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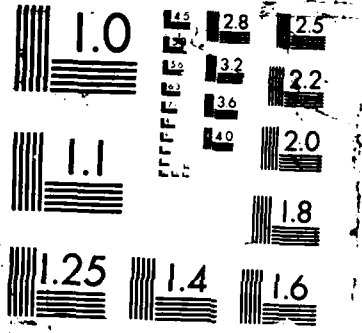
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The aim of this work was the construction of a bacterial plasmid carrying the genes for a small blue copper protein and for a small enzyme in close proximity, separated by a third genetic element, the loss of which may be easily detected, as an indicator of deletions in the system.

Although the first stage objective - cloning of a gene for a blue copper protein - has not been reached to-date, the final report exemplifies the work done on the problem, and the techniques that have been developed.

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A. STATEMENT OF THE PROBLEM STUDIED

The aim of this work was the construction of a bacterial plasmid carrying the genes for a small blue copper protein and for a small enzyme in close proximity, separated by a third genetic element, the loss of which may be easily detected, as an indicator of deletions in the system.

The plasmid so obtained would be a prerequisite for the systematic generation of gene fusion events between the metalloprotein gene and the enzyme gene. The chimeric proteins obtainable from such gene fusions could be used to test for the presence of allosteric effects involving the active site of the enzyme and the copper chelating site in the metalloprotein.

It was understood from the start that the desired plasmid might not be obtained within the contract period, at the activity level agreed upon, and that the work proposed would be executed on a best effort basis.

B. SUMMARY OF THE MOST IMPORTANT RESULTS

1. ATTEMPTS TO CLONE THE AZURIN GENE.

Azurin is a small (14,500 MW) blue copper protein present in certain genera of bacteria. It is thought to act as an electron carrier in their respiratory system, interacting with cytochrome c551. It has not been seen in Escherichia coli. The azurin of Pseudomonas aeruginosa ATCC 7700 has been studied crystallographically (Ref.1).

We extracted and purified DNA from bacteria of the above mentioned ATCC 7700 strain, digested it with specific restriction enzymes, fractionated it by size, and cloned selected size-fractions into a standard plasmid vector, pBR322.

Some 3,700 clones (1,800 after PstI digestion; 1,900 after BamHI digestion) were isolated and tested for their ability to synthesize azurin.

No convincing case of azurin production was observed. Those isolates that seemed possibly positive for azurin were reexamined several times by the same technique: the results were again unconvincing. Nevertheless, a few of the most promising isolates were grown up in large volumes, extracted and tested for the presence of azurin by gel chromatography and by HPLC. No convincingly positive results were obtained. The large majority of the clones studied have been saved (storage in glycerol at -75 C) and are available for further study.

Simultaneously, a group at the University of Illinois at Urbana (Dr. S. Sligar and Mr. P. Stayton) were also trying to clone the gene for Pseudomonas azurin. They examined their clones by the method of DNA probe hybridization and found ten clones that were positive in this test. They supplied us with these clones and we tested them for the presence of azurin with our methods. Again the results were negative. Considering the large numbers of clones examined in two laboratories, one might suspect that expression of the azurin gene in E. coli is either very poor or lethal to the cell.

In our experiments, we selected for cloning DNA fragments of relatively large size in order to avoid breaking the expression unit of the azurin gene. This might have been an unlucky choice. Quite recently, cloning of the azurin gene of Pseudomonas was reported in a poster by G.W. Canters at a meeting in Europe ("Protein Engineering '87", Oxford, April 5-8, 1987). The size of the DNA fragment was in this case quite small (1.3 kilobases).

The situation in regard to expression and recovery of the protein may be further complicated by the fact that, according to Canters, the azurin gene includes a signal sequence.

2. EXPERIMENTS WITH THE PLASTOCYANIN GENE.

Plastocyanin is another small blue copper protein (11 Kd), that is present in plants. It is involved in electron transfer in photosynthesis. The plastocyanin from poplar leaves has been studied crystallographically (Ref. 2). While a variety of plastocyanins have been documented, they are all rather similar in terms of amino acid sequence and resemble very much azurin in the subset of amino acids responsible for the Cu binding (Ref.3).

After our work on azurin started, the cloning of plastocyanin from a grass, Silene pratensis, was reported by a group in Holland (Ref.4). Dr. Smeekens was kind enough to let us have, under certain conditions, their isolate. This was supplied as a strain of bacteriophage M13 carrying a sequence of 301 base pairs including a large part of the gene for plastocyanin. Again, the situation is complicated by the fact that the plastocyanin gene also codes for a signal sequence, needed for the transfer of the protein from the cytoplasm to the chloroplast. Since bacteriophage M13 is not suitable for expression of a cloned gene, we have started experiments to transfer part of the cloned fragment to a plasmid vector, where the expression of the gene can be controlled.

3. OTHER MATERIAL.

Our proposal called for the eventual construction of a plasmid carrying three cloned genes in close proximity: those for lysozyme, for the tonB protein of E. coli, and for a blue copper

protein.

T4-lysozyme has been studied very intensively in several laboratories. Its gene is generally available in one or other cloned form.

At about the time our proposal was prepared, a report (Ref.5) appeared describing the sequencing of the tonB gene. We obtained from Dr. Postle the plasmid used for that purpose, pRZ540, and we tested it in our material. The product of tonB is an element of the E.coli cell envelope, which is required for the attachment of bacteriophages T1 and phi-80. We used still another phage, phiD-326 (Ref.6), which has similar properties. A derivative of E.coli C carrying a deletion that makes it resistant to those phages was transformed with pRZ540, selecting for neomycin resistance: the transformants were demonstrably sensitive to phiD-326. It thus seems that this material will fulfill the requirements of our scheme.

4. CONCLUSION.

Although the first stage objective - cloning of a gene for a blue copper protein - has not been reached to-date by us, the enclosed Appendix exemplifies the work done on the problem, and the techniques that have been developed.

C. TECHNICAL APPENDIX

1. Pseudomonas aeruginosa, ATCC 7700, was grown to full turbidity in BBL Trypticase broth, supplemented with yeast extract, at 37 C, with gentle shaking. The bacteria were harvested, washed in TE, pH 7.5, and frozen at -70 C.
2. After thawing, the bacteria were lysed with SDS and treated with either Pronase or Proteinase K. The pH was readjusted to 7.5 with NaOH. Deproteinization (twice with phenol, then twice with chloroform) followed. After treatment with RNase, the preparation was again extracted twice with phenol and twice with chloroform. The DNA was precipitated with ethanol.
3. The DNA was partially digested with restriction enzymes (1) PstI and (2) BamHI (Figure 1,A). The two samples (1) and (2) were layered on sucrose step gradients (6 steps, from 5% to 35% W/W) (technique as in Ref. 7 and 8), and centrifuged in Spinco Rotor SW28 for 24 hrs at 26,000 rpm. Fractions were collected from the bottom of the tube (about 2.3 ml per fraction), concentrated by ethanol precipitation, and individually tested by gel electrophoresis for DNA fragment size (Figure 1,B).

The DNA fragments covered a range from 20 to less than 0.4 kilobases (Figure 2). Some fractions were pooled and farther concentrated by means of Centricon membrane filters, 30,000 MW cut-off.

4. Vector pBR322 DNA (obtained from Sigma Corp.) was linearized by digestion with restriction enzymes (1) PstI and (2) BamHI. The transformation efficiencies for antibiotic resistance of these digests were 100 times lower than that of the undigested DNA. The digested preparations were then treated with phenol to deproteinize and, after dialysis, reconcentrated by ethanol precipitation. They were then treated with bacterial alkaline phosphatase (from IBI), deproteinized with phenol and chloroform (1:1) twice, then with chloroform, and again concentrated by ethanol precipitation. This treatment further reduced the transformation efficiency 100-fold. Exposure to T4 ligase did increase the transformation efficiency of the digested DNA more than 10-fold, but had very little effect, if any, on the digested-and-dephosphorylated DNA.
5. Standard techniques were used in transformation. The recipient strain used was E. coli C-1200 [F- rhamnose-methionine- histidine-] (Ref.9). From this strain a derivative was prepared, C-4046, that is lysogenic for a temperature inducible lambda phage: these bacteria will lyse after a brief exposure at 40 to 43 C.
6. Mixtures of digested, dephosphorylated vector DNA and Pseudomonas DNA fragments were left overnight at low temperature in the presence of T4-ligase then used to transfect either C-1200 or C-4046 (at 30 C), selecting for either ampicillin or tetracycline resistance (depending on the restriction enzyme used). The colonies obtained were picked and tested by replication for resistance to the other antibiotic. Those that were sensitive to the other antibiotic (between 2 and 41 % of all transformants, in individual ligation and transformation experiments) were the isolates presumably carrying a cloned Pseudomonas DNA fragment (Table 1). For several such clones, this was confirmed by examination of their plasmid DNA (Figure 3). The sensitive clones were studied further in attempts to demonstrate the presence of azurin antigens.
7. Sample of purified azurin were obtained from Dr. W.A.Toscano through Dr. S. Sligar of the University of Illinois, and from Dr. I.Pecht's laboratory. Additional azurin was prepared from Pseudomonas by a simplification of Toscano's method (Ref.10). This

involved chromatography on DEAE, ammonium sulfate precipitation, and separation on a polyacrylamide column (Bio-Gel P). The latter was twice as long as that used by Toscano and gave excellent separation of azurin (obviating the last step in Toscano's procedure, a carboxymethyl cellulose column fractionation).

8. Rabbit immune serum against pure azurin was prepared for us in the laboratory of Prof. J. Richards, California Institute of Technology. Its activity was measured by a capillary precipitation test (Figure 4).
9. Testing for azurin antigens in C-1200 derivatives was done on individual bacterial cultures and exposed to sonic disruption. Samples of the lysates were adsorbed onto paper (Schleicher & Schuell nitrocellulose membrane filters BA85) and treated with a 1:200 dilution of azurin-specific rabbit antiserum (from Miles Laboratories, Inc.), washed and treated with peroxidase-conjugated anti-rabbit goat serum (ELISA technique; details as in Ref.11). Preliminary experiments done with pure azurin showed that color (i.e. peroxidase activity) could be detected with as little as 8 ng azurin in 1 microliter, or 3.7×10^{11} azurin molecules). Similarly, spots of lysed Pseudomonas cultures were strongly positive. [Plastocyanin did not react in this test with anti-azurin serum.]
10. When using C-4046 in transformation, the technique could be greatly simplified as follows. Colonies were picked into broth (with the appropriate antibiotic) in 96-well assay plates and grown at 30 C; replicated (48 at a time) onto a double sheet of BA85 nitrocellulose paper, resting on top of a sheet of dialysis membrane, the latter resting on the nutrient agar (also with the antibiotic) of a Petri dish; incubated overnight at 30 C; shifted to 44 C for 20 minutes, then to 38 C for 2.5 hours. The middle layer nitrocellulose was processed for the ELISA test as described above. The top sheet (or a single sheet, in some experiments) was also used at times, but it was generally overloaded.
11. Various preparations of purified azurin were characterized by the following method. Samples were run electrophoretically in horizontal agarose gels; with the gel overlaid on a BA85 nitrocellulose membrane filter, they were blot-transferred, and then submitted to the the same ELISA procedure described above. Surprisingly, three bands were reproducibly detected (Figure 5). It is also of interest that not all these forms were present in extracts of Pseudomonas ATCC 7700 bacteria and that a new form, band D, appeared in the

extracts (Figure 5).

12. Pure azurin (Figure 6) could also be demonstrated by HPLC (Figure 7) and it could be likewise detected in Pseudomonas extracts by this method (Figure 7).
13. Both the above methods were applied to concentrates of cultures of presumptive azurin-producing clones, whether obtained by us in this work or supplied by the group at the University of Illinois. The results of these experiments were either negative or unconvincing.
14. The "mp" series of strains of the single stranded DNA bacteriophage M13 (Ref.12) are widely used for cloning and sequencing DNA. The strain received from Dr. Smeekens, which will be called here M13mp9::Pc, was purified through transfection and single plaque isolation. A carrier culture was established from Messing's E. coli strain JM101 and stored frozen (at -75 C, with glycerol) to be used as inoculum for phage production. A highly concentrated phage preparation was made (method as in Ref.13) and compared with a similar preparation of M13mp18. The DNA of the latter is known to be 7250 bases long; that of M13mp9::Pc is expected to consist of 7229 (vector) + 301 (insert) = 7530 bases, a difference of about 4%. In gel electrophoresis of whole virus particles (method of Ref.14) a lower mobility for M13mp9::Pc was observed (Figure 8), as expected from the difference in molecular weights.
15. For preparation of the replicative form (RF) of the DNA of M13mp9::Pc, a method for the "rapid plasmid DNA isolation for 10 ml culture" (Ref.15), appropriately scaled-up, was followed. The final product was further deproteinized with phenol, phenol+chloroform (1:1) and chloroform, and then concentrated by ethanol precipitation. Several bands were observed on agarose electrophoresis gels (Figure 9). The band identified as the covalently closed circles (CCC) of the RF (Figure 9,B) was purified by cutting it out of unstained agarose gels and extracting it with the "Geneclean" procedure (of Bio 101, Inc., La Jolla, CA).
16. This work is continuing.

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Table 1

E. coli isolates carrying fragments of Pseudomonas DNA examined for azurin production by an ELISA spot test procedure.

Restriction enzyme used	Fragment size	Total colonies tested
<u>PstI</u>	2 to 5 Kb	95
<u>PstI</u>	4 to 8 Kb	1,286
<u>PstI</u>	6 to 16 Kb	408
<u>BamHI</u>	6 to 14 Kb	24
<u>BamHI</u>	9 to 18 Kb	1,905

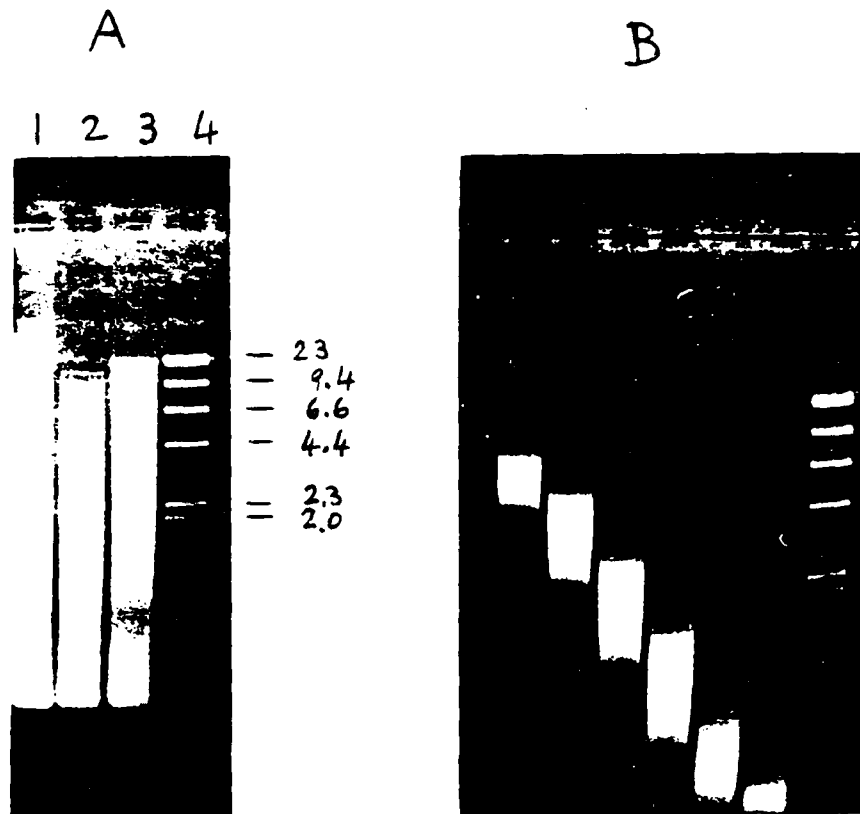


Figure 1

Size distribution of Pseudomonas DNA used for cloning (agarose gel electrophoresis).

- A. Before fractionation. Lane 1, undigested DNA. Lane 2, DNA after partial digestion with PstI restriction enzyme. Lane 3, DNA after partial digestion with BamHI restriction enzyme. Lane 4, DNA size standards, as indicated at right, in Kb.
- B. Example of size determination of partially digested DNA fractions obtained by sucrose gradient centrifugation.

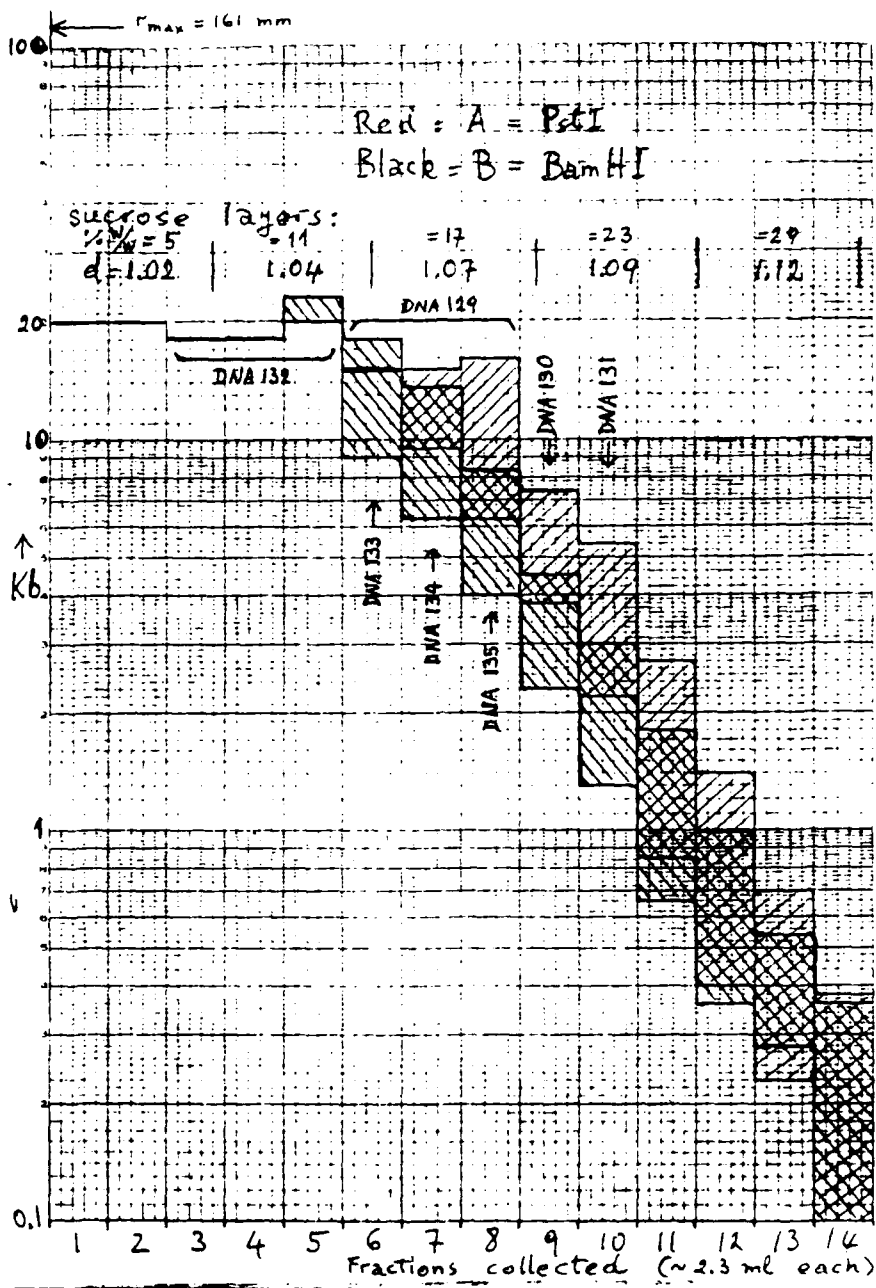


Figure 2

Fractionation of *Pseudomonas* DNA restriction fragments by sucrose gradient centrifugation. Abscissa: sequential fractions collected from centrifuge tubes. Ordinate: size range in Kb as determined on small samples from each fraction (as shown in Figure 1.B). Cross-hatching: SW-NE for DNA partially digested with *Pst*I restriction enzyme, SE-NW for DNA partially digested with *Bam*HI restriction enzyme.

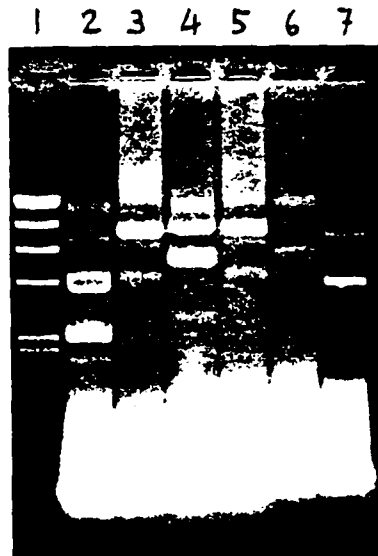
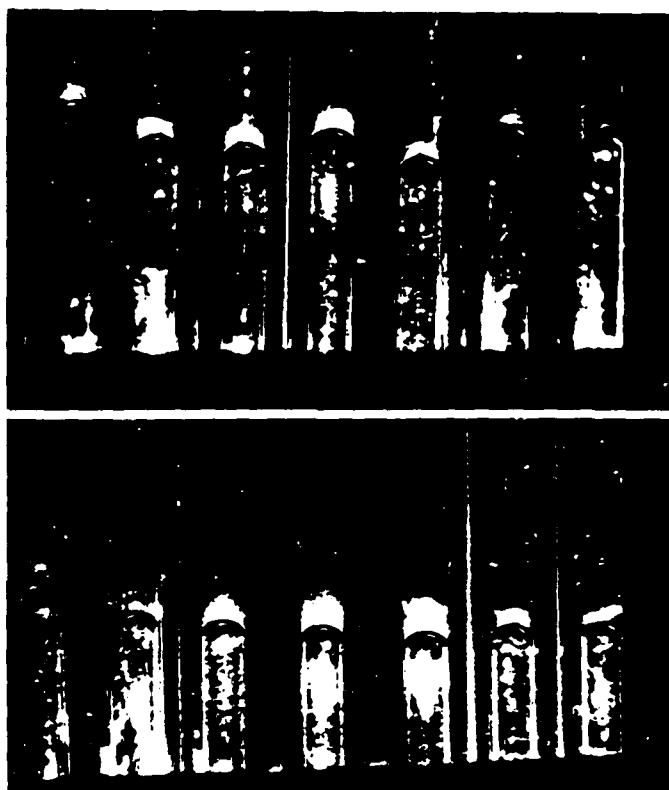


Figure 3

Example of plasmid DNA preparations from various transformed clones, analyzed by agarose gel electrophoresis.

Lane 1, molecular size standards (from top down: 23, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 Kb) Lane 2, control: pBR322. Lanes 3 through 7, five different clones of pBR322 carrying a Pseudomonas DNA insert. Independently of the complex distribution of the DNA between various molecular forms (covalently closed circles, linears and open circles; monomers and dimers), all five plasmids are clearly much larger than the control. (Lane 3 through 6 isolates contain PstI fragments from the 4-8 Kb fraction; the last isolate contains a PstI fragment from the 2-5 Kb fraction). The spots at the bottom are due to RNA that was not completely removed from these preparations.



A

B

Figure 4

Measurement of potency of anti-azurin rabbit serum by a microprecipitation method.

Capillary pipettes (20 μ L capacity) were loaded first with 10 μ L pure azurin, variously diluted in buffer (with added ampicillin to avoid growth of contaminants), and then with 5 μ L of antiserum, at an appropriate dilution, in such a way to have a perfect contact (but no immediate mixing) between the two liquids. The tubes were then sealed with putty, leaving some air between the serum and the seal. Where the reaction occurs, fine precipitate is first formed near the junction azurin-serum; it then falls and accumulates at the meniscus serum-air (white "cap" in the photographs). Magnification about 10X.

Total content of undiluted serum per tube: series A, 0.5 μ L; series B, 1 μ L.

Total content of azurin per tube, from left to right: 4.6 ng, 14 ng, 41 ng, 120 ng, 370 ng, 1.1 μ g, 3.3 μ g.

In the equivalence zone, 1 μ L of antiserum precipitates 0.5 μ g of azurin.

Figure 5

Protein blots transferred from a gel (0.8% agarose in 0.035 M Tris, 0.1M glycine buffer, pH 8.7) after electrophoresis (105 V) onto nitrocellulose filter paper and then demonstrated by the ELISA technique, using azurin-specific antiserum.

Lane 1. Pure azurin. Input: 67 picomoles. Three bands visible: A, B, C. (See also Lane 16)

Lane 5. Extract of Pseudomonas ATCC 7700 bacteria, grown in Toscano medium, with added Cu sulphate (1 mg/L). A 100 mL fully grown culture was concentrated 70-fold, frozen, thawed and extracted with 0.01 M phosphate buffer, pH 6.4, in the presence of RNase and DNase. Sedimentable material was removed by centrifugation (3 hours at 20,000 g). Band B is present, while A and C are not. In addition, a new band, D, is present.

Lane 9. Low molarity buffer extract (see under Lane 5) of a Pseudomonas culture in nutrient broth, after 12-fold concentration of the bacteria. Band A and B are present, but C is missing and D is strongest.

Lane 11. As above, but culture was grown in Toscano's medium. Band C is missing, and so are probably bands A and B. Band D is obvious.

Lane 16. Pure azurin, different preparation from that of Lane 1. Input: approx. 1.4 ug azurin (0.1 nanomoles).

1 ↓ 5 ↓



Gal 10/10

↓ ↓ ↓



↑ ↑ ↑
9 11 16

5
8
32

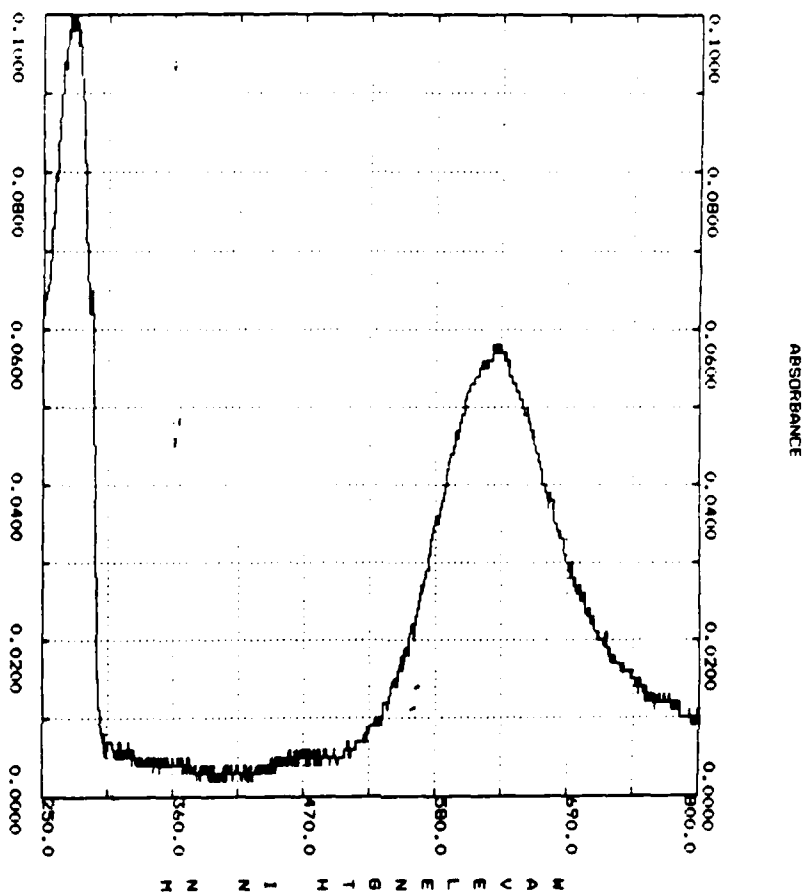


Figure 6

Absorption spectrum of pure azurin in Tris.HCl pH 7.2.
 Concentration: 1 μ M.

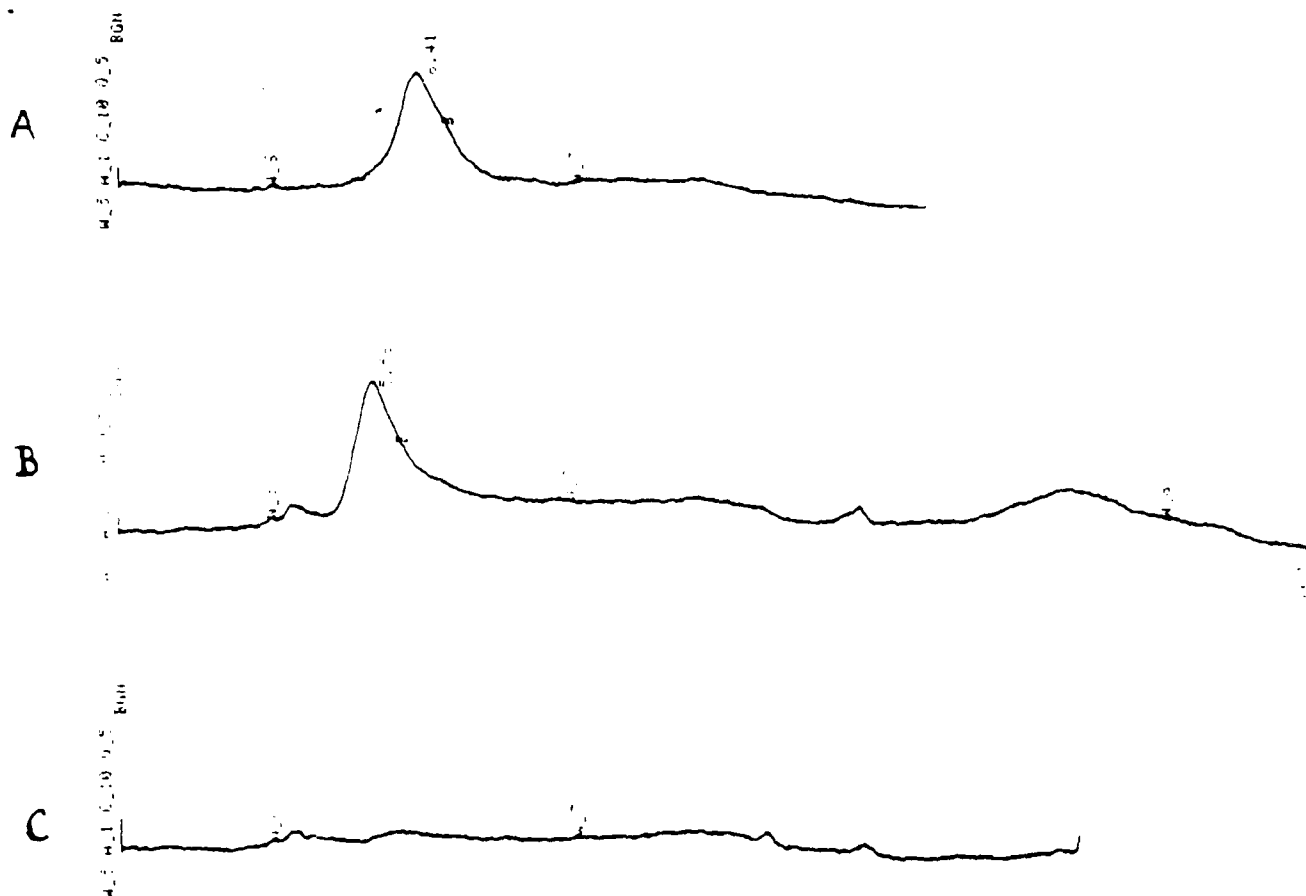


Figure 7

Ion exchange high performance liquid chromatography of azurin. Ordinates: absorbance at 620 nm. Same sensitivity setting for all graphs. Abscissa: retention time in minutes, shown as peak label. Column: Alltech Associates Synchronapak AX300, 25 cm x 4.6 mm I.D. Eluant A: 10 mM Tris.HCl, pH 7.9. Eluant B: same as A + 1 M NaCl. Gradient: 0 to 80% of B. Flow rate: 1 mL/min.

- A. Pure azurin (load: 200 picomoles) in presence of 10 mM Potassium Ferricyanide as oxidizing agent.
- B. Extract of Pseudomonas ATCC 7700 bacteria grown in Toscano's medium (prepared as for Figure 5, Lane 5) in presence of 10 mM Potassium Ferricyanide as oxidizing agent (load: 20 uL).
- C. Same extract as in B (same load), in presence of 5 mM Dithiothreitol (Cleland's reagent) as reducing agent.



Figure 8

Agarose gel electrophoresis of whole bacteriophage M13mp18 (lanes 1, 3, 4, 7) and M13mp9::Pc (lanes 2 and 5), showing retardation (i.e. larger molecular weight) of the latter.

The gel was dried and stained with the protein complexing dye Coomassie Blue.

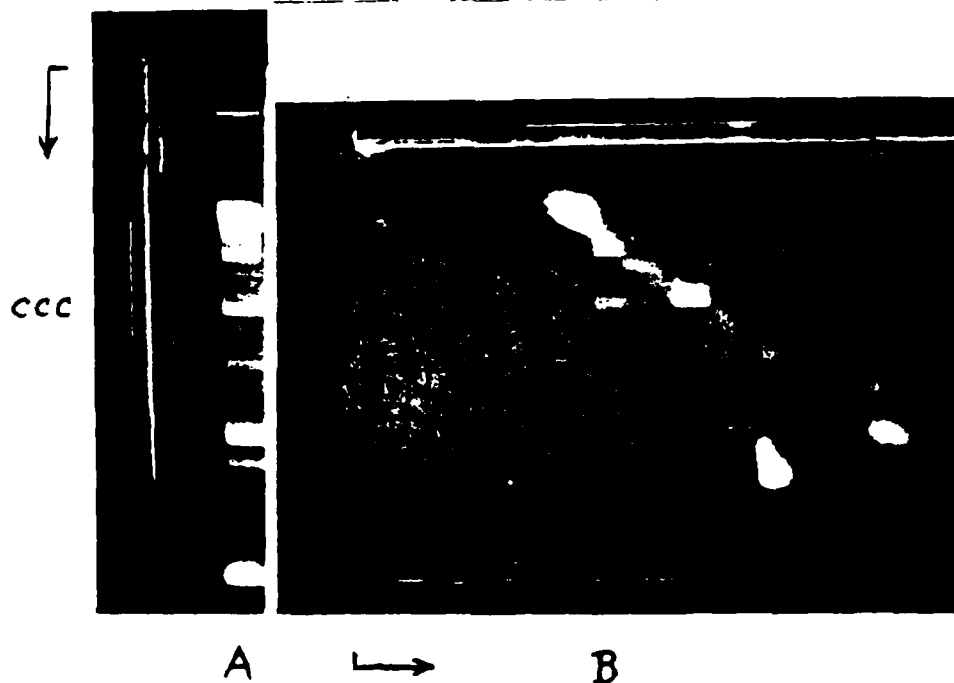


Figure 9

- A. Agarose gel electrophoresis of a preparation of replicative forms of DNA of M13mp9::Pc. The band presumed to consist of covalently closed circular forms is labeled (CCC).
- B. Demonstration of covalently closed DNA circles in the band indicated in A. The gel lane shown in A, was cut out, rinsed in buffer to remove excess ethidium bromide, irradiated with ultraviolet light (from a "germicidal" lamp, incident dose: 96 mW/sq cm), then embedded in agarose and run electrophoretically, perpendicularly to the first direction (method of Ref.16). The CCC band, as a result of UV irradiation, has generated a new band consisting of open DNA circles. (The irregular spot at the lower right is a photograph defect.) The arrows indicate the direction in electrophoresis.

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