Overproduction and purification of the connector protein of *Bacillus subtilis* phage φ29

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ABSTRACT
     A φ29 DNA fragment containing genes 10 and 11, coding for
the connector protein and the lower collar protein, respectively,
has been cloned in the pBR322 derivative plasmid pKC30 under the
control of the P$_L$ promoter of phage lambda. Two polypeptides with
the electrophoretic mobility of proteins p10 and p11 were label-
led with $^{35}$S-methionine after heat induction. The proteins were
characterized as p10 and p11 by radioimmunoassay and they repre-
sented about 10% and 7%, respectively, of the total E. coli pro-
tein after 4 hours of induction. These proteins represent less
than 1% of the B. subtilis protein in φ29-infected cells. Protein
p10 has been highly purified from the E.coli cells carrying the
recombinant plasmid. Antibodies raised against the purified pro-
tein p10 reacted with the connector protein produced in φ29-in-
fected B. subtilis.

INTRODUCTION
     The head-tail connecting region in bacteriophages  contains
a protein, the connector, that plays a key  role in the early
steps of prohead assembly. It has been shown recently in the  B.
subtilis phage φ29 (1,2) and in the E. coli phages T4 (3)  and
lambda (4) that the connector is an oligomer of a single protein
with 12-fold symmetry. This is, therefore,  the region at  which
the symmetry transition between the lower corner of the  phage
head (5-fold symmetry) and the tail (6-fold symmetry)  occurs.
According to Hendrix (5), the connector is also an active device
moving the DNA into the phage prohead. In addition, in phage φ29,
the connector is essential for the normal prohead elongation (6,7).
     Neck-tail complexes of phage φ29, composed of proteins p10
(upper collar or connector), p11 (lower collar), p12* (neck  ap-
pendages) and p9 (tail), have been isolated and the symmetry  of
the neck region (proteins p10 and p11) determined (1,2). To stu-
dy the structure of the isolated connector protein, p10, and  to

correlate it with that of the protein assembled in the phage necks, as well as to determine whether the protein has some catalytic activity that may be relevant to the process of DNA packaging we undertook the purification of the protein.

In this paper we describe the cloning of genes 10 and 11 in plasmid pKC30 (8) under the control of the $P_L$ promoter of phage lambda. Four hours after heat induction protein p10 (the connector) represents about 10% of the total E. coli protein whereas it amounts to less than 1% of the B. subtilis protein in Ø29-infected cells. The connector protein synthesized in E. coli has been isolated in a highly purified form and antibodies against the purified protein react with protein p10 synthesized in Ø29-infected B. subtilis.

MATERIALS AND METHODS

Bacterial strains, phage and plasmids

The E. coli strain N99 ($\lambda^+$) and plasmid pKC30 were obtained from M. Rosenberg (8). The E.coli $\lambda$ lysogen K-12$\Delta$trp ($\lambda N^-$cI857 $\Delta$ H1) (K-12$\Delta$H1$\Delta$trp) (9) was obtained from M. Zabeau. B. subtilis 110NA try$^-$spo A$^-$ was as described by Moreno et al. (10). The Ø29 mutant sus8(50011)sus14(1242) was as described by García et al. (11). The mutation sus14(1242) produces a delayed lysis phenotype (12).

Enzymes

Restriction endonucleases were from New England Biolabs and were used according to the supplier. The DNA ligase and nuclease Bal 31 were from Bethesda Research Laboratories, E.coli DNA polymerase I from Boehringer, DNase I from Worthington and fungal proteinase K from Merck.

DNA preparations and transformation

Plasmid and phage Ø29 DNA were isolated as described by Clewell and Helinski (13) and Inciarte et al. (14), respectively. The Ø29 DNA fragment Pvu I A (see Fig. 1) was isolated by centrifugation in a 5-20% sucrose gradient as described by Salas et al. (15). About 4 µg of the fragment were digested with 4 units of nuclease Bal 31 for 3 min at 30°C in a buffer containing 20 mM Tris-HCl, pH 8.1, 12 mM $CaCl_2$, 1 mM EDTA and 600 mM NaCl. After phenol extraction and ethanol precipitation the Bal

31-treated Pvu I A fragment (2 μg) was blunt-end ligated to Hpa I-linearized plasmid pKC30 (3 μg) by incubation with T4 DNA ligase as described by García et al. (16).

Transformation of competent E. coli cells and detection of colonies harbouring recombinant plasmids by hybridization to $^{32}$P-labelled ⌀29 DNA were carried out as described (16). The recombinant plasmids containing ⌀29 DNA sequences were further screened by restriction analysis to determine the size and the orientation of the insert.

Protein analysis in cells transformed by the plasmids

The cells were grown at 30°C in LB medium with ampicillin (50 μg/ml). At $A_{420}$ = 0.46 the bacteria were collected by centrifugation and resuspended in the same volume of minimal media (17). After 1 h at 30°C the temperature was shifted to 42°C and, at the indicated times, samples of 0.05 ml were labelled for 10 min with 10 μCi of $^{35}$S-methionine (1200 Ci/mmol, The Radiochemical Centre, Amersham). When indicated, after the 10 min pulse a 1000 fold excess of cold methionine was added and samples removed after different times. Samples were subjected to SDS-polyacrylamide gel electrophoresis as described below.

To determine protein accumulation the bacteria were grown in LB medium and samples were taken at different times after the shift at 42°C. The bacteria were concentrated 10-fold by centrifugation and resuspension in a buffer containing 100 mM Tris-HCl, pH 8.0, 200 mM KCl, 1 mM EDTA, 2 mM $CaCl_2$, 10 mM $MgCl_2$, and 5% glycerol and lysed by sonication. Bacterial debris were removed by centrifugation for 5 min at 8900 g and the samples subjected to SDS-polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis

SDS-electrophoresis was carried out either in slab gels containing 10% acrylamide (18) or in 10-20% acrylamide gradients (12). For the radioimmunoassay of protein p10 after electrophoresis, 4 M urea was present both in the stacking and the separation gels. The gels were either stained as described by Fairbanks et al. (19) or dried for autoradiography. Densitometric analysis was performed on the autoradiographs or on Kodalith orthofilm copies of the stained gels, using an Optronics digital microdensitometer with a 100 μm square raster, connected to a Digital

PDP 11/45 minicomputer.

Preparation of sera

The neck-tail complexes of Ø29, containing proteins p9,p10, p11 and p12* were isolated from phage particles as described by Carrascosa et al. (1). Protein p10 was purified from E. coli containing the recombinant plasmid pKC30 X16 as described in this paper.

Rabbits were immunized with two 100 µg doses of total protein, the first time in complete Freund's adjuvant. Serum was collected one week after the last injection.

Characterization of proteins p10 and p11 by radioimmunoassay

Cellular extracts of E. coli transformed with the recombinant plasmids prepared as described above were subjected to SDS-polyacrylamide gel electrophoresis and proteins p10 and p11 were detected by radioimmunoassay carried out as described (16) using anti neck-tail serum and $^{125}$I-protein A (a gift from I. Prieto).

B. subtilis 110NA was infected with the Ø29 mutant sus8(50011) sus14(1242) and labelled with $^{35}$S-sulfate (200 µCi/ml; 25-40 Ci/ mg, The Radiochemical Centre, Amersham) as described by Camacho et al. (20). After 60 min at 37°C the cells were lysed with ly-sozyme, treated with DNase I and RNase A and the cell debris were removed by low-speed centrifugation. A control of uninfec-ted bacteria was labelled and processed in the same way. A ra-dioimmunoassay was carried out following the procedure developed by J.A. Melero (personal communication). Briefly, protein A is bound to wells of a polystyrene multiwell plate followed by in-cubation first with anti-protein p10 serum and then with the $^{35}$S-labelled extracts of B. subtilis infected with mutant sus8 (50011)sus14(1242). The bound protein is analyzed by SDS-poly-acrylamide gel electrophoresis.


RESULTS

Isolation and characterization of recombinants

The restriction fragment Pvu I A containing the late region of Ø29 DNA, transcribed from left to right (see Fig. 1), was di-gested under controlled conditions with nuclease Bal 31 and blunt-end ligated to plasmid pKC30 linearized with Hpa I (8).E. coli N99 (λ$^+$) was transformed with the ligation mixture, the amp$^r$
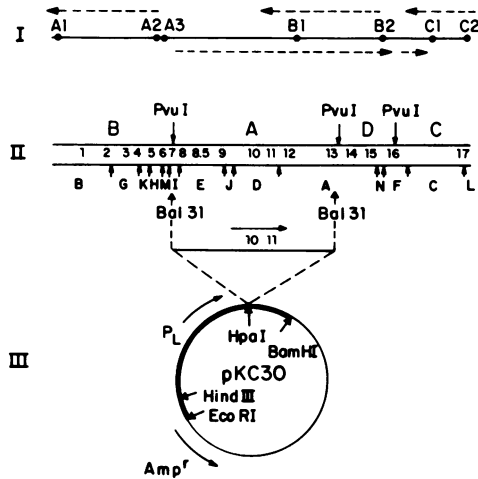
Fig.1. Construction of pKC30 recombinant plasmids containing the
Ø29 DNA fragment Pvu I A digested with Bal 31. (I) The filled
circles represent binding sites for B. subtilis RNA polymerase
on Ø29 DNA and the dashed lines with the arrows the extent and
direction of early transcription (top) and late transcription (bottom)
as described by Sogo et al. (21). (II) Genetic map of Ø29 DNA
taken from Mellado et al. (22). The location of the Pvu I  and
Hind III cuts has been taken from Yoshikawa and Ito (23). The Hind
III cuts are shown below the gene numbers with small arrows.(III)
Plasmid pKC30 showing the location of the Ø29 DNA insert. The
direction of transcription of the ampicillin gene and of the Ø29
genes 10 and 11 from the lambda $P_L$ promoter are indicated by arrows.


colonies were screened by hybridization to a $^{32}$P-labelled Ø29 DNA

probe and the positive clones were further checked by Hind III

digestion. Several recombinant plasmids containing fragment Hind

III D were  obtained, indicating the insertion of the Bal 31-di-

gested Pvu I A fragment (see Fig. 1). Insertions of a wide range

of sizes were obtained; in addition, Bal 31 digested asymmetri-

cally from each end of the Pvu I A fragment. Treatment of the

above recombinant plasmids with Hind III produced, besides the

Ø29 DNA Hind III D fragment, two other ones corresponding to the

fusion of the rest of the Ø29 DNA inserted and each of the two

pKC30 fragments of 4.7 and 1.6 kb which are present between the

Hind III site of the plasmid and the Hpa I insertion site (see

Fig. 1). The analysis of these fragments showed that in the re-

combinant plasmid pKC30 X16 the Ø29 DNA insert ($\sim$5.8 kb) was in

the correct orientation for transcription from the $P_L$ promoter
and in plasmid pKC30 X6 the insert ($\sim$5.9 kb) was in the opposi-
te orientation. Therefore, Bal 31 had digested about 100 and
2500 nucleotides, respectively, from the right and left ends of
the Pvu I A fragment in plasmid pKC30 X16 and about 300 and 2200
nucleotides, respectively, from the right and left ends of the
fragment in plasmid pKC30 X6.

Protein synthesis directed by the recombinant plasmids

      The recombinant plasmids were used to transform the lysogen
E. coli strain K-12ΔH1Δtrp with the thermosensitive cI857 muta-
tion in the lambda repressor. By raising the temperature  from
$30^O$ to $42^O$C the repressor is inactivated allowing the expression
of the genes under the control of the lambda $P_L$ promoter.  The
above cells, carrying the recombinant plasmids pKC30 X16 or pKC30
X6, were grown at $30^O$C as described in Materials and Methods and
at 30 min, 60 min and 120 min after induction at $42^O$C, they were
labelled with ^{35}S-methionine and the proteins analyzed by SDS-
polyacrylamide gel electrophoresis. As shown in Fig. 2, in the
cells transformed with the recombinant plasmid pKC30 X16, with
the ∅29 DNA insert in the correct orientation for transcription
from the $P_L$ promoter, one polypeptide with the same electropho-
retic mobility as the ∅29 protein p10 was clearly labelled after
60 or 120 min at $42^O$C. This polypeptide was not present in  the
cells transformed with the control plasmid pKC30. In the case of
plasmid pKC30 X6, with the ∅29 DNA inserted in  the opposite
orientation, the same polypeptide was labelled, although in smal-
ler extent, suggesting that a ∅29 promoter could be used to trans-
cribe this gene in the plasmid (see later). At $30^O$C, the synthe-
sis of this protein was much smaller than at $42^O$C in the cells
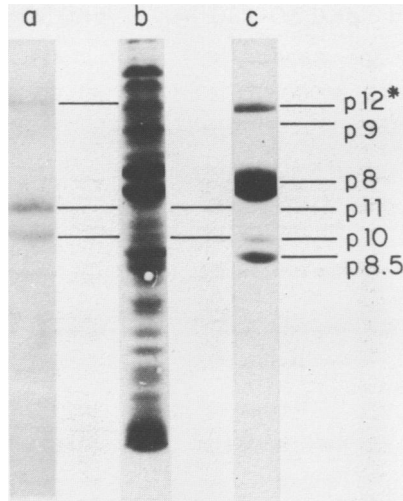transformed by either recombinant plasmid, pKC30 X16 or pKC30 X6.

      A polypeptide with the electrophoretic mobility expected
for protein p11 was also labelled after heat induction in the
cells transformed with both recombinant plasmids, pKC30 X16 and
pKC30 X6, although some protein of similar size was also label-
led in the cells transformed with the control plasmid pKC30 (see
Fig. 2). The evidence that the protein labelled in the cells
transformed by the recombinant plasmids was, indeed, p11, was ob-
tained by radioimmunoassay (see later).
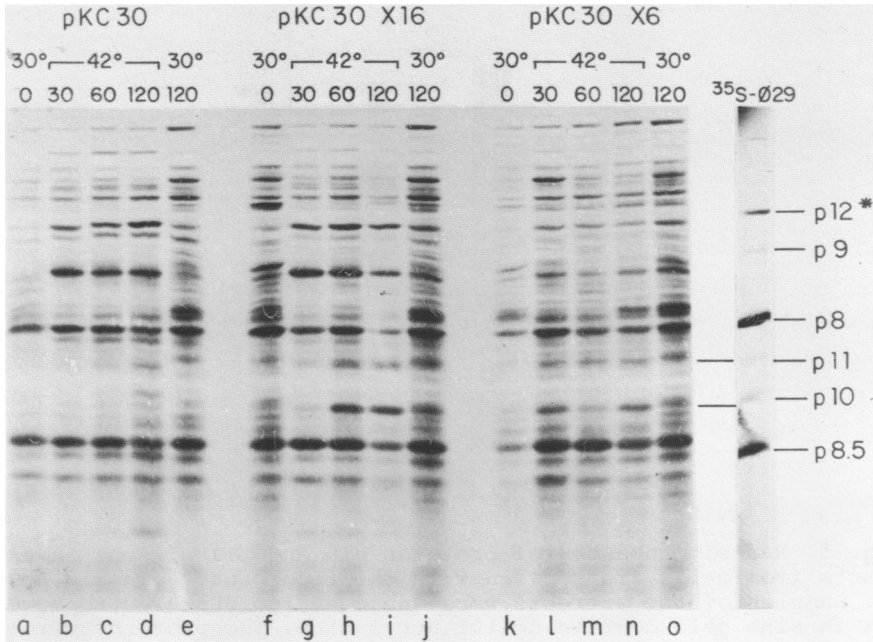
Fig. 2. SDS-polyacrylamide gel electrophoresis of extracts of cells transformed by the recombinant plasmids, labelled with 35S-methionine. The cells transformed with the recombinant plasmids pKC30 X16 or pKC30 X6 or with the control plasmid pKC30 were grown at 30°C and at 30, 60 and 120 min after the shift to 42°C, labelled and subjected to electrophoresis in 10% acrylamide gels as described in Materials and Methods. As a control, cells kept at 30°C were labelled at the time of induction and 120 min later. The starting time of each labelling period (in min) and the temperature of incubation are indicated above the slots. 35S-labelled Ø29 structural proteins were used as molecular weight markers (24).


Characterization of proteins p10 and p11 by radioimmunoassay

To determine whether the extracts of E. coli transformed with the recombinant plasmids contained indeed proteins p10 and p11, cells were induced at 42°C and, after 120 min, extracts were subjected to SDS-polyacrylamide gel electrophoresis and the proteins transferred to two cellulose nitrate papers. One of them was stained and the other one incubated first with anti-Ø29 neck-tail serum and then with 125I-protein A. Autoradiography showed the presence of labelled bands at the position of proteins p10 and p11 in the extracts from cells transformed by the recombi-

Fig. 3. Radioimmunoassay of proteins p10 and p11 present in ex-
tracts from cells transformed with the recombinant plasmid pKC30
X6. Samples of the extracts were subjected to SDS-electrophore-
sis in slab gels containing 10% acrylamide. The proteins were
transferred from the gel to two sheets of nitrocellulose paper
which were incubated with anti-neck-tail serum and $^{125}$I-protein
A (a) or stained with amido black (b), respectively, as descri-
bed in Materials and Methods. c, structural proteins of ∅29.

nant plasmid pKC30 X6 (Fig. 3a) or pKC30 X16 (not shown). In ad-
dition, a band with the electrophoretic mobility of p12* was
also labelled in the cells transformed by the recombinant plas-
mid pKC30 X6, suggesting the presence of gene 12 in the above
recombinant plasmid. This is in agreement with the fact that
Bal 31 digestion had only removed 300 nucleotides from the right
end of fragment Pvu I A present in the recombinant plasmid pKC30 X6.
Protein p10 was detected by staining but very low amount of pro-
tein p11 was present in the extracts of the transformed cells
(Fig. 3b) due to the fact that protein p11 sedimented after  low
speed centrifugation (see later).

Stability of proteins p10 and p11

      Proteins p10 and p11 induced in E. coli harbouring plasmid
pKC30 X16 were synthesized at a high rate at least up to 2 h af-
ter heat induction (see Fig. 2). To test the stability in E.coli
of the induced proteins, cells transformed with plasmid pKC30
X16 were shifted to 42°C, induced for 75 min, incubated 10 addi-
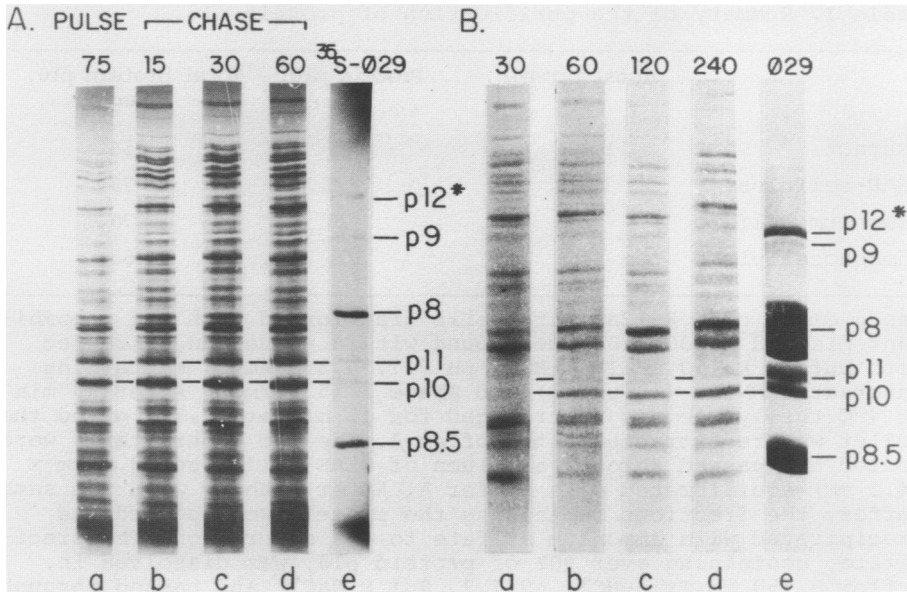
Fig. 4. Stability of proteins p10 and p11. A. E. coli cells ca-
rrying the recombinant plasmid pKC30 X16 were shifted to 42ºC
and, after 75 min, labelled with $^{35}$S-methionine for 10 min (a).
The labelled proteins were chased by addition of a 1000 fold ex-
cess of cold methionine and samples removed at 15 min (b), 30
min (c) and 60 min (d) and subjected to SDS-electrophoresis in
10% acrylamide slab gels. $^{35}$S-labelled Ø29 structural proteins
were used as molecular weight markers in (e). B. E. coli cells
carrying plasmid pKC30 ·X16 were induced at 42ºC and at 30 min
(a), 60 min (b), 120 min (c) and 240 min (d) samples were remo-
ved and the extracts, prepared as described in Materials and Me-
thods, were subjected to SDS-electrophoresis in 10% acrylamide
slab gels. Ø29 structural proteins were used in (e) as molecular
weight markers. After electrophoresis the gel was subjected to
autoradiography (A) or stained (B) as described in Materials and
Methods.

tional min in the presence of $^{35}$S-methionine and the labelled

polypeptides chased by addition of an excess of cold methionine.

Figure 4A shows that the two proteins, with the electrophoretic

mobility of p10 and p11, were stable after a chase of 1 h.

Taking into account the stability of the proteins and their high

rate of synthesis for at least 2 h it was expected to find pro-

gressive accumulation of proteins p10 and p11 after heat induc-

tion of cells harbouring the recombinant plasmid pKC30 X16. Fi-

Table 1. Summary of the purification of protein p10.

|  | Total protein, mg | Protein p10, % | Total protein p10, mg |
|---|---|---|---|
| Extract | 1,404 | 11 | 154 |
| DEAE-cellulose | 984 | 8 | 79 |
| Ammonium sulfate | 280 | 25 | 70 |
| Phosphocellulose | 63 | 95 | 60 |

Ten g of E. coli K-12ΔH1Δtrp cells transformed with the recombi-
nant plasmid pKC30 X16 were ground with alumina and extracted
with buffer A (50 mM Tris-HCl, pH 7.7, 0.3 M KCl), containing
pancreatic RNase (10 μg/ml) and DNase I (5 μg/ml). After 30 min
at 4°C the lysate was centrifuged for 15 min at 19,700 g and the
pellet was re-extracted with buffer A. The two supernatants were
pooled and passed through a column of DEAE-cellulose (3.6 cm x
24.3 cm) equilibrated with buffer A. After washing with the same
buffer, the fractions containing the protein were pooled and
precipitated with ammonium sulfate to 43% saturation. The preci-
pitate, containing over 90% of protein p10, was dissolved in
buffer B (50 mM Tris-HCl, pH 7.7, 0.1 M NaCl) and passed through
a column of phosphocellulose (2.9 cm x 24.6 cm) equilibrated
with buffer B containing 5% glycerol). The column was washed
stepwise with 4 volumes of buffer B containing 0.2 M, 0.4 M, 0.6
M, 0.8 M and 1.0 M NaCl, respectively. Protein p10 eluted at 0.6
M NaCl essentially free of contaminating proteins. SDS-polyacryl-
amide gel electrophoresis was carried out at the different puri-
fication steps to follow the presence of protein p10. Protein
concentration was determined by the method of Bradford (25).


gure 4B shows that there was a progressive accumulation of pro-

tein p10 up to at least 4 h, representing at this time about 10%

of the total E. coli protein. The amount of protein p11 was very

low due to the fact that most of p11 sediments after low-speed

centrifugation (see later).

Purification of protein p10

The amount of protein p10 synthesized in Ø29-infected B.sub-

tilis represents less than 1% of the total  bacterial protein

(data not shown). Since in the E. coli cells transformed with

the gene 10-containing recombinant plasmid pKC30 X16 protein p10

represents about 10% of the total protein after 4 h of heat in-

duction, we used these cells as a source of protein p10 for pu-

rification. Lysates were centrifuged as described in the legend

to Table 1. It can be seen  in Fig. 5, lanes b and c, that most

of protein p11 is lost by low-speed centrifugation of the lysate
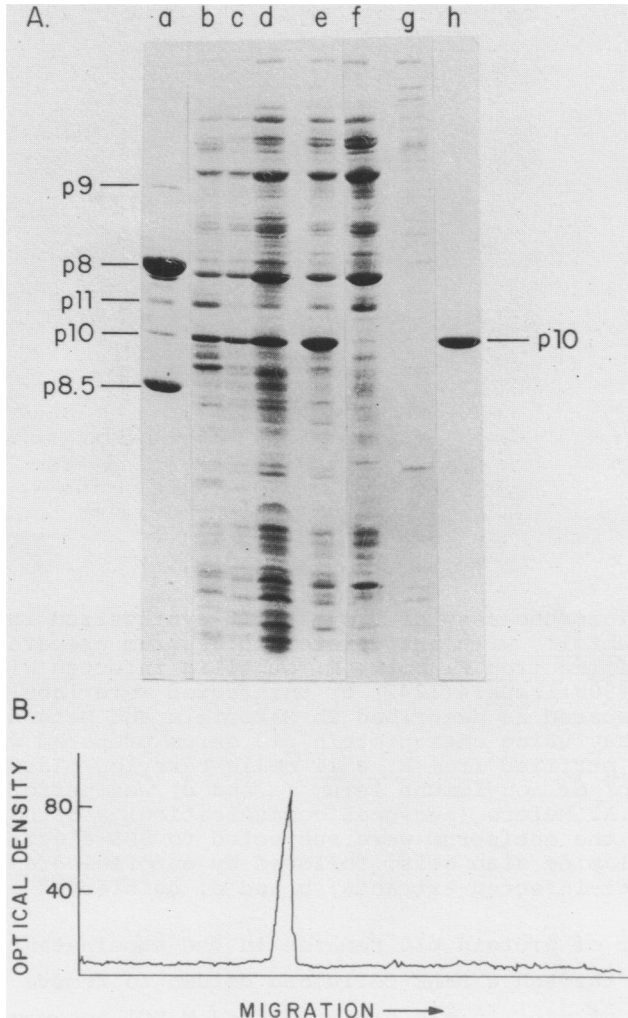
Fig.5. SDS-polyacrylamide gel electrophoresis of purified pro-
tein p10. A. Proteins at various purification steps were subjec-
ted to SDS-electrophoresis in slab gels containing a 10-20% acryl-
amide gradient. After electrophoresis the proteins were stained
as described in Materials and Methods. (a) structural proteins
of ⌀29 12⁻ particles. (b) lysate (48 µg); (c) extract, (36 µg);
(d) DEAE-cellulose (180 µg); (e) ammonium sulfate (140 µg); (f)
phosphocellulose, flow-through (80 µg); (g) phosphocellulose,0.4
M NaCl (14 µg); (h) phosphocellulose, 0.6 M NaCl (18 µg). B.Den-
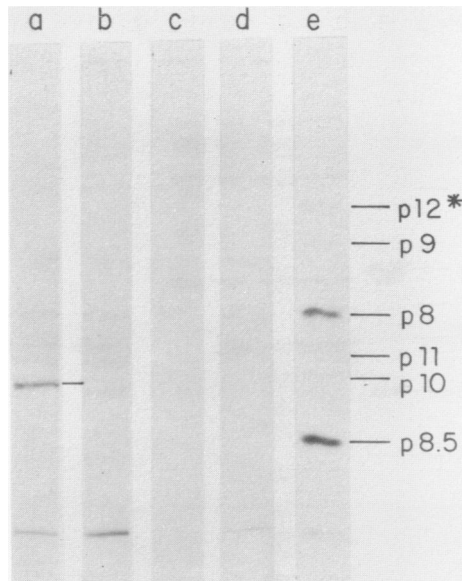sitometric analysis of purified protein p10 (phosphocellulose
fraction).

Fig. 6. Radioimmunoassay of protein p10 synthesized in ∅29-in-
fected B. subtilis with anti-protein p10 serum prepared with the
protein purified from E. coli. B. subtilis infected with the ∅29
mutant sus8(50011)sus14(1242) or uninfected were labelled  and
extracts prepared as described in Materials and Methods. The ra-
dioimmunoassay using anti-protein p10 serum prepared with  the
protein p10 purified from E. coli cells carrying plasmid pKC30
X16 (a and b) or non-immune serum (c and d) was performed as des-
cribed by J.A. Melero (personal communication) and the proteins
retained by the antiserum were subjected to SDS-electrophoresis
in 10% acrylamide slab gels, followed by autoradiography. a and
c, ∅29 mutant-infected extracts; b and d, uninfected extracts.

whereas most of protein p10 remains in the supernatant. Extracts

were passed through a DEAE-cellulose column to remove nucleic

acids. Most of protein p10 eluted at 0.3 M KCl and was precipita-

ted with ammonium sulfate to 43% saturation. Protein p10 was

further purified by phosphocellulose chromatography, eluting  at

0.6 M NaCl essentially free of contaminating proteins (Fig. 5).

Table 1 gives a summary of the purification. About 60 mg of puri-

fied protein p10 were obtained from 10 g of E. coli harbouring

the gene 10-containing recombinant plasmid pKC30 X16.

Crossreaction of protein p10 synthesized in ∅29-infected B. sub-
tilis with serum against protein p10 purified from E. coli carry-
ing plasmid pKC30 X16

        Protein p10 synthesized in E. coli containing plasmid pKC30

X16, purified as described above, was used to raise antibodies. A $^{35}$S-labelled extract of B. subtilis infected with a Ø29 mutant unable to assemble heads (to get p10 in soluble form) was reacted with the above anti-p10 serum. SDS-polyacrylamide gel electrophoresis of the proteins that were bound to this serum shows that the only protein specifically retained was p10 (Fig. 6a). Non-immune serum did not react with protein p10 from Ø29-infected B. subtilis (Fig. 6c). No band with the mobility of protein p10 was obtained from uninfected B. subtilis either with anti-protein p10 serum or non-immune serum (Fig. 6b and d).

DISCUSSION

Fragment Pvu I A contains the sequence coding for proteins p8 (major head protein), p8.5 (fibers), p9 (tail), p10 (connector), p11 (lower collar), p12 (precursor of the neck appendages) and probably part of the morphogenetic proteins p7 and p13 (22, 23). After digestion of the fragment with Bal 31 the resulting mixture was cloned in the Hpa I site of plasmid pKC30 under the control of the lambda $P_L$ promoter. Two recombinant plasmids were selected, pKC30 X16 and pKC30 X6, with the Ø29 DNA insert in the correct orientation for transcription from the $P_L$ promoter and in the opposite one, respectively. After heat induction of the cells carrying the recombinant plasmids, two proteins with the electrophoretic mobility of p10 (connector) and p11 (lower collar) were labelled, which were synthesized in smaller extent at 30°C or in the cells transformed with the control plasmid pKC30. The phage protein p12* might be synthesized, as shown by radio-immunoassay, but no other viral protein was synthesized, in agreement with the fact that nuclease Bal 31 had cut 0.1 kb from the right end and 2.5 kb from the left end of the Pvu I A fragment inserted in plasmid pKC30 X16 and 0.3 kb from the right end and 2.2 kb from the left end of the fragment inserted in pKC30 X6. The induced proteins were characterized as p10 and p11 by radioimmunoassay. It is known that E. coli RNA polymerase can recognize Ø29 late promoters in vitro (26). The synthesis of significant levels of the two proteins, p10 and p11, in the cells transformed with the recombinant plasmid pKC30 X6, with the Ø29 DNA insert in the opposite direction of transcription from the

$P_L$ promoter, suggests that a ∅29 late promoter or promoter-like sequence is present in the inserted fragment and could be recognized by E. coli RNA polymerase. Why the proteins with the electrophoretic mobility of p10 and p11 seem to be induced at $42^{O}C$ in the cells transformed with the recombinant plasmid pKC30 X6 is not understood at present.

The two proteins, p10 and p11, synthesized in E. coli transformed with plasmid pKC30 X16, were stable for at least 1 h as shown by a pulse and chase experiment. The accumulation of the two proteins in E. coli was determined by densitometry of the stained gels after separation by SDS-polyacrylamide electrophoresis of the proteins present at different times after the shift at $42^{O}C$. The maximal amount of proteins p10 and p11 accumulated after 4 h of induction was about 10% and 7%, respectively, of the total E. coli protein. These proteins represent less than 1% of the B. subtilis protein in ∅29-infected cells.

We have purified protein p10 from E. coli cells transformed with plasmid pKC30 X16. About 60 mg of highly purified protein p10 was obtained from 10 g of cells. Antibodies raised against the purified protein recognize protein p10 induced in ∅29-infected B. subtilis. Moreover, the purified protein p10 aggregates giving rise to the same type of structures found in the neck complexes isolated from phage ∅29 particles (unpublished results). The structural as well as the functional properties of purified protein p10 are presently being studied.

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