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Cloning of the Major Protein of the *Caulobacter crescentus* Periodic Surface Layer: Detection and Characterization of the Cloned Peptide by Protein Expression Assays

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A precisely ordered crystalline array is found on the surface of the bacterium *Caulobacter crescentus* CB15. Using an immunological assay, we identified recombinant bacteriophage clones expressing the predominant protein of this structure from a λ1059 library of *C. crescentus* CB15 DNA. A single 4.4-kilobase *Hin*dIII fragment encoded a polypeptide whose antigenic determinants, molecular weight, and peculiar solubilization properties were identical with those of the authentic predominant polypeptide (130K) of the surface array. The 130K protein was produced as a discrete product as a result of gene transcription initiated from a λ promoter; several experiments suggested that the *Caulobacter* promoter for this gene is not efficiently recognized by the *Escherichia coli* transcription machinery. Genomic Southern analysis revealed a single copy of the 130K protein gene per genome. The 130K protein gene was hybridized with DNA of two closely related laboratory strains of *C. crescentus* which have lost their ability to produce a surface array. One of these strains, CB2, possesses an homologous copy of the 130K gene, whereas DNA from the other strain, CB13B1a, showed a lesser degree of hybridization to the 130K gene probe; genomic fragments which did hybridize were of different sizes in CB13 as compared with those of CB15. These findings are discussed in relation to studies of the surface array function and its role in cellular morphogenesis in this stalk-forming bacterium.

Crystalline surface arrays of bacteria are ordered structures whose protein components are assembled in a precise fashion at the outer surface of the cell. The regulation of their biosynthesis, transport, and assembly at the external limits of the cell is not understood, and despite their widespread occurrence in aquatic bacteria, their functional relevance to the cell remains unknown (18). The *Caulobacter crescentus* surface array is atypically complex in its constituents and design, consisting of six member units whose regular arrangement seems determined by interconnecting fibers (20). Isolated array fragments contain three major peptides, designated 130K, 74K, and 20K, as compared with usual single polypeptides found in other bacteria. In an electron microscopic immunocytochemical study, we examined the pattern of surface array biogenesis and determined that the structure enlarges by a diffuse intercalation of new array proteins over the body of the cell (19). However, the growth of the membranous stalk of this organism and the formation of its division plane are accompanied by a completely de novo assembly of the surface array on these regions. Thus, in addition to the requirement to form a highly ordered matrix, the pattern of surface array assembly is coordinated with membrane morphogenesis and cell development.

The complex characteristics of *Caulobacter* surface array synthesis and assembly raise intriguing questions with respect to surface array formation which may be addressed through molecular genetic techniques. As an extension of the structural and biochemical analysis of the surface array, we developed methods to isolate the genes which encode the proteins of this layer.

The only criterion available for isolation of surface array proteins was their antigenic determinants. Accordingly, we used antibody-based screening methods to detect recombinant bacteriophage λ1059 clones which were capable of producing the 130K gene product in infected *Escherichia coli* strains. This paper describes the characterization of the cloned sequence and the examination of related *Caulobacter* strains which do not express a crystalline surface structure.

MATERIALS AND METHODS

Bacterial strains and growth media. *C. crescentus* and *E. coli* strains used in this report are listed in Table 1. Except where noted, *C. crescentus* was grown in a peptone-yeast extract medium (11) at 30°C. L broth medium (6) was used for growing *E. coli* strains at 37°C. When plating lambda bacteriophage (λ1059 and derived recombinant phage), the yeast extract was omitted (T medium). *E. coli* cells used for phage infection were grown in L broth with 0.2% maltose, pelleted by centrifugation, and suspended in 10 mM $MgSO_4$.

Purification of 130K protein and preparation of antisera. *C. crescentus* strain CB15, grown in glucose-glutamate-mineral salts, produces insoluble red-pigment aggregates which contain surface array polypeptides (21). These aggregates were solubilized in 2% sodium dodecyl sulfate (SDS) and chromatographed on a Sephacryl S200 gel (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) filtration column in the presence of SDS as previously described (21). The initial fractions of the void volume, which were greater than 95% 130K protein (data not shown), were pooled, dialyzed extensively to remove SDS, and used to prepare antiserum in a New Zealand White female rabbit. An initial injection of 1 mg in complete Freund adjuvant was divided between intramuscular and subcutaneous sites. After 21 days, three booster injections of 0.5 mg of protein in incomplete Freund adjuvant were given at 7-day intervals; maximal immunological activity was found in blood taken at 35, 42, and 49 days postinjection. Immunoglobulin fractions were prepared by standard procedures (2) and maintained at concentrations of

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TABLE 1. Strains

Isolate	Genotype	Comments	Reference or source
Caulobacter sp.			
CB15		ATCC 19089, a wild-type strain	
CB15 NY106		CB15 derivative strain used to isolate surface array fragments	12
CB13B1a		Does not produce a surface array structure	11
CB2-A		A version of strain CB2 that does not produce a surface array structure	
CB2 NY66R		A version of strain CB2 that does produce surface array structure	13
E. coli			
Q358	*hsdR hsdM supF* $\phi 80^r$		4
Q359	*hsdR hsdM supF* $\phi 80^r$ (P2)	Host for cloning λ1059 recombinants	4
159	*uvr sup gal*		15
159 Ind$^-$	*uvr sup gal* (λ i^{434} T)		8
K12ΔHΔTrp	M72 Sm^r *lacZ*(Am) Δ*b10*$^-$ *uvrB*, F$^-$ Δ*trypEA2* (λ *N*am7 *N*am53 *c*I857 Δ*H1*)	Host for pLC28	16
CSR603	*recA1 uvrA6 phr-1 thi-1 leuB proA2 argE3 rpsL31 thr-1*		17
MS100[a]	Δ*lacU169 proA*$^+$ Δ*lon araD139 rpsL* SupF [*trpC22*::Tn*10*]		This paper; 28
Plasmids and phage			
λ1059			4
pBr328	Ap^r Tet^r Cam^r		22
pLC28	Ap^r	p_L promoter vector	16

[a] This strain is Y1090 (28) after elimination of plasmid pMC9 by acridine orange treatment (7).

about twice the original serum concentration. The antibody titer was estimated by a double-diffusion assay (10).

To prepare antiserum against whole cells, a similar immunization, bleeding, and serum fractionation protocol was followed. *C. crescentus* CB15 cells containing 10 mg of protein were disrupted by sonication and used for initial immunization in complete Freund adjuvant. Booster injections contained 5 mg of protein in incomplete Freund adjuvant. The preparation of antiserum to isolated fragments of the *Caulobacter* surface array has been previously described (19).

Cloning in bacteriophage lambda. A λ1059 library of *C. crescentus* CB15 DNA, partially digested with *Bam*HI or *Sau*3A restriction endonucleases, was prepared by standard procedures (6). Approximately 4,800 independent recombinant plaques containing *Sau*3A-digested DNA or 3,200 recombinant plaques resulting from *Bam*HI-digested DNA were pooled for two libraries. For most experiments, the recombinant phage were propagated in strain Q358. To isolate DNA from recombinant λ phage, 200 ml of high-titer lysate (described below) was centrifuged for 6 to 7 h at 12,000 × *g*. The phage pellet was suspended in 1 to 2 ml of SM (50 mM Tris [pH 7.5], 10 mM $MgSO_4$, 0.01% gelatin, 100 mM NaCl) and briefly treated with DNAse I (1 μg), followed by standard DNA isolation procedures (6).

Screening phage libraries for 130K production. Rather than assaying *E. coli* lysogens, a procedure was developed to directly screen λ1059 bacteriophage plaques for the presence of *Caulobacter* DNA-dependent products which reacted with specific antisera. Fresh overnight recombinant phage plaques were prepared in plates (15 cm) containing T medium at a density of ca. 3,000 PFU/plate. The plates were overlaid with dry nitrocellulose membranes (Schleicher & Scheull, Inc., Keene, N.H.) and incubated for 2 to 4 h at 4°C. The residual protein-binding capacity of the membranes was saturated by treatment for 30 min at room temperature in 50 mM Tris (pH 7.5)–150 mM NaCl–0.025% gelatin and 0.05% Nonidet P-40 detergent (NET) containing 1% goat serum and 1% bovine serum albumin. The membranes were then transferred to NET containing 0.5% goat serum (NET-GS), 3 mM NaN_3, and antiserum and incubated overnight at room temperature. Generally, 25 ml of the above solution containing 5 to 15 μl of antiserum was used. After several washes with NET-GS, goat anti-rabbit antibody coupled to horseradish peroxidase (Antibodies Inc.) was added (10 to 15 μl/50 ml of NET-GS) and incubated for 1 to 2 h. The membranes were then washed several times with 50 mM Tris–150 mM NaCl (TES); peroxidase-antibody complexes were visualized by enzyme-catalyzed precipitation of the chromophore 4-chloro-1-naphthol (19).

Plaques that gave rise to a positive reaction were removed with a sterile Pasteur pipette and suspended in SM. Replating and screening was repeated until all plaques reacted in the assay.

High-titer phage lysates were prepared by inoculating 100 ml of L broth medium containing 5 mM $CaCl_2$ with one to two fresh (8 to 16 h after plating) plaques and associated bacteria with a Pasteur pipette. The culture was incubated on a rotary shaker at 37°C for 8 to 16 h, and titers of 10^{10} PFU/ml were routinely obtained.

Immunoblot analysis of recombinant phage-infected cells. A late log culture of host cells was diluted to 5 × 10^8 cells per ml, and recombinant phage was added to 4 ml of cells at a multiplicity of infection of 10. Phage were allowed to adsorb for 20 min at room temperature; the mixture was then incubated for 80 min at 37°C. After centrifugation at 8,000 × *g* for 10 min, 75 μl of water was added to the pellet; samples could then be stored at −20°C. In preparation for gel electrophoresis, the pellets were thawed and treated with 2 μl of DNase I (1.8 mg/ml) for 30 min at room temperature; an

equal amount of gel electrophoresis sample buffer (containing SDS) was then added. The mixture was boiled for 10 min or left at room temperature for 10 to 15 min (see below), and SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (5, 27). After electrophoresis, the separated fractions were transferred to a nitrocellulose membrane, and immunoblots were prepared as previously described (19). Background activity to *E. coli* or lambda phage proteins was significantly reduced by the preincubation of antiserum with an equal volume of a high-titer λ1059 lysate which itself had been treated with 20 mM EDTA, heated to 60°C for 10 min, and disrupted by sonication. After incubation for several hours at 4°C, the antibody-phage lysate mixture was centrifuged at 15,000 × *g*, and the supernatant was used in the immunoblot procedure.

Bacterial colony expression assays for 130K. To assay for 130K synthesis from plasmid subclones of the 130K gene region, the colony expression assay described by Henning et al. (3) was used. To examine 130K production with subclones of the gene cloned in pLC28 (a p_L promoter vector grown in K12ΔHΔTrp, a lambda lysogen host [16]), fresh (overnight) colonies were induced at 42°C for 6 h before transfer to filters.

RESULTS

130K purification and antibody production. The 130K protein represents 5 to 7% of the total cell protein (20). Pigmented particles, produced by some high-density cultures of *C. crescentus* CB15 (21) which entrap shed surface structures, provided a ready source of surface layer fragments and, in turn, 130K protein. As described above, size fractionation by gel filtration of SDS-solubilized particles yields milligram quantities of 130K protein of >95% purity. When antibody raised against these 130K protein preparations was used in immunoblots of the entire surface array preparation, 130K was the only immunoreactive component (see Fig. 2 and 4).

130K also has unusual biochemical properties which were exploited for identification of the recombinant DNA-encoded product. When preparing 130K samples for SDS-PAGE, 130K will become soluble and migrate normally only if it is derived from intact crystalline surface array, e.g., by being boiled in the presence of SDS. If surface array preparations are disrupted mechanically or by EDTA before being boiled in SDS, very little of the 130K will migrate in SDS-PAGE. However, in such preparations, 130K does efficiently enter the gel and migrate if exposed to SDS at less than ca. 40°C. Once 130K is denatured by SDS or other strong denaturants (e.g., guanidine hydrochloride) after the removal of the denaturant, it will not even penetrate the 5% acrylamide stacking layer in SDS-PAGE, regardless of the temperature treatment. Although the protein is not visibly insoluble, it apparently aggregates in an irreversible manner. Its temperature-dependent solubilization properties seem to

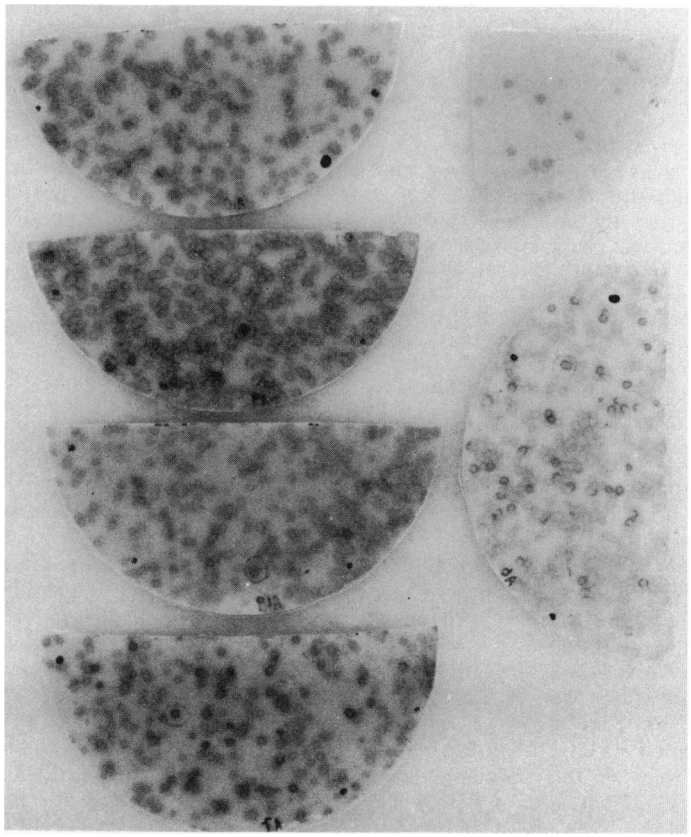

FIG. 1. Antibody screening of recombinant λ1059 phage plaques. Shown are nitrocellulose membranes which have been contacted on bacterial lawns containing phage plaques, treated with antiserum, and a peroxidase-based methodology to reveal the position of bound antibody. Pictured are several instances of an intermediate stage of antibody-reactive plaque purification. Thus, both positive and negative responses are commonly seen on the filters. The antiserum used here was directed to whole *Caulobacter* cells for reasons discussed in the text. The 1/4 filter is a nonrecombinant λ1059 control.

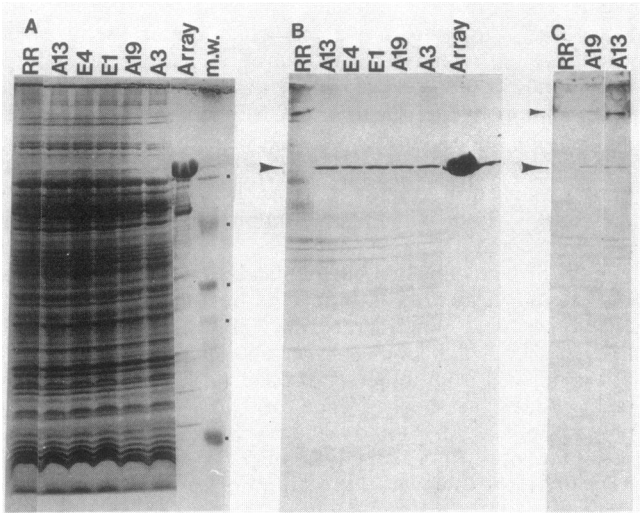

FIG. 2. Immunoblot analysis of cell cultures infected with recombinant phage. Cells were infected with putative 130K containing recombinant phage as described in the text. The cell populations were then solubilized with SDS-PAGE sample buffer at room temperature, electrophoresed in a 10 to 17% gradient acrylamide gel, and electroblotted onto a nitrocellulose membrane for the immunoblot procedure. Panel A, Coomassie blue-stained gel after the electroblotting procedure. Much of the protein does not leave the acrylamide gel during the transfer process and serves as a convenient way to distinguish immunoreactive and nonreacting peptide bands. RR, Randomly chosen recombinant phage that does not react with any of the *Caulobacter* antisera. A13, E4, E1, A19, and A3 are recombinant phage containing the 130K gene. Array, fragments of shed surface layer prepared as previously described (20); m.w., molecular weight standards (phosphorylase *b* [92,500], bovine serum albumin [66,000], alcohol dehydrogenase [41,000], DNase I [31,000], and RNase A [13,700]). Panel B, Immunoblot resulting from the gel shown in panel A labeled with anti-130K antibody whose location is determined by a peroxidase-catalyzed staining method. The arrow indicates the position of the 130K peptide. Note that all of the recombinant phage except the control result in production of an immunoreactive peptide that comigrates with the 130K protein in the surface array preparation. Panel C, immunoblot prepared in the same way as panel B, except the phage-infected cells were boiled in sample buffer. Note that only a very small amount of immunoreactive peptide is seen at the normal position for 130K, but that a higher-molecular-weight immunoreactive band (small arrow) is present in variable amounts.

be manifest when 130K is separated from other elements (including at least two other proteins [20]) of the surface layer.

Identification of cloned 130K by expression. Phage plaques were screened with anti-130K antibody to identify those recombinant λ1059 clones which were expressing the 130K polypeptide. For the initial screening, two filters were prepared from each plate; one filter was processed with specific anti-130K antibody; the second filter was treated with a combination of one antiserum directed against whole cells and another directed against shed surface array fragments. This procedure was adopted because this broad-spectrum antiserum gave a stronger anti-130K response which was useful for confirming the faint positives scored with anti-130K antibody. Figure 1 shows several filters from an intermediate stage of the plaque purification procedure and represents an experiment in which a relatively strong signal was obtained. However, considerable variability in the colorimetric reaction was noted from experiment to experiment, which biases the assay to more strongly reacting clones. The polypeptides expressed by recombinant phage scored as positive in the plaque assay were analyzed by the immunoblot method to determine whether the 130K antibody was reacting with a peptide of the appropriate size. Recombinant phage-infected *E. coli* identified by this procedure were indeed expressing a peptide which comigrates precisely with authentic 130K in SDS-PAGE and is specifically recognized by anti-130K antibody (Fig. 2). Furthermore, the electrophoretic behavior of the cloned peptide was altered when prepared for SDS-PAGE by boiling; only very small amounts of the cloned polypeptide migrated at the expected position. Instead, a slower moving, antibody-positive band sometimes appeared; this may have been a dimer of 130K or an unusual conformation which migrated more slowly than did the completely denatured protein. Peptide bands which migrated in SDS-PAGE at the same position as did the altered forms were also seen in other preparations of 130K which did not solubilize in boiling SDS solutions. Thus, by the criteria of antibody specificity, molecular weight, and distinctive solubilization characteristics, recombinant bacteriophage λ clones were isolated which contained genetic material specifying the 130K protein.

Characterization of the 130K gene fragment. Each of six recombinant phage clones which expressed the 130K polypeptide were isolated, and the DNA of each was mapped and compared by restriction enzyme analysis. Assuming that there is a single copy of the 130K gene, the DNA sequence encoding 130K must be shared by all of these clones. By aligning overlapping regions of the cloned DNA fragments, we were able to localize the 130K gene to a 4.4-kilobase (kb) region bordered by *Hin*dIII and *Bam*HI sites (Fig. 3). Although the protein designation is 130K, more recent determinations have indicated a molecular weight of ca. 105,000. Minimally, therefore, 3 kb of DNA is required to encode the translated portion of the 130K protein; there were no indications that 130K is first produced as a larger precursor form. The 4.4-kb *Hin*dIII-*Bam*HI fragment which spans the gene was subcloned and used as a 130K gene probe.

Although there are significant differences between clones in the amount of *Caulobacter* DNA flanking the 130K gene region, the insert DNAs of all six clones were oriented identically with respect to the bacteriophage vector DNA. This apparent orientation requirement suggested that factors in the adjacent λ DNA may be necessary for the expression of the 130K gene. Because all of the clones produced only a protein of appropriate size and in uniform amounts, it was unlikely that the reason for the required orientation was at the level of translation (i.e., the necessity to form fusion peptides between 130K and lambda proteins). Therefore, we examined the role of promoters on adjacent lambda DNA in 130K production. In addition, *Caulobacter* 130K promoter recognition in *E. coli* was evaluated by subcloning into plasmid vectors.

To determine whether the cloned DNA contained a functional promoter that acts independently of adjacent lambda DNA, the orientation of the cloned segment in one of the recombinant phage was reversed relative to the original clone. Phage clone A19 DNA was digested with *Bam*HI, religated, and packaged into phage particles. One-half of the resulting phage particles should contain the insert DNA in reverse orientation from A19. Twelve phage plaques were selected at random from the new preparation, and the infection and immunoblot process was performed. Of the 12

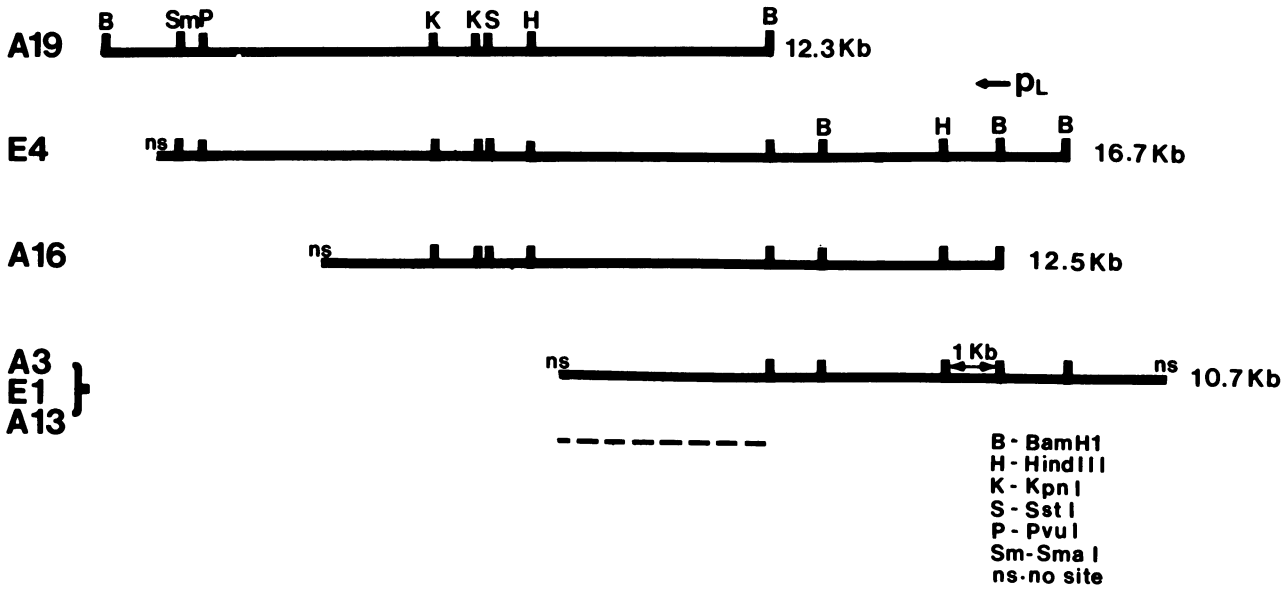

FIG. 3. Six recombinant phage, all capable of eliciting expression of 130K, were subjected to restriction enzyme site analysis. This allowed aligning the cloned insert DNA regions as shown. The right and left portions of the λ1059 DNA are not shown in this figure, but we note that all of the DNA segments shown are oriented in the same direction in the cloning vehicle. The common region of all DNA inserts, and the presumed locus of the gene, is indicated by the dotted line. The "no site (ns)" designation is the result of ligating *Bam*HI and *Sau*3A "sticky ends" during the cloning procedure, which often does not regenerate a *Bam*HI restriction site. The designation p_L refers to the fact that the p_L promoter is located on the right portion of the cloning vector ca. 2 kb from the junction between phage and cloned DNA.

isolates, 4 showed levels of 130K expression similar to the original clone. In the remaining clones, there was either an absence of expression or very much reduced levels. These results suggested that the 130K promoter is not functional in *E. coli* and demonstrated that there indeed is a required orientation of the insert within the lambda genome for expression.

To directly determine whether 130K mRNA is transcribed from an adjacent λ promoter, several of the recombinant phage were used to infect *E. coli* strain 159 Ind$^-$, which contains a noninducible lambda lysogen (8, 15); high levels of the lambda cI protein produced by this strain inhibited transcription from lambda promoters. If 130K transcription was initiated from outside the insert, in a region with λ promoter activity, this host should suppress 130K expression from these clones. Figure 4 indicates that the phage infection does not result in 130K expression in this host, although expected levels of expression were obtained in the control, suggesting that expression requires a functional lambda promoter. Since replication of the phage genome is also repressed by the presence of the lysogen, the possibility that the absence of detectable expression was due to lower copy numbers of the 130K gene could not be entirely ruled out.

The activity or presence of the 130K promoter was directly tested by subcloning the phage A19 insert DNA in plasmid pBR328. These constructs were used to transform either "maxicells" (CSR603) (17) or MS100, an *E. coli* strain which is deficient in degrading some foreign peptides (Deg$^-$), and were assayed for their ability to produce 130K polypeptide; none of the plasmid constructions resulted in detectable amounts of 130K. Taken together, these results strongly suggest that most or all transcriptions which result in 130K production originate from a promoter within adjacent lambda DNA.

A priori, the best candidate to initiate transcription would be p_L, a strong promoter which resides on the right arm of the lambda cloning vector ca. 2 kb from the junction between lambda DNA and the cloned DNA. To test whether this was the case, we inserted the *Hin*dIII-*Hin*dIII fragment of phage A16 that spans the 130K gene (see Fig. 3) into pLC28. This plasmid is a p_L promoter vector with a multiple restriction site region adjacent to p_L (16). The promoter is regulated by a lambda lysogen host specifying a temperature-sensitive cI gene product. Thus, the promoter is fully repressed at 30°C and active at temperatures above 37°C. Transformants resulting from this plasmid were screened by the colony expression assay for 130K production. Approximately one-half of the transformants expressed 130K and did so only when heat induced to derepress p_L. Examination of the plasmid of an expressing clone by restriction enzyme site analysis revealed that the orientation with respect to p_L was the reverse of that found in the lambda clones. The *Hin*dIII-*Bam*HI fragment that spans the 130K gene was also subcloned in pLC28. Because of an appropriate order of restriction sites in the plasmid vector, the only possible orientation of insert with respect to p_L was the same as that found in the lambda clones. In agreement with the previous result, this construction did not result in 130K expression. Thus, in six of six instances, recombinant phage capable of 130K expression have the 130K gene oriented from left to right (Fig. 3), precluding transcription initiation from p_L.

Genomic hybridization studies. *C. crescentus* CB15 DNA was digested with several restriction enzymes whose sites had been mapped in the cloned 130K gene. These DNA digests were electrophoresed, transferred to nitrocellulose, and hybridized with radiolabeled, nick-translated phage A19 DNA. Figure 5 demonstrates that a *Bam*HI fragment of the same size as the A19 insert (12.3 kb) is present in the genome. Digestion with *Hin*dIII and *Sst*I, which cleave within the A19 insert region, results in two hybridizing bands, whereas *Eco*RI, which does not cleave within the

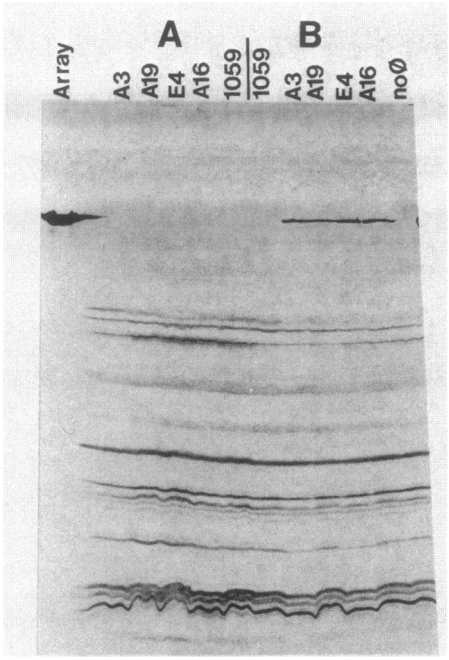

FIG. 4. Application of the immunoblot procedure to test the effect of a λ lysogen host on recombinant phage-directed synthesis of 130K protein. Anti-130K antiserum was used to detect synthesis of the cloned gene protein. Panel A, *E. coli* strain 159 Ind⁻, which contains a noninducible λ lysogen, was the host for infection with the recombinant phage indicated and a λ1059 control. Note the absence of 130K protein. Panel B, Strain 159, which does not contain a λ lysogen, was infected with the same battery of phage, and these lanes serve as positive controls. The lane "no phage (no ϕ)" contains the results obtained with an uninfected cell population. At left are the results obtained with a lane containing the surface array preparation similar to that shown in Fig. 2.

cloned sequence, yields a band larger than the A19 insert. These data indicate that there were no major rearrangements of DNA during the cloning process and that only a single copy of 130K is present in the genome. That is, because the A19 probe used in these experiments has a significant amount of flanking DNA, additional bands would be expected if 130K were in more than one genomic context.

Surface arrays are found on many *Caulobacter* isolates, but in some the structure disappears after a period of maintenance in laboratory cultures. We wished to know whether the surface arrays of various isolates are genetically similar and what types of defects result in the loss of expression. In strain CB2, some stocks produce a surface array (see Fig. 7), whereas others do not. CB13B1a appears to have produced a regular surface structure in earlier reports (14, 20), but at present there are no known stocks which engage in similar production. DNA from these strains and CB15 were digested with several restriction enzymes, blotted to nitrocellulose, and hybridized with a radioactive probe prepared by nick translation of the *Hin*dIII-*Bam*HI region of the A19 insert (Fig. 3). In this way, only regions homologous to the 130K gene and very little surrounding genome are examined. The stock of strain CB2 which does not make a surface layer or 130K (CB2-A) demonstrated a hybridization pattern identical to that of CB15 (Fig. 6). Based on examination of three restriction enzyme digests, the CB2-A version of 130K appears to be very similar to that of CB15, both in number and context within the genome. In contrast, CB13B1a showed marked differences. There was considerably less hybridization between CB13B1a DNA and the 130K gene probe than with CB2-A or CB15, and the single hybridizing region was located on a different size genomic fragment in each of the digests, relative to CB15.

DISCUSSION

We present here the cloning and initial characterization of the gene for the dominant protein of the *C. crescentus* regular surface structure. It represents the beginning of a study with molecular genetic approaches to learn how the organism secretes and assembles this intricate membrane assembly. With *Caulobacter* strains there is the additional opportunity to study the coordination of surface array biogenesis with the dynamic process of morphogenesis in this organism (19).

By screening a bacteriophage lambda library of *C. crescentus* DNA with specific antisera to the dominant protein of the *Caulobacter* crystalline surface array, we have identified clones responsible for the production of an immunoreactive peptide. The cloned product demonstrated the same apparent molecular weight as did the authentic 130K protein, reacted with anti-130K antibody on immunoblots, and had the same unusual solubility characteristics as did the surface array protein. By these criteria, the 130K gene was cloned and made capable of heterologous expression in *E. coli*.

The unusual solubility characteristics of the 130K protein aided in identification of the cloned product but were not completely without precedent. The lactose permease of *E.*

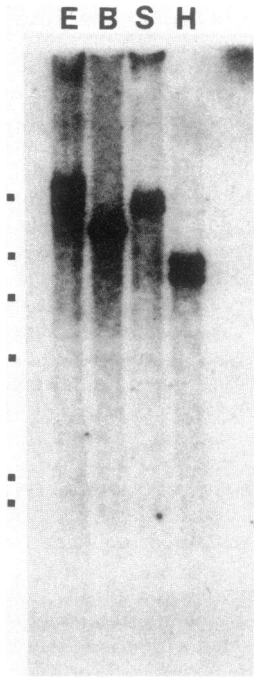

FIG. 5. Genomic Southern analysis (23) of *C. crescentus* CB15 DNA. The radioactive probe for this experiment was prepared by nick translation of the entire phage A19 (see the legend to Fig. 3). Shown are the results obtained by digestion of CB15 DNA with *Eco*RI (E), *Bam*HI (B), *Sst*I (S), and *Hin*dIII (H). The results are discussed in the text. The marks at left indicate the positions of the molecular weight standards (*Hin*dIII digest of λ DNA) in kilobase pairs: 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0.

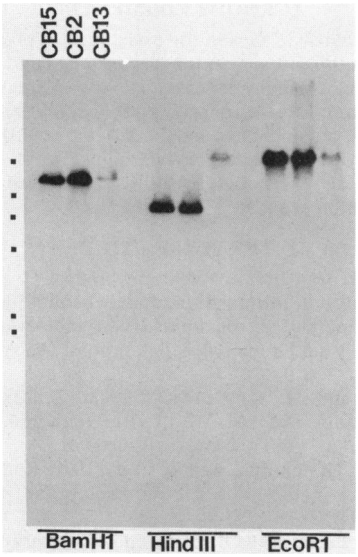

FIG. 6. Genomic Southern analysis comparing the chromosome homology with the 130K gene in *C. crescentus* strains CB15, CB2-A, and CB13B1a DNAs. The radioactive probe for this experiment was prepared from the 4.4-kb *Hin*dIII-*Bam*HI fragment of phage A19 that spans the presumed locus of the 130K gene. Hybridization and subsequent washing of the blot were done under high stringency conditions, permitting mismatch of no greater than ca. 17%. The restriction enzymes used are shown, and the marks at left indicate the position of the size markers (see the legend to Fig. 5).

coli must also be solubilized for SDS-PAGE at a low temperature (26). A similar phenomenon was observed with the *rodA* gene product involved with shape determination in *E. coli* (24). However, we know of no instance in which a protein migrates properly in SDS-PAGE under one set of denaturing conditions, but then fails to migrate after the removal of the denaturant. We have not ruled out the possibility that a polymerization is occurring; however, concentrated (10 mg/ml) solutions showed no precipitates and no increase in viscosity of 130K solutions after a denaturation step was noted. Hence, polymerization seems unlikely. We are tentatively assuming that the protein is aggregated in some way after denaturant removal to produce stable associations, with a molecular weight of at least several million.

It is likely that the 130K promoter is not functional in *E. coli*. Instead, one or more promoters on the left arm of the cloning vector were ultimately responsible for expression of the 130K gene. None of the experiments testing promoter function have distinguished whether the 130K promoter is inactive in *E. coli* or simply is not present in any of the cloned sequences. However, if the latter case is true, the promoter must reside greater than 9 kb upstream from the beginning of the coding sequence as part of an operon, of which only the 130K gene is expressed in *E. coli*. In addition, inability of *E. coli* to efficiently recognize the *Caulobacter* 130K promoter would not be unexpected. Winkler et al. (27a) noted that the promoter for several *Caulobacter* auxotrophic marker genes were poorly recognized in *E. coli*. Similarly, studies in our laboratory with the cloned *Caulobacter* flagellin genes failed to detect significant levels of flagellin expression in *E. coli*. The flagellar hook gene also appears to require fusion to an *E. coli* promoter for efficient expression (9). A lack of *Caulobacter* promoter recognition in *E. coli* may be a general phenomenon which must be considered when developing cloning or gene complementation strategies for additional *Caulobacter* genes. However, a corollary of our findings with the 130K gene is that once a message is transcribed, translation signals are recognized accurately and efficiently enough for immunochemical screening.

The promoter studies also indicate that transcription of 130K mRNA can be initiated only from the left arm of the lambda cloning vehicle from an as-yet-unidentified promoter. It is not clear why the p_L promoter of λ1059 is not used for the expression of 130K. Although there is a transcription termination site for p_L between the promoter and the right-hand *Bam*HI cloning site of λ1059 (25), as far as we can determine, normal antitermination factors are present in λ1059 and the bacterial host used. In addition, the ability to achieve p_L-controlled expression with pLC28 indicates no

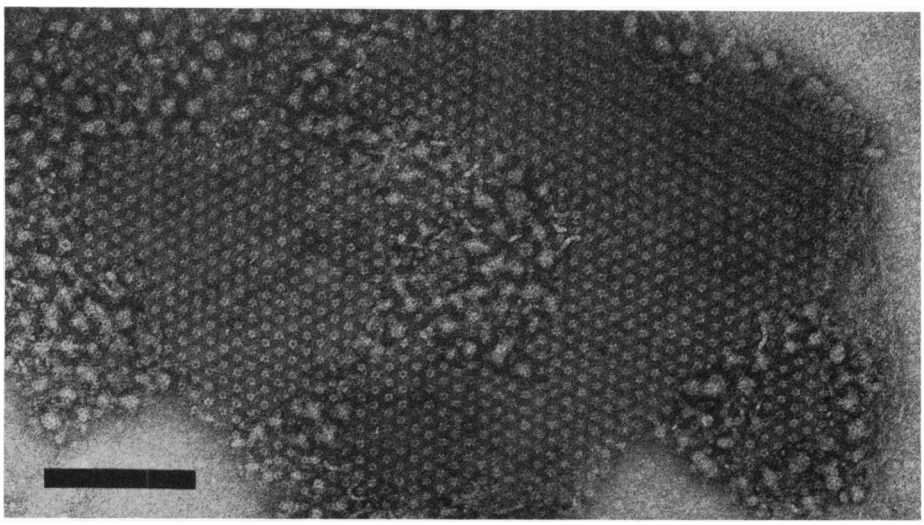

FIG. 7. Electron microscopy of *C. crescentus* strain CB2 NY66 surface array fragments. Shed surface array fragments were isolated and negatively stained with ammonium molybdate as previously described (20). The general appearance of the repeated elements and the center-center spacing are very similar to surface array preparations derived from strain CB15. Bar = 0.2 μm.

unforseen problems pairing the p_L promoter to the 130K gene.

The DNAs of two other independently isolated *C. crescentus* strains were compared with strain CB15 by the Southern blot method. CB2-A appears to contain a gene indistinguishable from the 130K gene of CB15. In fact, SDS-PAGE (data not shown) and electron microscopy of isolated CB2 surface array fragments (Fig. 7) (prepared from substrain CB2NY66R) indicate that the surface array in general is indistinguishable from that of CB15. It is likely that the arrayless stock of CB2-A has lost 130K and subsequent array production, due to a comparative minor genetic alteration (e.g., point mutation) which results in the halted production of 130K.

The results with strain CB13B1a, however, give a somewhat different view. Assuming that CB13B1a at one time did produce a surface layer, it did so with a distinctly different set of genes, or the elimination of surface array production during laboratory culture was the result of a significant genetic rearrangement and elimination of the 130K gene equivalent. Examination of older stocks of CB13B1a with hybridization probes may help resolve this point.

The similarity in surface layers between CB15 and CB2 contrasts with the fact that they are unquestionably different strains of *C. crescentus*. Each was isolated at different times and places and each has different patterns of phage sensitivity (11). Southern blots of these two strains were hybridized with a flagellin gene probe which detects all known flagellin genes in the multigene family of CB15 (data not shown). Although some basic similarities in number and size of hybridizing bands were seen, the profiles were not the same, confirming the separate identity of these strains. Thus, the particular surface layer we have been examining is most likely not a chance acquisition by one strain of *C. crescentus* but rather may have developed as a fundamental character of the organism in the natural environment, providing specific protective or competitive advantages. In fact, in preliminary experiments with ca. 20 isolates of marine *Caulobacter* strains, all showed significant hybridization with the 130K gene probe, suggesting that they all may have similar surface array structures (J. Smit, manuscript in preparation). Clearly, additional study is needed to clarify the genetic relatedness of the *Caulobacter* group and the requirements for a regular surface array in the natural environment.

Southern analysis of the CB15 genome was done in part to determine whether there were multiple copies of the 130K gene within the genome. The interest in the 130K gene copy number extends to the study of *Caulobacter* cell development. A long-term goal has been to discover how *Caulobacter* strains are able to produce a stalk at a very specific region on the cell with the same membrane materials, including the surface array, used to construct the remainder of the cell boundary. One possible mechanism of differential assembly of similar components could be via a duplicate set of some genes whose temporal and spatial control is different from the set of genes responsible for general membrane synthesis. This hypothesis is rendered unlikely by the apparent finding of a single gene copy. Thus, this single-surface-layer gene can be viewed as capable of expressing multiple roles or positional fates during the *Caulobacter* life cycle. How this dichotomous expression is accomplished constitutes a fundamental question in developmental biology research. The cloned surface array gene enables many molecular genetic approaches into the study of this intriguing problem.

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LITERATURE CITED

1. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of protein from sodium docecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
2. **Heide, K., and H. G. Schwick.** 1978. Self fractionation of immunoglobulin. *In* D. M. Weir (ed.), Handbook of experimental immunology, vol. 1. Blackwell, Oxford.
3. **Henning, U., H. Schwarz, and R. Chen.** 1979. Radioimmunological screening method for specific membrane proteins. Anal. Biochem. **97**:153–157.
4. **Karn, J., S. Brenner, L. Barnett, and G. Cesarani.** 1980. Novel bacteriophage cloning vector. Proc. Natl. Acad. Sci. U.S.A. **77**:5172.
5. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
6. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1983. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Murialdo, H., and L. Siminovitch.** 1972. The morphogenesis of bacteriophage lambda. IV. Identification of gene products and control of the expression of the morphogenetic information. Virology **48**:785–823.
9. **Ohta, W., L. S. Chen, and A. Newton.** 1982. Isolation and expression of cloned hook protein gene from *Caulobacter crescentus*. Proc. Natl. Acad. Sci. U.S.A. **79**:4863–4867.
10. **Ouchterlony, O.** 1949. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. **26**:507–515.
11. **Poindexter, J. S.** 1964. Biological properties and classification of the *Caulobacter* group. Bacteriol. Rev. **28**:231–295.
12. **Poindexter, J. S.** 1978. Selection for nonbuoyant morphological mutants of *Caulobacter crescentus*. J. Bacteriol. **135**:1141–1145.
13. **Poindexter, J. S., and L. F. Eley.** 1983. Combined procedure for assays of poly-β-hydroxybutyric acid and inorganic polyphosphates. J. Microbiol. Methods **1**:1–17.
14. **Poindexter, J. S., P. R. Hornack, and P. A. Armstrong.** 1967. Intracellular development of a large DNA bacteriophage lytic for *Caulobacter crescentus*. Arch. Microbiol. **59**:237–246.
15. **Ptashne, M.** 1967. Isolation of the lambda phage repressor. Proc. Natl. Acad. Sci. U.S.A. **57**:306–313.
16. **Remaut, E., P. Stanssens, and W. Fiers.** 1981. Plasmid vectors for high-efficiency expressor controlled by the p_L promoter of coliphage lambda. Gene **15**:81–93.
17. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. **137**:692–697.
18. **Sleytr, U. B.** 1978. Regular arrays of macromolecules on bacterial cell walls: structure, chemistry, assembly and function. Int. Rev. Cytol. **53**:1–64.
19. **Smit, J., and N. Agabian.** 1982. Cell surface patterning and morphogenesis: biogenesis of a periodic surface array during *Caulobacter* development. J. Cell Biol. **95**:41–49.
20. **Smit, J., D. A. Grano, R. M. Glaeser, and N. Agabian.** 1981. Periodic surface array in *Caulobacter crescentus*: fine structure and chemical analysis. J. Bacteriol. **146**:1135–1150.
21. **Smit, J., M. Hermodson, and N. Agabian.** 1981. *Caulobacter crescentus* pilin: purification, chemical characterization and NH_2-terminal amino acid sequence of a structural protein

regulated during development. J. Biol. Chem. **256**:3092–3097.
22. **Soberon, X., L. Covarrubias, and F. Bolivar.** 1980. Construction and characterization of new cloning vehicles. IV. Deletion derivatives of pBr322 and pBr325. Gene **9**:287–305.
23. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
24. **Stoker, N. G., J. M. Pratt, and B. G. Spratt.** 1983. Identification of the *rodA* gene product of *Escherichia coli*. J. Bacteriol. **155**:854–859.
25. **Szybalski, W., D. Drahos, K.-C. Luk, and G. Somasekhar.** 1983. Antitermination: the lambda system, p. 35–38. *In* D. Schlessinger (ed.), Microbiology—1983. American Society for Microbiology, Washington, D.C.
26. **Teather, R. M., B. Muller-Hill, U. Abrutsch, G. Aichele, and P. Overath.** 1978. Amplification of the lactose carrier protein in *Escherichia coli* using a plasmid vector. Mol. Gen. Genet. **159**:239–248.
27. **West, D., C. Lagenaur, and N. Agabian.** 1976. Isolation and characterization of *Caulobacter crescentus* bacteriophage ϕCd1. J. Virol. **17**:568–575.
27a. **Winkler, M. E., P. V. Schoenbein, C. M. Ross, J. T. Barrett, and B. Ely.** 1984. Genetic and physical analyses of *Caulobacter crescentus tip* genes. J. Bacteriol. **160**:279–287.
28. **Young, R., and R. Davis.** 1983. Yeast RNA polymerase II genes; isolation and antibody probes. Science **22**:778–782.