmodesmata and that fluorescein is too large to permeate these channels. It is also possible that sequestration of the fluorescein compounds within cells, possibly within the intra-thylakoid compartment or other compartments of *Anabaena* cells (if that is where the dyes had been deesterified) or by binding of the charged carboxyl group to cellular components, prevents movement of the dyes.

Heterocyst differentiation is known to be accompanied by extensive changes in cellular physiology. Lack of esterase activity in the heterocyst, which could have prevented labeling of the heterocyst, coupled with the slow loss of the dye out of all cells may explain why heterocysts are not labeled even when vegetative cells are labeled prior to the formation of heterocysts. However, failure to visualize labeling of the heterocyst might also be due to other differences associated with the heterocyst, such as the possible presence of compounds that can quench the fluorescence of fluorescein. Some of these possible explanations may be verified or refuted by isolating some of the cellular components and determining whether they show any preferential labeling by the dyes. Isolated heterocysts could be used to check for the presence of any compounds that may quench fluorescence of fluorescein, or dyes with different spectral properties could be used.

The only way to prove definitively that cytoplasmic continuity exists will be

to find a probe that cannot be specifically transported by cells but can move between cells. A probe with a radius that is smaller than or equal to that of sucrose would determine whether sucrose or other disaccharides can diffuse between cells. Unfortunately, bacterial cells are notoriously difficult to infuse with cytosol-specific dyes (i.e., polar dyes) because i) the small size of bacterial cells precludes the use of microinjection techniques, ii) the complex bacterial cell wall prevents the use of liposome fusions, and iii) very few dyes exist that are membrane-permeable and modified to a polar form within cells. To my knowledge, fluoresceins and umbelliferones are the only dyes available in membrane-permeable, hydrolyzable forms that can label the cytoplasm. I have shown that fluorescein and carboxyfluorescein do not move between cells and that umbelliferones diffuse out of cells too rapidly to allow analysis of their intercellular movement. In the case of probes of membrane potential, such as the rhodamine dye used by Severina et al. (1988), the electrical coupling that is observed lends strong support to the presence of aqueous channels, but is correlative and not definitive as ion currents could also propagate through contiguous membranes. However, if probes of membrane potential are used in conjunction with genetic and ultrastructural analysis, it may be possible to prove, or disprove, that electrical coupling occurs via microplasmodesmata.

Use of ^{14}C-labeled substrates has shown that substances do move between cells (Wolk, 1968); however, the method does not provide information as to how the movement occurs. It is possible that the movement is directed by specific transport processes. The gene that is disrupted in a heterocyst

developmental mutant, M7 (Ernst et al., 1992), in which the protoplasts of differentiating cells do not mature, has been found to encode a protein that is similar to the ATP-binding component of binding-protein-dependent transporters (I. Maldener, personal communication). Therefore, maturation of heterocysts may require the uptake of compounds from adjacent vegetative cells, with the uptake mediated by specific pumps rather than by diffusion through aqueous channels. Recently, Maddock and Shapiro (1993) showed that chemoreceptor complexes are specifically targeted to the cell poles in *E. coli*. They suggested that a structure at the cell pole, such as the periseptal annulus made during septation (Cook et al., 1987), may provide the target for localization. The septa of cells of *Anabaena* lie at the cell junctions. Therefore, a similar mechanism of protein targeting could place specific transporters at the junctions, and the transporters could mediate intercellular communication. The localization of porins at cell poles, where the cell wall is relatively thin, might also provide for electrical coupling between cells. A porin protein from *Anabaena variabilis* has been partially characterized (Benz and Böhme, 1985). It would be of interest to determine whether this porin, or other porins, is present primarily at the cell septa, and whether porins are required for heterocyst development. A mutant of *Anabaena* sp. has been found that does not adsorb a particular bacteriophage, and is incapable of aerobic nitrogen fixation (I. Khudyakov, personal communication). Perhaps the binding site for the phage is a porin protein that is required for heterocyst maturation.

In conclusion, the presence of a protein that contains a peptide

immunologically related to a gap junction protein, and the structural similarities found between microplasmodesmata, plasmodesmata, and gap junctions, make further studies on intercellular communication in *Anabaena* very attractive. However, the existence of microplasmodesmata has still not been proven. Nonetheless, the potential exists to utilize the genetic manipulability of *Anabaena* to examine the involvement of the putative microplasmodesmatal protein in cell-cell communication and perhaps to provide a model system for examining how plasmodesmata and gap junctions function. This study has shown that a cytosolic dye different from the ones used in this study, or a membrane potential dye, will have to be used in order to measure intercellular connectivity. Membrane potential dyes may also be useful, along with genetic analysis, in examining whether porins, which may provide interperiplasmic connections, rather than microplasmodesmata, which would provide intercytoplasmic connections, are required for intercellular electrochemical coupling.

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