MICROBIAL DEVELOPMENT

Edited by

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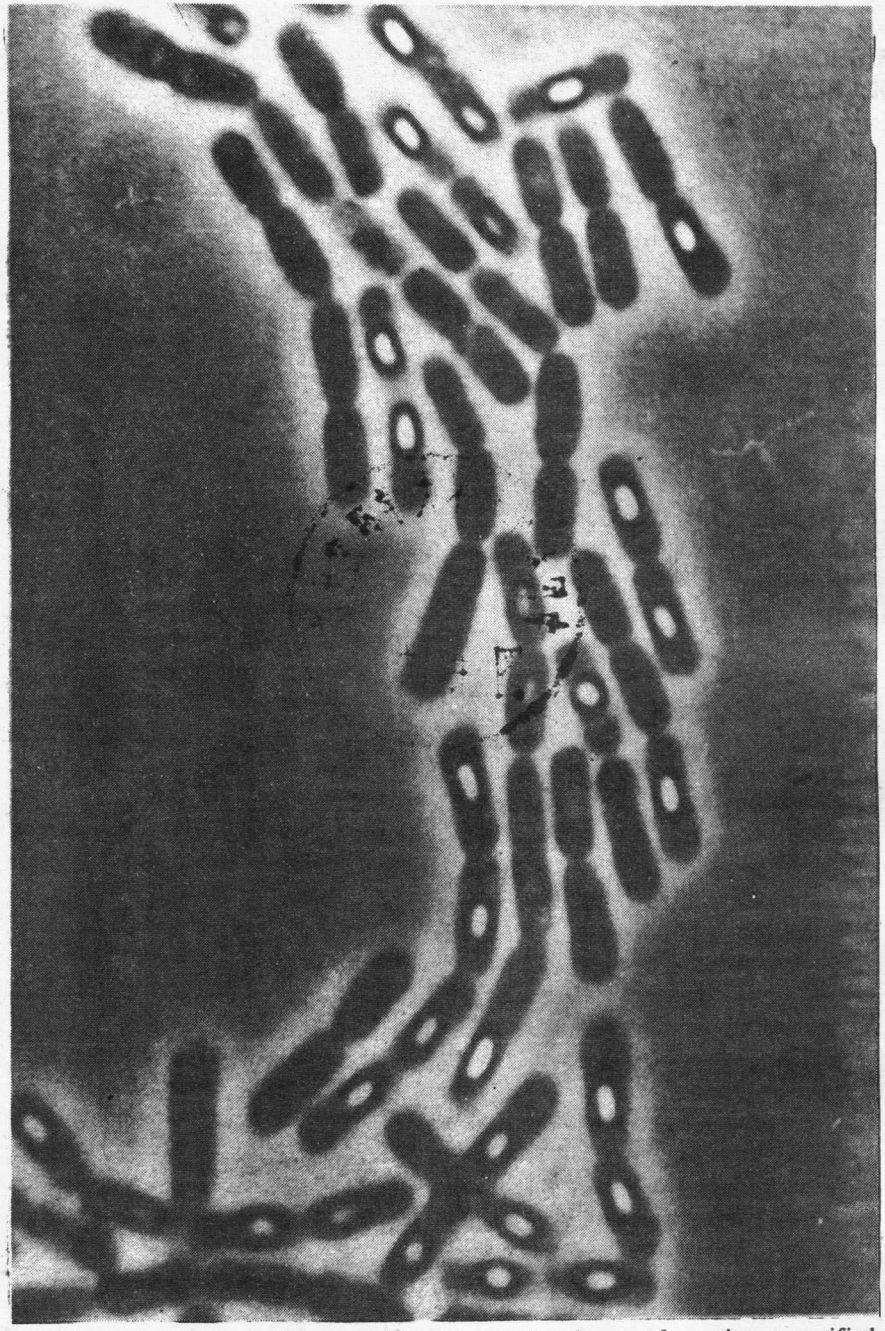

Cells of *Bacillus cereus* at early and late stages of endospore formation, magnified 3900 × under phase-constast microscopy. Developing prespores are visible as phase-bright bodies within the sporangia. (Courtesy of P.C. Fritz-James, University of Western Ontario, Canada.)

Preface

Microorganisms are exceptionally attractive systems in which to study development because of the facility with which they can be manipulated by genetic and biochemical techniques. This book brings together, in a single volume, reviews on prokaryotic and eukaryotic microbes that have proved to be successful systems for addressing general problems of cellular differentiation, cell-cell communication, and morphogenesis. A special feature of this volume is the comparison of similar biological problems posed by very different experimental systems, such as cell division in *Escherichia coli* and *Caulobacter*, spore formation in *Bacillus* and *Streptomyces*, cell-cell communication in *Streptococcus* and *Saccharomyces cerevisiae*, and social behavior in myxobacteria and *Distyostelium*. Despite this apparent diversity, a coherent set of themes pervades each paper: the role of nutritional signals in triggering development and adaptation, the way in which sets of genes are activated in ordered sequences during differentiation, polarity and the role of positional information, cell communication, and the transduction of sensory information.

This monograph derives in part and was indeed motivated by the Conference on Microbial Development held at Cold Spring Harbor Laboratory in the Spring of 1983. The success of that meeting and the sense of excitement felt by its participants was due to the fact that workers in diverse systems found themselves on common ground not only in the questions they ask and in the experimental approaches they employ, but in the manner by which both prokaryotes and eukaryotes have apparently solved the problems inherent in developing systems. Limitations of space have forced us to be eclectic in the systems that we have been able to represent here. Our bias has been to emphasize systems in which genetic manipulations are particularly facile and/or for which a sufficiently large community of investigators has made rapid

progress possible. We regret not being able to include several very important systems, such as heterocyst formation in *Anabena*, pattern formation in *Pseudomonas*, spore formation in *Aspergillus*, and cell division and differentiation in *Physarum*, which were the subjects of excellent presentations at the conference. (In this regard, we note here with great sadness the untimely loss to the *Physarum* field of its leading geneticist, Ned Holt, whose laboratory contributed mightily to rendering the acellular slime mold accessible to experimental manipulation.)

This monograph is organized to progress from relatively autonomous (in the sense of being internally driven and entirely self-contained) developmental systems, such as cell division, to environmentally induced adaptations, such as spore formation and chemotactic behavior and, finally, to relatively complex systems involving interactions between cells, such as mating, social behavior, and symbiosis. Ely and Shapiro and Donachie et al. set out the principal features of the cell-division cycles of *Caulobacter* and *E. coli*. *Caulobacter* is distinctive for the asymmetry of its binary fission process, which results in the formation of a sessile stalked cell and a motile swarmer cell. A unique feature of the swarmer daughter cell is its polar flagellum, a complex structure whose synthesis and assembly can be exploited, as reviewed by Ely and Shapiro, by genetic and biochemical means to approach the important problem of the role of positional information in cellular differentiation. Though lacking the positionally regulated structures of *Caulobacter, E. coli* offers extraordinarily powerful genetic tools and a wealth of background information on the structure and biochemistry of its cell envelope. Donachie shows how these tools are used to delve into the surprisingly intricate organization of genes ("morphogenes") that determine septum formation. It is worth noting that even in organisms as well studied as *Caulobacter* and *E. coli*, an understanding at the molecular level of a problem as basic to biology as cell division remains a formidable task.

In contrast to cell division, the developmental cycles of *Bacillus* and *Streptomyces* represent elaborate responses (leading to the formation of spores) to environmental signals (conditions of nutrient deprivation). *Bacillus* and *Streptomyces* differ dramatically in their morphology and in the way they differentiate; yet in evolutionary terms, these organisms are relatively closely related (both being gram-positive bacteria). In their reviews, Losick and Youngman and Chater emphasize the distinguishing features of development in *Bacillus* and *Streptomyces*, respectively, but at the same time the authors raise several issues common to both organisms, such as the role of nutritional deprivation in triggering differentiation and the course of events required to transform a vegetative cell into a spore. In addition, certain characteristic features of *Bacillus* and *Streptomyces* differentiation are extremely pertinent to developmental life cycles of other microorganisms considered in this volume. For example, asymmetric septum formation, a hallmark feature of *Bacillus* development, is strongly reminiscent in the questions it poses to the

polarity of the cell-division process in *Caulobacter* and budding yeast; likewise, the mycelial habit of *Streptomyces* is well suited to the degradation of insoluble organic debris through the accumulation of high local concentrations of extracellular hydrolytic enzymes, a situation strategically comparable to swarming in myxobacteria (the "microbial wolf pack effect") for the purpose of feeding on complex food sources in the soil.

Another kind of adaptive response to environmental cues is the movement of individual cells in response to chemical gradients (chemotaxis), the subject of the paper by Stock and Koshland. Chemotaxis is principally studied in unicellular bacteria, but this kind of behavior is also relevant, at least in a formal sense, to the coordinated movement of myxobacteria cells and *Dictyostelium* amoeba into aggregation centers. Indeed, as noted by Stock and Koshland, the selective pressures for optimal chemotatic responses of individual cells in a swarm of bacteria could promote the evolution of developmental systems that depend upon the integrated movement of groups of cells.

The microorganisms considered in the remaining six papers introduce a higher level of complexity in that development is shown to be dependent in part upon interactions between cells as mediated by chemical signals and/or by direct contact. Two simple examples (in the sense of involving small numbers of cells) are the mating interactions of *Streptococcus* and *Saccharomyces cerevisiae*, in which specific chemical signals, sex pheromones, induce developmentally appropriate responses leading to genetic exchange among aggregated cells in the case of bacteria and fusion to a diploid state in the case of yeast. As reviewed by Clewell et al. and Klar et al., research on these organisms is directed at understanding the way in which mating type is determined by mobile genetic elements (conjugative plasmids in the case of *Streptococcus* and transposable cassettes of mating-type information in the case of yeast). Yeast is especially remarkable for the many ways in which its mating system has come to be pertinent to general problems of development. As examples, the coupling of mating-type conversion to cell lineage is an important model system for understanding the way in which changes in cell type can be determined by programmed changes in genetic information; the selective capacity of experienced mother cells, but not of newly budded daughter cells, to undergo mating-type conversion as a result of the partitioning of the product of homothalism gene *HO* is a powerful system in which to study problems of polarity and positional information; finally, the ability of the products of yeast mating (diploid cells) to transform themselves into haploid ascospores in response to conditions of nutrient deprivation is, of course, a recurring theme of this volume.

Myxobacteria and *Dictyostelium* offer even more complicated examples of cell-cell interaction, in which the formation of complex multicellular structures (fruiting bodies) is dependent upon communication between and coordinated movement among many thousands of cells. Even though these microorganisms are separated by the prokaryotic/eukaryotic evolutionary divide,

myxobacteria and *Dictyostelium* are similar in many aspects of their social behavior, which represents one of the more notable examples of convergent evolution in biology. Nutrient deprivation induces cells of these microbes to aggregate into large masses, which are capable of integrated movement. These cell masses, in turn, form elaborate fruiting bodies, which are composed of two specialized cell types: stalk cells and spore cells. From an experimental point of view, an important focus of interest in these organisms is the way that differentiation among many cells is coordinated by means of intercellular communication during the course of development. The papers by Kaiser, Chisholm et al., and MacWilliams and David emphasize three distinct but complementary experimental approaches to this problem. As documented by Kaiser, an important advantage of *Myxococcus xanthus*, the most studied of the myxobacteria, is its accessibility to the techniques of classical and molecular genetics. Kaiser emphasizes the way in which these tools, as well as the remarkable phenomenon of extracellular complementation, can be used to investigate the basis of cell-cell communication during development. Important findings from this line of research are the identification of genetic loci responsible for specific kinds of intercellular communication and the identification of cell-envelope (peptidoglycan) components as a class of intercellular developmental signals. *Dictyostelium* has become a fruitful experimental system for studying cell-cell interaction because of its ready accessibility to physical manipulations, such as disaggregation and regeneration of slugs, histological staining methods, which serve to identify subsequent differentiated cell types, and the ability to isolate its differentiated cell types in quantity. Chisholm et al. show how molecular and biochemical techniques have provided information about cell-cell contact and chemical signaling (by cyclic AMP) and insights into the patterns of gene expression characteristic of cells destined to become stalk or spore (so-called "prestalk" and "prespore" cells). MacWilliams and David are also concerned with how the developmental decision to become prestalk and/or prespore is made, but they approach this as a classic problem in pattern formation in which the physical manipulations of slugs, the isolation of mutants altered in the proportion of each cell type within the slug, and mathematical modeling are used to gain insight into the rules by which the abundance and distribution of prestalk and prespore cells are determined.

One of the most sophisticated examples of microbial development is the formation of root nodules by the symbiotic interaction of *Rhizobium* with its leguminous plant host, the subject of the final review in this monograph. Nodulation is a complex multistep process, which undoubtedly involves a series of interactions between the infecting bacterium and cells of the plant host. These interactions enable *Rhizobium* to penetrate into the plant root, proliferate, and then differentiate into a bacteriod, a specialized cell type capable of fixing molecular nitrogen. Ausubel shows how the application of molecular genetics, including transposition mutagenesis, has made possible

rapid progress in the identification of genes involved in the nodulation process. Surprisingly, despite the elaborate morphological features of nodulation and the complex signaling that must take place between bacterial and plant cells, the genetic information for nodulation appears to be considerably simpler and more compactly organized than that required for many other microbial developmental processes, such as cell division, spore formation, and chemotaxis.

We thank Jim Watson for hosting the Microbial Development meeting and for encouraging us to edit a monograph based on the conference. We thank our meeting co-organizer Amar Klar and the meeting participants whose ideas had a strong influence on the formulation of this volume. We are grateful to Gladys Kist and Maureen Berejka for their skillful assistance in coordinating the conference. We also thank Nancy Ford, Director of Publications, and her staff, particularly Nadine Dumser for her expert editing of this monograph, Michaela Taylor for production assistance, and Adrienne Guerra and Mary Cozza for help in manuscript preparation. The conference was supported by funds from Abbott Laboratories, American Cyanamid Company, Biogen S.A., Cold Spring Harbor Laboratory, Eli Lilly Company, the National Science Foundation, Monsanto, Monsanto Agricultural Products Company, and the Upjohn Company.

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INTRODUCTION

The ability to differentiate is a fundamental function of most, if not all, forms of life. The term differentiation connotes a change in cellular structure or function due to an altered program of gene expression. The onset of a differentiation process may reflect the apparent expression of an internal clock that functions independent of environmental changes. Differentiation may also be initiated by extracellular fluctuations, which include chemical signals sent between cells or abrupt changes in available nutrients. An overriding theme in the expression of cellular differentiation, independent of the mechanism of initiation, is the temporal coordination of large numbers of genes involved in the event. In many instances the simple differentiation event culminates in the formation of subcellular structures such as the F pili in *Escherichia coli* (Sambucetti et al. 1982), flagella and pili in *Caulobacter* (Poin-

dexter 1964; Shapiro 1976), and endospores in *Bacillus* (Losick and Youngman, this volume) or altered morphologies as seen in the formation of slugs in *Dictyostelium* (MacWilliams and David, this volume), shmoos in yeast (Klar et al., this volume), fruiting bodies in *Myxobacterium* (Kaiser, this volume), or aerial mycelium in *Streptomyces* (Chater, this volume). A particularly cogent issue in all of these systems is whether the mechanisms that regulate *when* to express a given set of gene products contribute to the control of the position of these gene products within the cell or the position of the differentiated cell within the organism. Questions of how cells are able to translate genetic information into spatial organization are fundamental to our understanding of all of developmental biology, yet are most difficult to approach.

A group of laboratories have begun to analyze these questions in the bacterium *Caulobacter crescentus*. This paper presents the manner in which the *Caulobacter* cell cycle has been manipulated both genetically and biochemically to uncover the cascade of reactions that culminates in localized subcellular differentiation events. The formation of a flagellum and pili at a specific cell pole during a defined time in the cell cycle affords access to a clearly defined event that can be defined structurally, protein components that can be identified, and the genes encoding these proteins that can be isolated and analyzed within the context of the rules that govern their differential expression. The temporally regulated expression of specific proteins and their assembly at defined locations on the cell surface have been related to both chromosome replication (Osley and Newton 1977, 1980; Osley et al. 1977; Sheffery and Newton 1981; Shapiro et al. 1982) and membrane biogenesis (Contreras et al. 1979, 1980; Mansour et al. 1980, 1981; Letts et al. 1982; Hodgson et al. 1984b,c). The synthesis of the components of the flagellum have been studied in conditional mutants altered in DNA initiation, DNA elongation, and cell division. The clear dependence of flagellar protein synthesis on the replication of the chromosome has allowed Osley and Newton (1980) to suggest that the replication of the chromosome functions as a clock that regulates the synthesis of differentially expressed proteins (Osley et al. 1977; Osley and Newton 1980; Sheffery and Newton 1981). Analysis of membrane lipid and protein synthesis has revealed that the synthesis of several differentially expressed flagellar and chemotaxis proteins is tightly coupled to both DNA replication and membrane biogenesis (Contreras et al. 1980; Shapiro et al. 1982; Gomes and Shapiro 1984). Based on the concept that the cell membrane might function as a receptor to help position the newly synthesized flagellar proteins, we have suggested that the mechanisms that regulate temporal expression might be ultimately related to those that control spatial organization. The following sections of this paper are specifically focused on the structure and synthesis of the flagellum, the chemotaxis apparatus, and the pili as a function of DNA replication and membrane biogenesis during the cell cycle.

THE CELL CYCLE

The *Caulobacter* cell cycle is characterized by a series of stage-specific morphogenic events. Because these events occur at a constant fraction of the generation time, independent of the growth rate, they appear to be the consequence of an internal timing mechanism (Poindexter 1964; Newton 1972; Shapiro 1976). The developmental program occurs during balanced growth and is not a response to environmental stress. The direct observation of single cells in microculture (Poindexter 1964) has provided evidence that cell contact or chemical communication is not involved in the differentiation process but, rather, that this process is an intrinsic property of the cell. A schematic representation of the *C. crescentus* cell cycle is shown in Figure 1. The motile swarmer cell carries a single polar flagellum. Receptors for

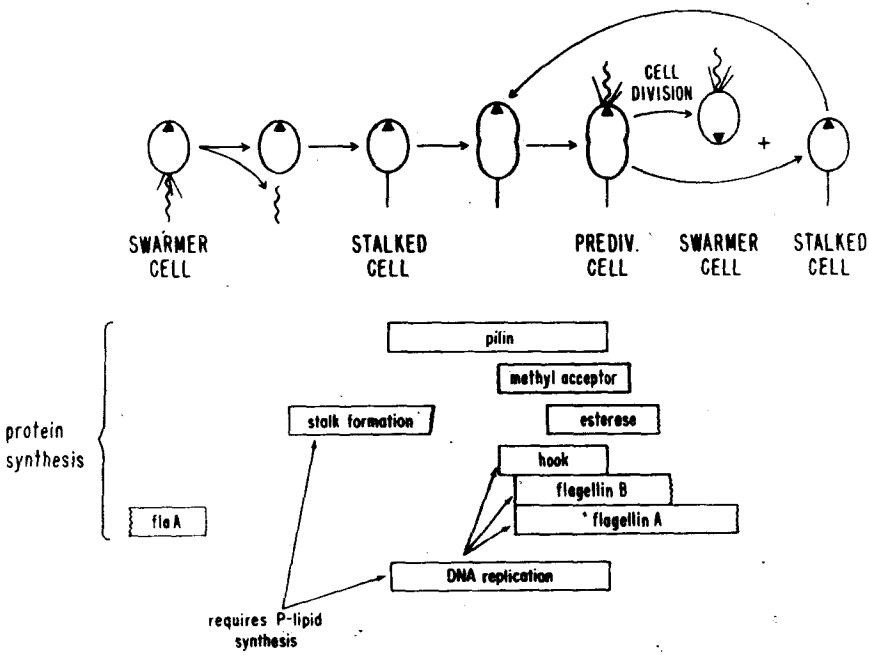

Figure 1 Schematic of the *C. crescentus* cell-division cycle. Shown below the diagram are the periods during the cell cycle when each of the indicated proteins are synthesized, when the DNA is replicated (Degnan and Newton 1972), and when stalk formation is initiated. The time of synthesis of the pilin is from Smit and Agabian (1982); the chemotaxis methyl-acceptor proteins and the methylesterase are from Gomes and Shapiro (1984); and the flagellar components, hook, flagellin A, and flagellin B are from Osley et al. (1977) and Agabian et al. (1979). Arrows indicate those events dependent on phospholipid synthesis (Contreras et al. 1980; Mansour et al. 1981; Shapiro et al. 1982) and DNA replication (Osley et al. 1977; Sheffery and Newton 1981).

the DNA phage ϕCbk (Agabian-Keshishian and Shapiro 1971) and pili, which are receptors for *Caulobacter*-specific RNA phage (Schmidt 1966; Bendis and Shapiro 1970; Shapiro and Agabian-Keshishian 1970), are also positioned at the flagellar pole. The swarmer cell differentiates into a sessile stalked cell by shedding the intact flagellum (Shapiro and Maizel 1973) and growing a stalk at the site previously occupied by the flagellum. The pili and DNA phage receptors are lost coincident with the release of the flagellum (Shapiro and Agabian-Keshishian 1970; Smit and Agabian 1982). The new stalk, whose cell envelope is continuous with that of the cell body (Poindexter 1964), is formed by localized cell-wall and membrane synthesis (Schmidt and Stanier 1966). Mutants that are unable to synthesize membrane phospholipid were found to be unable to carry out stalk formation (Mansour et al. 1981). The new stalked cell goes on to follow a temporally regulated program that is initiated with the onset of DNA replication (Degnan and Newton 1972). The stalked cell elongates and, upon completion of a round of DNA replication (Osley and Newton 1980), a new flagellum, pili, DNA phage receptor sites, and chemotaxis methylation proteins (Gomes and Shapiro 1984) can be detected in the incipient swarmer cell. Because these morphogenetic events occur at only one portion of the predivisional cell, cell division yields two different daughter cells: a stalked cell and a flagellum-bearing swarmer cell. The progeny stalked cell continues to function as a stem cell giving rise to a new swarmer cell at each division. Chromosome replication is blocked in the progeny swarmer cell but not in the progeny stalked cell (Degnan and Newton 1972; Evinger and Agabian 1974; Iba et al. 1977). As described above, the subsequent transition of the swarmer cell into a new stalked cell signals the onset of DNA replication.

Temperature-sensitive mutants that affect the cell cycle have been isolated and used to determine the order of events in the DNA replication and cell-division pathways (Osley and Newton 1977). Temperature-shift experiments demonstrated that there were five discrete steps in the DNA replication and cell-division pathways: DNA initiation, DNA elongation, completion of DNA replication, initiation of cell division, and cell separation (Osley and Newton 1977, 1980). Reciprocal temperature-shift experiments, similar to those used by Jarvick and Botstein (1973) and by Hereford and Hartwell (1974), were used by Newton's laboratory to determine whether any two steps in these pathways were dependent, independent, or interdependent. These studies led to the proposal that there are at least two circular pathways of dependent steps: one for DNA replication and one for cell division (Osley and Newton 1980; Nathan et al. 1982).

The *Caulobacter* cell cycle provides an elegant system in which to study the establishment of cell polarity in a unicellular organism. The clearest and most accessible expression of cell polarity is the biogenesis in the predivisional cell of the flagellum and pili at the cell pole opposite the stalk. The site of assembly of these newly synthesized structures is under strict control

and likely reflects the concerted action of a large number of genes. Mutants with altered flagellar or pili locations have not, thus far, been obtained in any laboratory studying this organism. The cell pole that hosts these newly assembled structures was originally the site of the cell-division event, and it may be that the onset of the cascade of events that ultimately directs the flagellar and pili components to this pole is initiated at the time of division. Furthermore, it seems probable that the placement of the flagellum and pili at the cell pole is the first visible "polar" event and that the ensuing biogenesis of the stalk at the same pole, following the release of the flagellum, is simply a consequence of the same regulatory cascade. A glimpse into the conditions required for correct placement of the polar organelles was provided by the observation that pili encoded by the RP4 plasmid are arrayed randomly on the *C. crescentus* cell surface (Ely 1979). Therefore, the assembly of surface structures can occur at locations other than the cell pole. Specific controls must be exerted to place the genome-encoded pili and flagellum at the cell pole, and it may be that the chromosomal location of the genes involved in their biogenesis might play a role in their placement on the cell.

GENETIC ANALYSIS AND PERTURBATION OF THE CELL CYCLE

Despite the fact that the characteristics of the *C. crescentus* cell cycle provide easy access to the temporal and spatial regulation of cell differentiation and that much can be inferred from direct observation and biochemical analysis, study of the organism would flounder without the ability to obtain mutants and to perform genetic analyses. The mechanisms used to regulate cell differentiation can be clearly defined only if genetic tools exist to gently perturb the system. There are two avenues of entry into the genetic analysis of this complex phenomenon: classical genetics and molecular genetics. The latter is ultimately self-limiting in the absence of the former. Fortunately, *C. crescentus* has proven to be amenable to genetic analysis. Mutants have been isolated by a variety of techniques. For instance, a number of studies have involved mutants identified by screening survivors of nitrosoguanidine treatment or irradiation with ultraviolet light (Jollick and Schervish 1972; Newton and Allebach 1975). These procedures have been quite effective for obtaining specific kinds of mutants. However, nitrosoguanidine is notorious for causing multiple mutations (Guerola et al. 1971), and ultraviolet light causes the induction of an error-prone repair system (R. Bender, in prep.) that results in the occurrence of multiple mutations (Johnson and Ely 1977). The high frequency of multiple mutations increases the probability of finding a strain containing the desired mutation but can interfere with subsequent genetic analyses. Often mutants can be isolated without resorting to mutagenesis. For instance, antibiotic-resistant mutants have been obtained by direct selection (Jollick and Schervish 1972; Barrett et al. 1982b), and motility mutants and auxotrophs have been obtained after enrichment procedures (Johnson and Ely

1977, 1979). More recently, a variety of mutants have been isolated following transposon mutagenesis with Tn*5* (Ely and Croft 1982). Transposon mutagenesis assures the presence of a single mutational event per cell and also provides a selectable antibiotic-resistance phenotype associated with the mutation. Furthermore, the inserted transposon generally eliminates the gene product of the mutated gene so that one does not observe effects caused by altered gene products.

Two of the standard methods of gene transfer in bacterial genetics, transduction and conjugation, are available in *C. crescentus*. Transduction provides a highly efficient means of introducing a small defined piece of DNA into an organism using a bacteriophage. The bacteriophage used in *C. crescentus* genetics is ϕCr30 (Ely and Johnson 1977). This bacteriophage can transfer segments up to approximately 4% of the chromosome at frequencies of 10^{-5} to 10^{-8}. Larger pieces of the chromosome can be transferred using RP4-mediated conjugation (Ely 1979). RP4 is a broad host-range plasmid that can cause the transfer of a copy of part of the host chromosome to a recipient cell. In *C. crescentus* transfer of chromosomal genes occurs at a frequency of 10^{-6} per donor cell. The maximum size of a transferred piece appears to be less than 20% of the genome (Barrett et al. 1982b). Thus, ϕCr30-mediated transduction can be used to transfer relatively small pieces of the chromosome, whereas longer pieces can be transferred by RP4-mediated conjugation.

To date, a method of transformation has not been devised for *C. crescentus*. However, DNA can be transformed into *E. coli* and then transferred from *E. coli* to *C. crescentus* by RP4-mediated conjugation (Bryan et al. 1984; P.V. Schoenlein et al., unpubl.). Both the P-type and N-type plasmids have been shown to have transfer systems that will mediate plasmid exchange between *E. coli* and *C. crescentus* (Table 1; Ely 1979). In contrast, the F-type plasmids are not capable of being transferred to *C. crescentus*. Small plasmids generally lack a transfer system, but some can be mobilized and transferred by *tra*$^+$ plasmids. In addition, some nontransmissible plasmids have been made transmissible by the addition of *tra* genes from a transmissible plasmid. In the case of pRK2013, containing the *tra* genes from RK2 and a ColE1 replicon (Ditta et al. 1980), transfer of the plasmid from *E. coli* to *C. crescentus* has been demonstrated, but the plasmid is not maintained in *C. crescentus* even under selective conditions (Table 1). This property has been used as a means of introducing transposons into *C. crescentus* on an unstable vector for transposon mutagenesis (B. Ely, unpubl.). Two other small plasmids, containing the Q-type or P15A replicons, are capable of being replicated and maintained in *C. crescentus* (Table 1; P.V. Schoenlein et al., unpubl.). These observations have led to the construction of clone banks containing *C. crescentus* DNA inserted into either RP4 or the Q-type plasmid R300B. Transfer of these plasmids from *E. coli* into a collection of over 100 *C. crescentus* mutants has resulted in the identification of plasmids that complement spe-