

# GENERATION OF ASYMMETRY DURING DEVELOPMENT

## Segregation of Type-Specific Proteins in *Caulobacter*

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### ABSTRACT

An essential event in developmental processes is the introduction of asymmetry into an otherwise undifferentiated cell population. Cell division in *Caulobacter* is asymmetric; the progeny cells are structurally different and follow different sequences of development, thus providing a useful model system for the study of differentiation. Because the progeny cells are different from one another, there must be a segregation of morphogenetic and informational components at some time in the cell cycle. We have examined the pattern of specific protein segregation between *Caulobacter* stalked and swarmer daughter cells, with the rationale that such a progeny analysis would identify both structurally and developmentally important proteins. To complement the study, we have also examined the pattern of protein synthesis during synchronous growth and in various cellular fractions.

We show here, for the first time, that the association of proteins with a specific cell type may result not only from their periodicity of synthesis, but also from their pattern of distribution at the time of cell division. Several membrane-associated and soluble proteins are segregated asymmetrically between progeny stalked and swarmer cells. The data further show that a subclass of soluble proteins becomes associated with the membrane of the progeny stalked cells. Therefore, although the principal differentiated cell types possess different synthetic capabilities and characteristic proteins, the asymmetry between progeny stalked and swarmer cells is generated primarily by the preferential association of specific soluble proteins with the membrane of only one daughter cell. The majority of the proteins which exhibit this segregation behavior are synthesized during the entire cell cycle and exhibit relatively long, functional messenger RNA half-lives.

KEY WORDS differentiation · polarity ·  
unequal protein distribution · progeny analysis

From the beginnings of experimental embryology, the study of development has been concerned with the mechanisms by which cells generate and

maintain structural and functional differences. The molecular basis for the generation of these differences is not known, but implicit in this view of differentiation is a requirement that at some period in the development of an organism a special subclass of cellular components is asym-

metrically or unequally distributed among the cell progeny. The establishment of this asymmetry presumably enables daughter cells to initiate and respond differentially to a variety of developmental signals.

In complex metazoan systems, the differentiation patterns of a specific cell type are generally under the additional influences of extracellular and intercellular interactions. It is therefore extremely difficult to determine cause and effect relationships in the developmental pattern of such cells. For this reason, we have chosen to study the generation of asymmetry in a simple bacterial system, *Caulobacter crescentus*, which is uniquely suited for these studies.

*Caulobacter* differentiation is independent of environmental triggers, and cell division results in the formation of morphologically and physiologically distinct daughter cells (Fig. 1; for review, see references 18, 21, 27). The swarmer cells possess a single flagellum, several pili, and cell surface receptors for DNA bacteriophage, all of which are located at the swarmer cell pole. The stalked cells are characterized by the possession of a polar stalk. At the time of cell division, these surface structures are segregated between the daughter cells; each of the progeny cells then follows a different sequence of development. The progeny

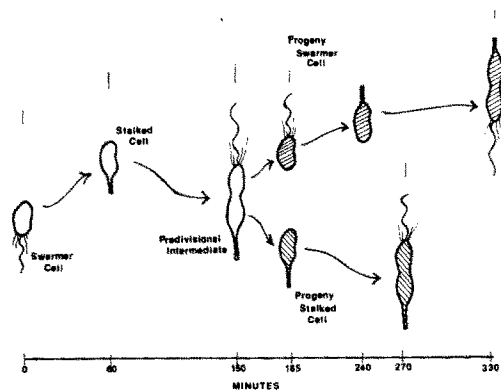


FIGURE 1 The *Caulobacter* cell cycle. Two generations of the *Caulobacter* cell cycle are represented. The temporal sequence of differentiation is shown in minutes and corresponds with the differentiation cycle in Figs. 2-4. The progeny of cell division are shaded and correspond to the symbols of the cell types analyzed in the experiment shown in Fig. 5. It should be noted that the developmental sequence of each progeny cell type is different. The progeny cells are of opposite polarity; the orientation maintained is such that the cell poles formed at division become the differentiating poles in the subsequent cell cycle.

stalked cells reinitiate the cycle of elongation, DNA replication, and swarmer cell production, while the progeny swarmer cells exhibit a free-swimming phase of development during which DNA replication is postponed (5, 6). Differentiation of the swarmer into a stalked cell occurs by the outgrowth and compartmentation of cell-envelope and cytoplasmic constituents to form the polar stalk structure.

Because the morphological alterations which characterize the *Caulobacter* cell cycle occur at specific polar locations on the cell surface, the expression of these structures (*i.e.*, stalk, bacteriophage receptor sites, flagella, and pili) requires that their constituent proteins are either synthesized or translocated into the polar regions at the appropriate period of development. Unequal distribution of cellular components is thus reflected in the shape and surface structures of these daughter cells, the properties of their chromosomes (6), and their pattern of development.

In this study, the patterns of protein synthesis of each of these cell types has been assayed during synchronous cell growth. The experiments reported below were designed: (a) to identify proteins made predominantly by a given cell type (stage-specific proteins), and (b) to follow the distribution of specific proteins among the cell progeny. We reasoned that such an analysis should define both developmentally and positionally important proteins. These experiments demonstrate that a subclass of cellular proteins, which may also be related to polar surface structures, exhibit an unusual pattern of segregation and compartmentation between *Caulobacter* stalked and swarmer daughter cells.

## MATERIALS AND METHODS

### *Bacterial Strains and Culture Conditions*

*C. crescentus* CB15 (ATCC 19,089) was grown in minimal glucose medium (HMG) (29) at 30°C with rotary shaking. Synchronous swarmer cells were prepared by the Ludox procedure (6) in 26 ml gradient volume.

For the isolation of progeny stalked and swarmer cells, synchronous swarmer cells were prepared as previously described, suspended in HMG (30°C), and grown until cell division. The dividing population was harvested at 12,000 g for 10 min at 4°C and separated on 13-ml Ludox gradients of the same composition as described previously (6). Swarmer cells and dividing cells formed two widely separated tight bands in the gradient. Stalked cells formed an intermediate, more

diffuse band. The purity of cell populations was assessed in fixed samples by phase-contrast microscopy. Approx. 95% of the cells divide between 160 and 200 min (6).

### *Isotopic Labeling and Gel Electrophoresis*

Cells were pulse-labeled with 1  $\mu\text{Ci/ml}$  of  $^{14}\text{C}$ -amino acid mixture (reconstituted protein hydrolysate, algal profile [Schwarz/Mann Div., Becton, Dickinson, & Co., Orangeburg, N. Y.]) for 8-min periods. Isotopic label was incorporated linearly for 20 min under these conditions, and the specific activity of incorporation did not change during synchronous growth. Pulse-labeling was terminated by the addition of 70  $\mu\text{l/ml}$  of peptone-yeast extract medium (PYE) (1, 21) at 0°C.

In pulse-chase experiments, cells were labeled for 15 min as described above; the radioactivity was chased by the addition of 70  $\mu\text{l/ml}$  PYE medium. This reduced the incorporation of  $^{14}\text{C}$ -amino acids by 80–85% in 15 min and did not change the membrane protein composition. Increased amounts of PYE were not used because the membrane protein profile is altered when CB15 is grown in rich medium (1).

SDS-polyacrylamide slab gel electrophoresis was performed by the procedure of Laemmli (12), modified as described previously (32). Gels were 15 cm long and contained 9–13% or 10–13% gradients of acrylamide as indicated. Equal counts per min of acid-precipitable  $^{14}\text{C}$ -labeled protein fractions were loaded per slot in the gel. Radioactive protein profiles were detected by autoradiography with Kodak X-Omat R x-ray film.

### *Preparation of Membrane Fractions*

Membrane fractions were prepared by the method of Gudas et al. (7), except that after cells were suspended in 0.01 M Tris-HCl, pH 6.8, they were lysed by adjusting the solution to 0.01 M EDTA and 50  $\mu\text{g/ml}$  lysozyme (Sigma Chemical Co., St. Louis, Mo.) and then further disrupted by repeated (3  $\times$ ) passage through a 26-gauge needle. Membrane protein profiles obtained from cells treated with 1% Sarkosyl (Chemical Additives Co., Farmingdale, N. Y.) were the same as those obtained from total membranes fractionated by sucrose density gradient centrifugation (1).

## RESULTS

The patterns of membrane and soluble protein biosynthesis in synchronous *Caulobacter* populations were examined to assess the periodicity and extent of stage-specific gene expression in this system. Particular attention was paid to the membrane fraction because the major morphological changes are seated in this cellular fraction. In the following figures, the letters W, T, and PD refer to the cellular proteins for which the major period of synthesis is during the swarmer, stalked, or

predivisional intermediate stage of the cell cycle, respectively. The numerical designation after the letters corresponds to apparent molecular weights  $\times 10^{-3}$  of the protein. Proteins synthesized uniformly during the cell cycle are designated by their apparent size in kilodaltons (Kd). Thus W108 is a protein of 108,000 daltons which is synthesized predominantly in swarmer cells while protein 74Kd is synthesized throughout the cell cycle and has an apparent mol wt of 74,000 daltons.

Synchronous swarmer cells were prepared by the Ludox procedure (6) and pulse-labeled for 8 min with  $^{14}\text{C}$ -amino acids at 10-min intervals throughout the cell cycle. Each fraction is designated by the time of the initiation of the pulse period. The membrane fraction, separated into outer (Fig. 2) and inner (Fig. 3) membranes, and the soluble fraction (Fig. 4) were prepared as described in Materials and Methods and analyzed by SDS-polyacrylamide gel electrophoresis and subsequent autoradiography. Equal counts were assayed per gel sample, and therefore the band intensity may be assumed to be proportional to the rate of protein synthesis. In all of the following experiments, differentiation begins with swarmer cells at 0 min and follows the temporal sequence of differentiation outlined in Fig. 1. Table I summarizes the proteins of interest in this paper, their cellular location, and periodicity of synthesis.

### *Protein Synthesis in the Membrane Fraction*

The pattern of synthesis of outer membrane proteins is shown in Fig. 2. Proteins synthesized primarily by a specific cell type include proteins W108, W95, T94, T91, T87, and T73, PD27.5, and PD25. Proteins T73, PD27.5, and PD25 have been identified by radioimmune assay as components of the *C. crescentus* flagellar organelle (15). The hook protein monomer, T73, is found only in the outer membrane; PD27.5 and PD25 correspond to flagellin subunits (13, 14, 20, 30).

Changes in the relative rates of outer membrane protein synthesis occur at discrete intervals during the cell cycle (Fig. 2 and Table I). In the outer membrane, these occur almost exclusively in proteins  $\geq 74,000$  daltons. W108 is exceptional as it is synthesized during the entire swarmer cell stage (0–60 min), in contrast to proteins W95 and W88 which predominate during the first half of the swarmer differentiation period (0–40 min). It has been shown that transcription during the first half

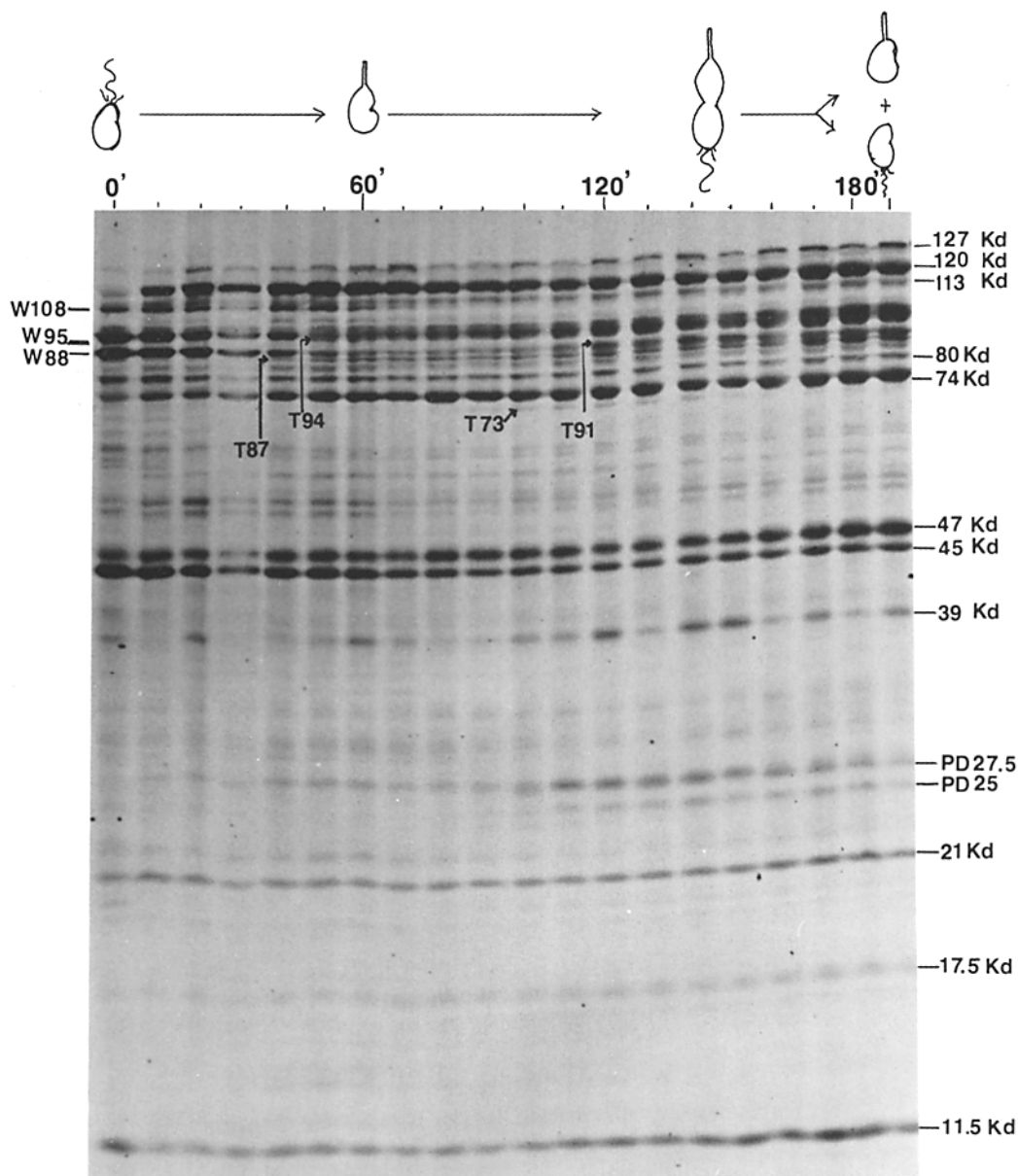


FIGURE 2 Pattern of outer membrane protein synthesis in synchronous cells. Synchronous cells were pulsed-labeled for 8 min with  $^{14}\text{C}$ -amino acids at 10-min intervals. The outer membrane fraction was prepared and analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The temporal sequence of differentiation may be compared with the schematic drawing in Fig. 1. Approx. 18,000 cpm of  $^{14}\text{C}$ -labeled protein was applied to each gel sample.

of the swarmer cell cycle is required for the subsequent loss of cellular motility and differentiation into a stalked cell (17). Proteins T94 and T87 are first expressed at 40–50 min and continue to be synthesized until cell division (190 min). T91

first appears in the outer membrane at ~110 min in the cell cycle; its appearance is concurrent with the period of maximal DNA replication in the stalked cell. T73, the flagellar hook monomer (15), is synthesized from ~100 to 150 min. The

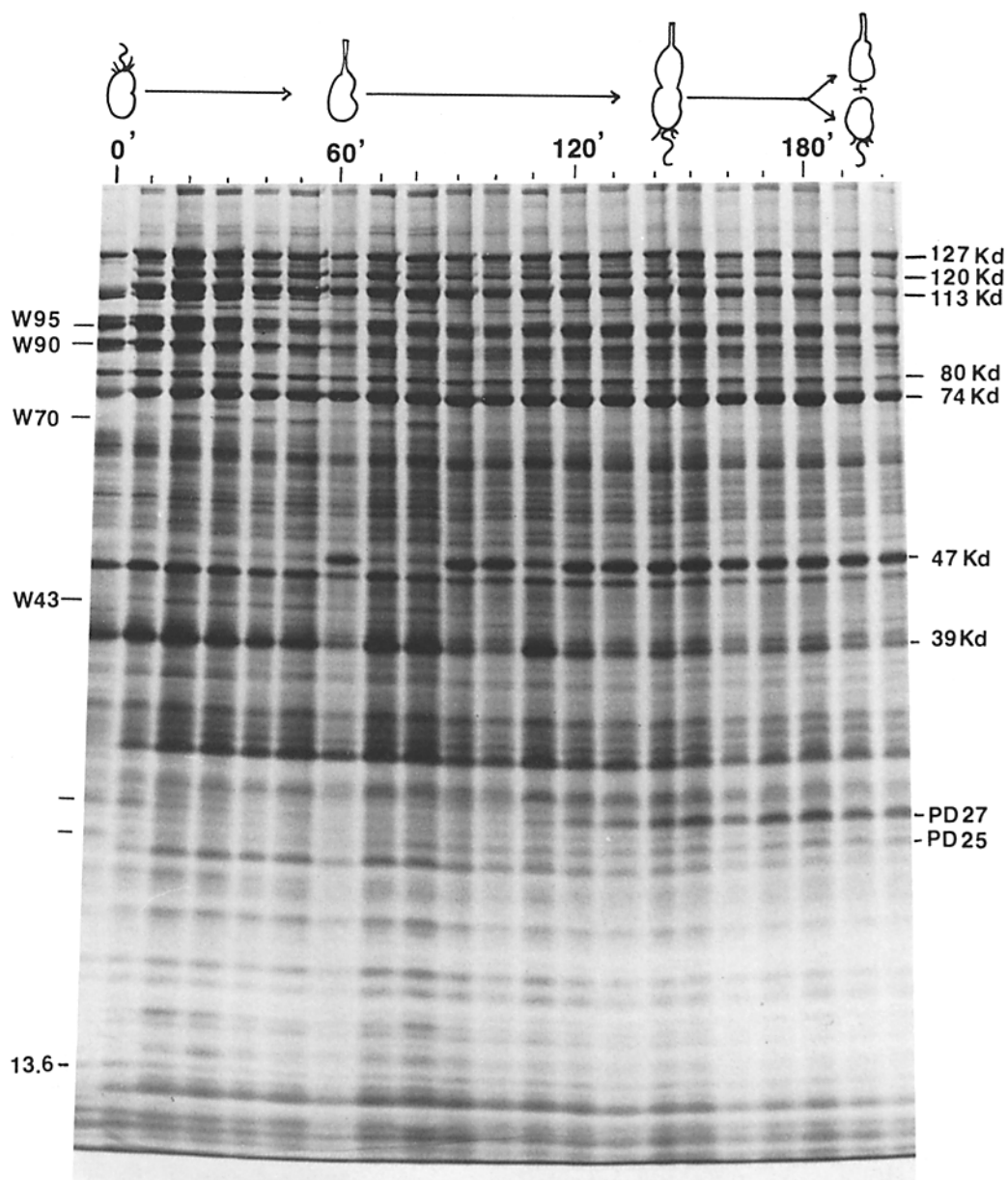


FIGURE 3 Pattern of inner membrane protein synthesis in synchronous cells. Inner membrane fractions were prepared from the same synchronous cell population as in Figs. 2 and 4 and analyzed as described in the legend to Fig. 2. Each gel sample contained  $\sim 8,000$  cpm of  $^{14}\text{C}$ -labeled protein.

major period of flagellin (PD27.5 and PD25) synthesis in the outer membrane occurs during the predivisional cell stage (100–150 min); however, low levels of PD27.5 and PD25 are detectable at earlier times in the cell cycle. Synthesis of the

remaining outer membrane proteins proceeds at a relatively uniform rate; proteins of interest in this study include those designated 120Kd, 80Kd, and 74Kd. The inverse correlation between the amounts of 47Kd and 39Kd protein is accounted

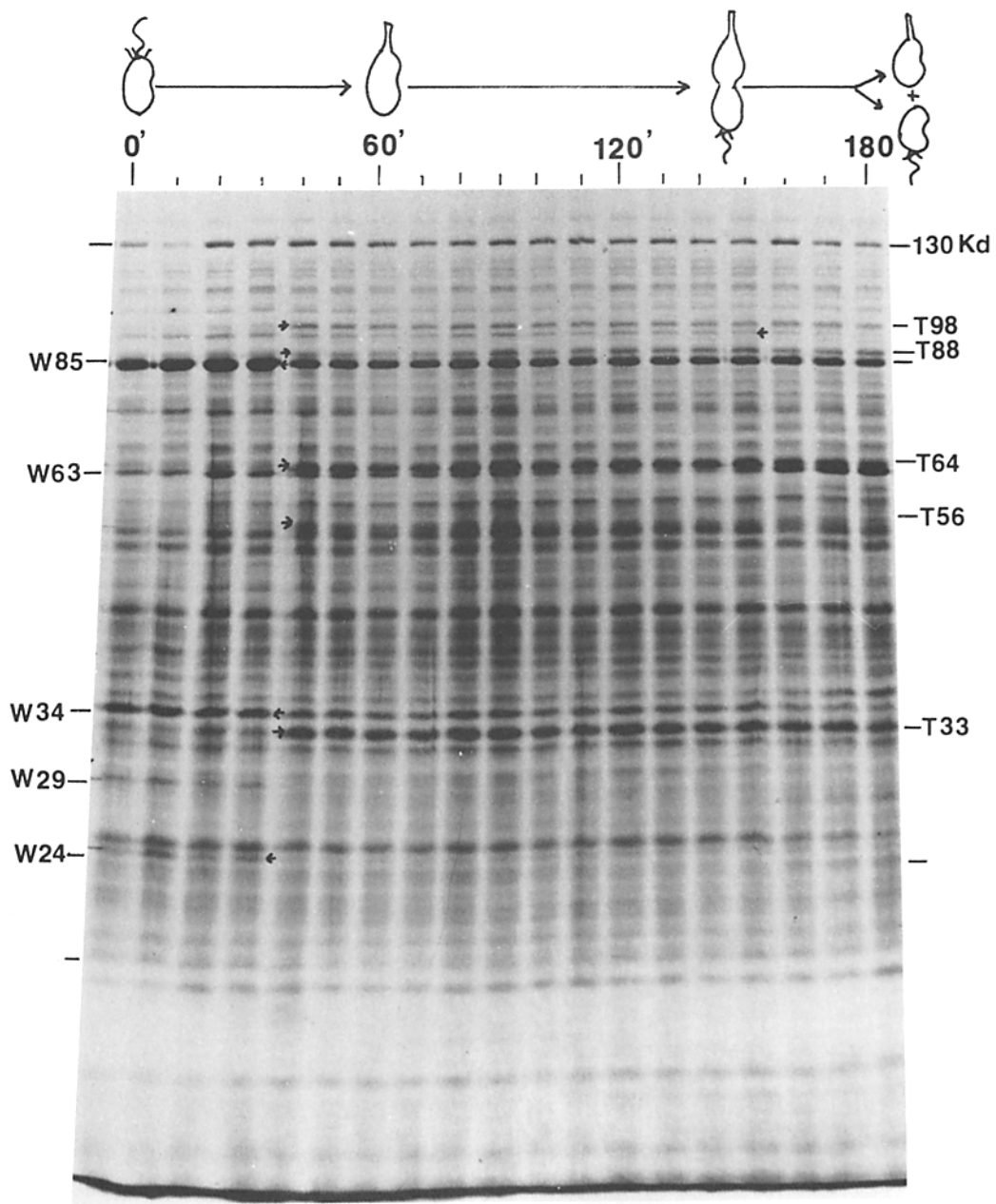


FIGURE 4 Pattern of soluble protein synthesis in synchronous cells. Soluble fractions were prepared from the same synchronous cell population as in Figs. 2 and 3 and analyzed as described in the legend to Fig. 2. Each gel sample contained  $\sim 8,000$  cpm of  $^{14}\text{C}$ -labeled protein.

TABLE I  
Periodicity of Protein Synthesis

Protein	Location	Relative Rates of Synthesis*				
		0-40'	40-60'	60-110'	110-150'	150-180'
W108	O.M./I.M.‡	+++	+++	±	0	0
W95	O.M./I.M.	+++	++	±	±	+
W90	I.M./O.M.	+++	++	+	+	+
W88	O.M./I.M.	+++	++	±	±	+
W85	SOL	+++	++	++	++	++
W70	I.M.	++	++	++	0	0
W63	SOL	++	+	+	+	++
W43	I.M.	+	+	+	0	0
W34	SOL	+++	++	++	++	++
W29	SOL	++	0	0	0	0
W24	SOL	++	0	0	0	0
T98	SOL	0	++	++	++	++
T94	O.M./I.M.	0	+	+	+	+
T91	O.M./I.M.	0	+	+	+	+
T88	SOL	0	++	++	++	++
T87	O.M./I.M.	±	+	+	+	+
T73	O.M.	0	0	0	+	0
T64	SOL	0	++	++	++	++
T56	SOL	0	++	++	++	++
T33	SOL	+	+++	+++	+++	+++
PD25	§	+++	+	±	++	+++
PD27.5	§	0	0	0	+	++

\* Relative rates of protein synthesis are indicated by the notations: 0, ±, +, ++, and +++. Times indicated correspond to the temporal sequence of differentiation outlined in Fig. 1.

‡ Several *Caulobacter* membrane proteins are found in both the outer membrane (O.M.) and inner membrane (I.M.) (1). The location listed first is that in which the protein is most abundant. SOL refers to the soluble fraction.

§ The periodicity of synthesis and the localization of PD27 and PD25 have been determined by radioimmune assay. The values reported represent the overall relative rates of synthesis of these proteins during the *Caulobacter* cell cycle and are derived from the study by Lagenaur and Agabian (15).

for by the fact that the apparent molecular weight of this polypeptide is heat modifiable.<sup>1</sup>

The relative rates of synthesis of proteins located in the inner (cytoplasmic) membrane fraction during synchronous cell growth are shown in Fig. 3. Proteins found exclusively in this cellular

fraction and synthesized at specific periods of the cell cycle are designated W70 and W43. W70 and W43 are synthesized in swarmer and early stalked cell stages (10-90 min). Protein 113Kd, a major component of the inner membrane, is synthesized continuously. As reported previously (1), a number of membrane proteins are found in both the inner and outer membrane fractions; for example, protein 80Kd and 74Kd appear to be present in approximately the same ratios relative to one another in both inner and outer membrane preparations, although enzymatic and immunological assays indicate that the inner and outer membranes are well-separated (1). Protein 120Kd, on the other hand, appears to be enriched in outer membrane fractions relative to the inner membrane. Appreciable amounts of proteins 127Kd and 130Kd are not found in the outer membrane.

<sup>1</sup> When these experiments were done, we were unaware of the fact that the 47,000- and 39,000-dalton membrane proteins were heat modifiable forms of the same polypeptide. The behavior of this protein is analogous to the heat-modifiable 28,500-dalton protein in the *Escherichia coli* membrane which is converted to an apparent mol wt of 33,400 upon heating in SDS-containing solutions (26). The combined synthesis of the 47/39Kd proteins appears uniform during the cell cycle. (DeMartini, M., and N. Agabian, manuscript in preparation).

Proteins W108, W95, W90, W88, and PD25 exhibit similar patterns of expression in the inner membrane as those shown in the outer membrane, but in general the striking periodicity of protein synthesis is not readily apparent in this cellular fraction. Because of the complexity of the inner membrane proteins, it is likely that multiple polypeptides of similar molecular weight are obscuring some changes. Further analysis by two-dimensional gel electrophoresis is in progress.

#### *Protein Synthesis in the Soluble Fraction*

Fig. 4 represents the changes in the relative rates of protein synthesis in the soluble cell fraction during the differentiation cycle. Proteins synthesized predominantly by the swarmer cell include W85, W63, W29, and W24. Their major period of synthesis is during the first half of the swarmer cell differentiation period (0–40 min). Proteins designated T98, T88, T64, T56, and T33 form a second group of polypeptides which appear to be expressed together at 40 min in the cell cycle. Although there are other proteins of interest in this cellular fraction, such as T123, 97Kd, and 130Kd, the salient feature of these patterns is the abrupt change in proteins synthesized at the 40-min period of differentiation.

Some of the soluble proteins identified here are likely to be similar to those described by Cheung and Newton (4) who measured soluble protein synthesis during major periods of the cell cycle. The use of [<sup>35</sup>S]methionine in these studies, however, substantially alters the protein pattern so that direct comparison between data cannot be made accurately.

#### *Distribution of Proteins Among Progeny Cells*

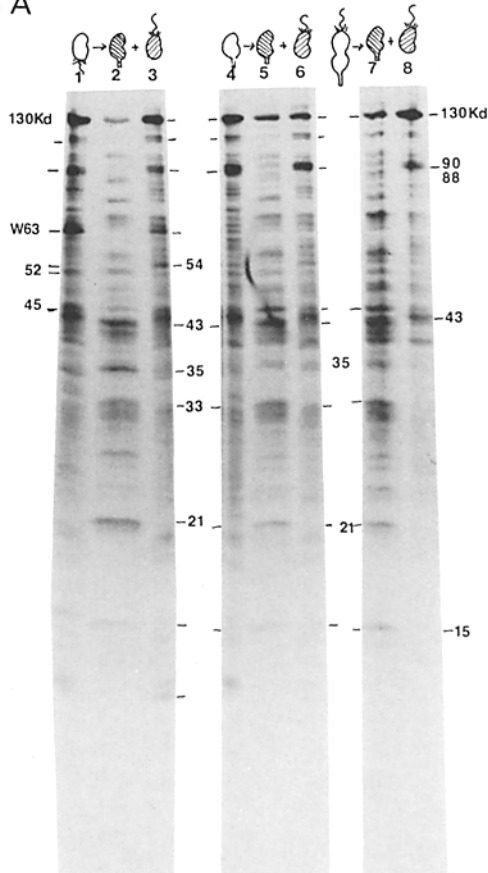
A specific advantage of the *Caulobacter* system is the ability to distinguish and isolate the progeny of cell division. In the following experiments, we have used this property to determine the pattern of segregation of specific proteins during subsequent differentiation events. Synchronous CB15 swarmer, stalked, and predivisional cells were pulse-labeled for 15 min with <sup>14</sup>C-amino acids, and the radioactivity was chased as described in Materials and Methods. At the end of the pulse-labeling period, aliquots of stalked and swarmer cells (parent, see Fig. 1) were removed and the remaining cell populations were permitted to grow, differentiate, and divide. Predivisional in-

termediate cells divided by the end of the pulse-labeling period. The progeny stalked and swarmer cells from each of these pulse-labeled parent cell types were then re-isolated by a modification of the Ludox procedure (Materials and Methods). Pulse-labeled parent cells and each of their respective progeny cells were separated into soluble, inner and outer membrane fractions; the proteins in each sample were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as in the previous experiments. The results of these experiments are shown in Fig. 5. (Note that the symbols in these experiments are equivalent to those used in Fig. 1 in which shaded cells are progeny cell types.)

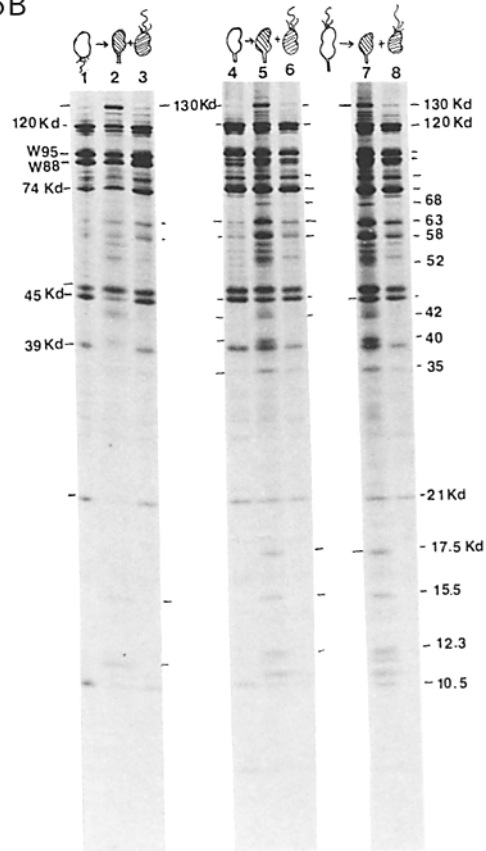
In the soluble fraction (Fig. 5A), proteins which are unequally distributed between both progeny stalked and swarmer cells are easily identified. These include a protein doublet at ~88,000 daltons and proteins of 63,000 and 54,000 daltons which segregate with the progeny swarmer cells; those of 42,000, 35,000, 33,000, 21,000, and 15,000 daltons segregate with the progeny stalked cells. In the case of some of these proteins, such as the 63,000-dalton protein which is synthesized predominantly during the swarmer stage, the protein segregation pattern is reflected only in one cycle of the experiment. The parent and progeny samples in these experiments were all prepared from a single population of synchronized cells. The finding that soluble proteins are asymmetrically distributed between both stalked and swarmer progeny is experimentally important because, in the outer membrane (Fig. 5B), the pattern of protein segregation is substantially different from that of the soluble fraction. Several pulse-labeled outer membrane proteins are segregated with the stalked daughter cells, although there appear to be none which segregate with the swarmer daughter cells. Proteins of 130,000, 68,000, 63,000, 58,000–52,000, 42,000, 40,000, 35,000, 17,500, 15,000, and 12,000–10,000 daltons are all segregated with the outer membrane fractions of the progeny stalked cell. Furthermore, a number of the proteins found segregating with the progeny stalked cell outer membrane are not found in the pulse-labeled outer membrane of the parent cell types (Figs. 2 and 5B). For the most part, stage-specific outer membrane proteins such as W95 and W88 are distributed uniformly to the progeny cells (Fig. 5B), thus indicating that swarmer proteins are retained during the swarmer to stalked cell differentiation process. This obser-



5 A



5B



5C

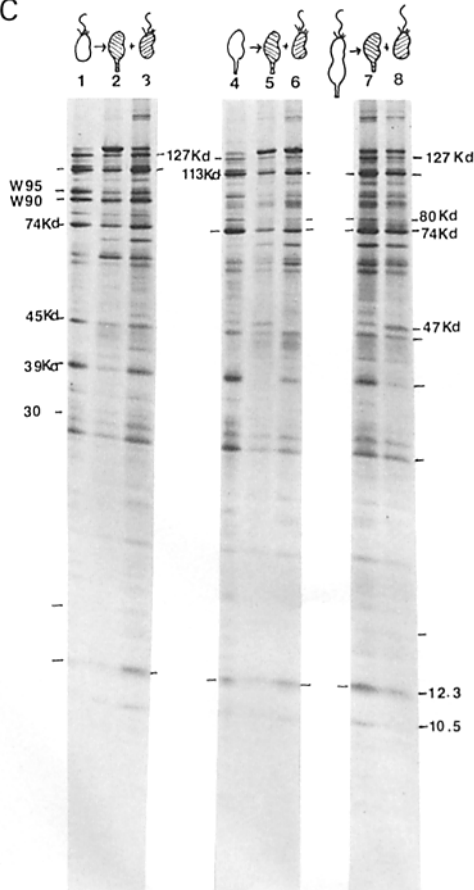


FIGURE 5 Segregation of cellular proteins among daughter cells. Synchronous cells were pulse-labeled with  $^{14}\text{C}$ -amino acids for 15 min and chased as described in Materials and Methods; aliquots were removed for preparation of parent cell fractions, and the remaining cells were chased until cell division. Progeny cells were re-isolated as described in Materials and Methods. Fig. 5 A, B, and C are the soluble, outer and inner membrane fractions obtained from the same cell aliquots. The cell figures above the gel slots correspond to those depicted in Fig. 1. In each of these gel samples, the numbers above the gel slots are correlated with cell types as follows: 2 and 3—Progeny stalked and swarmer cells, respectively, of the pulse-labeled swarmer (parent) cell 1. 5 and 6—Progeny stalked and swarmer cells, respectively, of the pulse-labeled stalked cell 4. 7 and 8—Progeny stalked and swarmer cells of the pulse-labeled predivisional intermediate cell. Pulse-labeled predivisional intermediate cell samples analogous to 1 and 4 were not prepared because cells were dividing by the termination of the pulse-labeling period. Proteins are identified according to the nomenclature outlined in the Results. Proteins that could not be unequivocally identified are designated by a number which corresponds to molecular weight  $\times 10^{-3}$ .

variation is consistent with that in which stained protein profiles of stalked and swarmer cell outer membranes were shown to have the same overall protein composition (1). In contrast to these striking differences in the soluble and outer membrane protein profiles, the inner membrane fraction reveals few qualitative differences in the distribution of parental protein among cell progeny (Fig. 5 C).

In these experiments, many proteins are distributed among the progeny stalked and swarmer cells in a manner different from that which might have been predicted as a result of the pulse-labeling experiments described in the previous section. Stage-specificity arises not only as a result of differential protein synthesis, but also as a result of the segregation of a protein among the daughters of cell division. This is particularly true for proteins which are asymmetrically distributed to progeny stalked cell outer membranes (Fig. 5 B). Protein 130Kd illustrates this particular segregation behavior.

The synthesis of 130Kd may be detected in the soluble cell fraction of both parent and progeny cells; in fact, it is synthesized continuously during the cell cycle (Figs. 4 and 5 A). However, if cells are pulse-labeled and the inner and outer membranes are prepared at the end of the pulse period, 130Kd is not found in either the inner or outer membrane fractions of the parent cells (Fig. 5 B and C). On the other hand, if the pulse-labeled parent cells are allowed to grow and divide, the 130Kd protein is located preferentially in the stalked cell outer membrane (Fig. 5 B), thus suggesting that association of this protein with the outer membrane of the cell occurs after a delay of one generation. Comparison of the molecular weights of proteins which segregate asymmetrically in the soluble and outer membrane fractions would suggest that protein-130Kd behavior is representative of a class of proteins which segregate with the stalked cell outer membrane. Proteins of 63,000, 35,000, and 21,000 daltons may also exhibit such behavior; they are synthesized in the soluble cell fraction of swarmer cells and segregate with the outer membrane of the progeny stalked cell.

#### *Stability of Messenger RNA for Specific Proteins*

Some outer membrane proteins in gram-negative bacteria are synthesized from messenger RNA (mRNA) which has a longer functional half-

life than the bulk of the mRNA population. The most widely known example is the murein lipoprotein of *Escherichia coli* which has a functional mRNA half-life of 11.5 min (8). The effect of rifampicin on the functional half-life of *Caulobacter* mRNA was examined to determine whether any of the proteins which exhibit unusual segregation behavior were related by being synthesized from stable mRNAs. As shown in Fig. 6, after addition of rifampicin, cells were pulse-labeled for 10 min with  $^{14}\text{C}$ -amino acids at the intervals described in the legend. The relative rates of amino acid incorporation into cellular proteins after rifampicin treatment, as compared with control cultures, were quantitated by densitometry of this autoradiograph. These values provide an estimate of the functional stability of the mRNA for specific protein species. Each of these proteins, except flagellin, is a unique polypeptide, as determined by two-dimensional gel electrophoresis.

Most cellular protein synthesis is immediately affected by rifampicin inhibition of mRNA synthesis, consistent with the 2.5-min mRNA half-life reported for *Caulobacter* mRNA (19), although synthesis of several proteins continued for as long as 20–30 min after rifampicin addition.<sup>2</sup> Proteins

<sup>2</sup> Iba et al. (9) have reported functional mRNA stability between 0.4 and 5.8 min in a mutant *Caulobacter* derived from strain CB13. CB15, another *C. crescentus* strain, is used in the studies reported here. When we measured functional mRNA stability in the parent CB13 strain, a population of proteins which were synthesized from mRNAs with apparent functional stability, equivalent to those described herein for CB15, were also detected. These CB13 proteins were similar in molecular weight to those described by Iba et al. (9) in their mutant strain as having extended mRNA half-lives. The relative proportions of these proteins in our studies and those previously reported (9) were significantly different, a result which may be partially explained by the use of [ $^{35}\text{S}$ ]methionine in the earlier study (9) and  $^{14}\text{C}$ -amino acids by Agabian. The kinetics of mRNA decay calculated for the  $\beta\beta'$ -subunits of RNA polymerase in the studies of Iba et al. and in our own were comparable. Thus, it appears that there is a differential effect on the stability of specific mRNA populations in our respective studies. In the absence of detailed knowledge of the particular strain used by those authors (9), and differences in sampling technique, we cannot simply account for this disparity in results. However, we would like to point out that the high levels of methionine (200  $\mu\text{g}/\text{ml}$ ) used in the experiments previously reported (9) are toxic to the CB15 strain used in the studies reported here and result in an immediate and drastic (50%) growth inhibition.

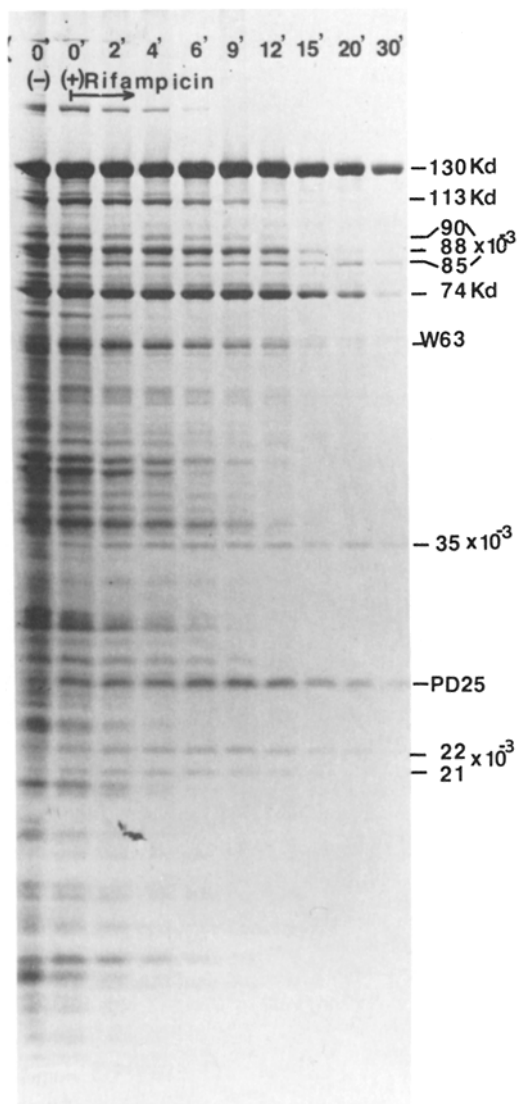


FIGURE 6 Autoradiogram of proteins synthesized after rifampicin treatment of cells. Heterogeneous populations of cells (o.d. = 0.35) were treated with rifampicin (5  $\mu\text{g/ml}$ ) and pulse-labeled for 8 min with  $^{14}\text{C}$ -amino acids at 0, 2, 4, 6, 9, 12, 15, 20, and 30 min after the addition of the inhibitor. Labeled cells were pelleted at 12,000  $g$  for 10 min and prepared for electrophoresis as described in Materials and Methods; 30  $\mu\text{l}$  of sample were loaded per gel slot. The rates of protein synthesis were quantitated by densitometry of these autoradiograms of SDS-polyacrylamide gels. Soluble and membrane proteins were identified from similar experiments with synchronous or heterogeneous cells which had been fractionated as described in Materials and Methods. Proteins which could not be unequivocally identified (i.e., W88 vs. T88) were designated by molecular weight  $\times 10^{-3}$ .

synthesized from mRNA with functional half-lives between 5–6 min include W63 and proteins of 90,000, 88,000, and 21,000 daltons. Proteins synthesized from mRNA with a functional half-life of 10–15 min include proteins 130Kd, 74Kd, and PD25, and proteins of 85,000, 35,000, and 22,000 daltons. Thus, several, but not all, of the proteins which become associated with the progeny stalked cell outer membrane are synthesized from mRNAs with a relatively long functional stability.

We are aware of the possibility that each of the proteins which appear to become membrane-associated in the stalked progeny represents more than a single polypeptide species. Without immunological or functional criteria, it is not possible to unequivocally determine the periodicity or fate of some of these proteins. For example, proteins of 88,000 and 85,000 daltons which segregate with the swarmer progeny cells (Fig. 5A) may or may not be related to proteins W88, T88, or W85. Two-dimensional gel analysis of proteins synthesized after the addition of rifampicin, however, indicates that the protein species of 130,000, 88,000, 85,000, 74,000, 63,000, 35,000, 22,000, and 21,000 daltons are all unique polypeptide species with similar isoelectric points and molecular weights in both the membrane and soluble fractions of the cell. Of those proteins made from relatively stable mRNA, only two are expressed at specific stages of development. The flagellins are synthesized predominantly by the predivisional intermediate and early swarmer cells (15, 20), and W63 is synthesized predominantly during the swarmer and predivisional intermediate cell stages. The remainder of the proteins are uniformly synthesized during the cell cycle.

## DISCUSSION

Analysis of the pattern of synthesis and segregation of proteins during the *Caulobacter* differentiation cycle indicates that stage-specificity results not only from the periodicity of protein synthesis, but also from the distribution of proteins at the time of cell division. As demonstrated in Figs. 2–4, the principal differentiated cell types possess different synthetic capabilities. These are expressed as discrete intervals in which the relative rates of synthesis of specific classes of proteins are modulated. A major period in the shift in protein profile occurs during the swarmer to stalked cell transition at 40 min in synchronous growth (Table I). This change is seen in both the soluble (Fig. 4)

and outer and inner membrane (Figs. 4 and 5) fractions. Examination of the changes in the protein profile indicates that very few proteins are synthesized uniquely by one or the other cell type, but rather that the rates of specific protein synthesis increase and decrease from one stage to another. Two-dimensional gel electrophoresis of the soluble cell fraction (4) and whole cell extracts<sup>3</sup> further indicates that modulation of the rates of protein synthesis are more pronounced than absolute differences in protein profile in both soluble and membrane fractions of the cell. Similar changes in the relative rates of synthesis rather than absolute differences in protein profiles have been described for early events in *Dictyostelium* development (2). As discussed below, it appears, then, that many of the molecular differences between developmental stages are caused by the manner in which proteins are segregated between the cell progeny.

There are two different periods in the cell cycle during which the fate of proteins synthesized at specific stages of development may be regulated in a different manner. The first is during the swarmer to stalked cell transition, and the second is during the formation of the predivisional intermediate cell. As swarmer cells differentiate into stalked cells, the proteins specifically synthesized by swarmer cells may be either discarded or retained in the differentiation process. If retained, these proteins can either remain associated with the region of the cell which will eventually become a swarmer cell, or be evenly distributed among the progeny. The period of formation of the predivisional intermediate cell imposes an additional requirement in the developing cell with respect to the fate of its membrane proteins. The predivisional intermediate must not only synthesize but also assemble specific proteins at different cellular locations. At the same time, each of the daughter cells is provided with the molecular information to proceed with its individual developmental program (Fig. 1).

The problems regarding the fate of specific proteins during development may be addressed in the type of experiment where each of the cell progeny may be isolated and identified. Such a progeny analysis of the proteins of *Caulobacter* is shown in Fig. 5. The patterns are very complex;

however, with some exceptions, several generalizations may be drawn. For the most part, membrane proteins are equally distributed among the cell progeny. This is true both for proteins which are synthesized uniformly during the cell cycle, such as 80Kd, 74Kd, 47/39Kd, and 45Kd, and for others which are stage-specific, such as W88. We believe that proteins which are distributed in this fashion are likely to be important structural proteins of the *Caulobacter* gram-negative membrane. The equivalent distribution of W88 to both progeny stalked and swarmer cells (Fig. 5B, samples 1-3) indicates, first, that this protein is retained during differentiation and that there is no preferential segregation of a swarmer-specific protein with the progeny swarmer cell. Thus, one can say that the *Caulobacter* membrane of either cell type represents a composite of proteins synthesized at various stages of the cell cycle.

The observation that some stage-specific proteins are retained during differentiation is in contrast to the regulation of flagellar organelle expression in *Caulobacter*. It has long been known that the flagellar filament, hook, and rod structures are simply expelled from the cell during the differentiation of the stalked cell (21, 24, 30). The flagellum is not an obligatory structural component of the cell, as W88 may be, and can be deleted by mutation without consequence to the developmental cycle (13, 20, 30).

Although the majority of cellular proteins are equally distributed between daughter cells, several membrane-associated and soluble proteins are segregated asymmetrically (Fig. 5). One of these proteins, flagellin (PD25), has been studied extensively; PD25 is a component of the flagellar filament which is assembled at the swarmer cell pole and is segregated with the swarmer cell at the time of division (Fig. 1). Osley *et al.* (20) have shown that flagellin is synthesized from a relatively long-lived mRNA, and inhibitor studies with the drug rifampicin suggest that the period of mRNA synthesis is in the predivisional intermediate cell. The stability of PD25 mRNA is similar to that of the mRNAs of several proteins which are segregated asymmetrically at the time of cell division (Fig. 6). Studies in our laboratory (15) suggest that the ability to synthesize PD25 after division is restricted to the swarmer progeny cell. Thus, it would appear that there is a functional segregation of flagellin (PD25) mRNA with the progeny swarmer cell. Because flagella are shed shortly after cell division, the flagellin segregation pattern

<sup>3</sup> Milhausen, M., and N. Agabian, manuscript in preparation.

is not resolved in the experiments reported in this paper.

The regulation of flagellin synthesis may be a special case in *Caulobacter*, however, because the progeny analysis shown in Fig. 5 indicates that the majority of proteins which segregate with either daughter cell are not necessarily those that are products of stage-specific protein synthesis. A more interesting and unusual outcome of the progeny analysis experiments (Fig. 5) is the indication that a subclass of soluble proteins, which are synthesized throughout the cell cycle, is found in the outer membrane fraction obtained from progeny stalked cells. Asymmetry between the progeny stalked and swarmer membranes thus appears to be generated by the preferential association of a soluble protein (i.e., 130Kd) with the membrane of only one of the daughter cells. This finding further implies that there is a subclass of proteins which has alternative fates: these proteins are found in the cytoplasm of all cell types, but, subsequent to or coupled with cell division, a fraction of these proteins become fixed in the outer membrane of the progeny stalked cell.

The mechanism by which these soluble proteins become associated with the stalked cell membrane is not known, nor is it known whether these are intrinsic membrane proteins or merely proteins which become transiently bound or otherwise associated with this part of the cell. Their presence in the soluble fraction would seem to indicate that association occurs by some post-translational mechanism. Physical entrapment seems unlikely because these proteins are isolated with the outer membrane on the basis of either density in sucrose or resistance to solubilization in 1% Sarkosyl, indicating at least a firm association with the outer membrane. One hypothesis explaining the differential association of some proteins with the progeny stalked cell is generated by the observation that in *Caulobacter* a specialized membrane system at the junction of the stalk and cell body is formed before cell division (23, 28). The structural and/or functional association of specific soluble proteins with this polar organelle at the time of cell division could thus result in the generation of asymmetry in the progeny stalked cell.

In the case of the *E. coli* murein lipoprotein (10), bacteriophage f1 coat protein (3), and alkaline phosphatase (31), insertion into the membrane or secretion into the periplasm occurs simultaneously with their translation. In *E. coli*, the mRNAs of several membrane proteins are stabi-

lized relative to the rest of the cellular mRNA (8, 16), and it appears from work in *E. coli* that membrane proteins are translated by membrane-bound polyribosomes (25, 31). The interaction of some specific mRNAs or proteins with the polar organelle or the cell pole is further suggested by the following correlation of other asymmetrically segregating proteins with polar morphogenesis.

First, a class of mutants has been isolated that pinch off or abscise their stalks at the stalk-cell junction. The stalks which are produced have a small cellular bleb at the point of abscission, giving them the appearance of stalked mini-cells (22). Unlike stalks sheared from the cell, abscised stalks contain protein 130Kd and proteins of 88,000 and 91,000 daltons (perhaps T88/T91?), suggesting that these proteins may be located in the stalk-cell junction (Agabian, N., unpublished observations.). Secondly, it has been shown that guanosine 3',5'-monophosphate will inhibit polar morphogenesis in *Caulobacter*. Under conditions where polar flagella, pili, and bacteriophage receptors in the swarmer cell are not expressed, the synthesis of two membrane proteins, protein 74Kd (cited 73,000 daltons in reference 11) and a membrane protein of 63,000 daltons (11), is specifically inhibited.

In *Caulobacter*, the new cell poles formed as a result of cell division will be those which differentiate in the subsequent cell cycle (Fig. 1). Polar morphogenesis might, for example, result from an event coupled with the formation of a cell septum which determines the site of future differentiation events. Such a trigger or assembly site might be provided by the primordium of the polar organelle, a few molecules of a specific protein such as 130Kd, or a region of the cell membrane which is otherwise specialized for binding or compartmentalizing specific mRNAs. The expression of polar events would then be regulated by the differentiation program of each daughter cell. The initiation of polar development occurs in the (new) progeny stalked cell pole before that of the progeny swarmer cell pole. Thus, the asymmetry generated in the membrane of the cell progeny may be a reflection of the temporal differences in stalked and swarmer development.

Additionally, by identifying proteins which are segregated in a particular fashion with either daughter cell, we believe that we have identified positionally and developmentally important molecules. It is probable that some aspects of polar morphogenesis in *Caulobacter* involve mecha-

nisms that have evolved from those which are responsible for the formation of cell poles in other gram-negative bacteria. Taken together, these data provide a strong case for examining the relationship between proteins which segregate asymmetrically in *Caulobacter* and polar differentiation.

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