Refractile Granular Inclusions in a Temperature-sensitive Mutant of *Escherichia coli* K-12

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Abstract

At the restrictive temperature, cells of a temperature-sensitive mutant of *E. coli* K-12 lyse, and the culture medium is seen to contain ghosts of cells containing large inclusions that are refractile when viewed by phase-contrast microscopy. The addition of sodium chloride (1%) or sucrose (12·5%) protects the cells from lysis and under these conditions the refractile inclusions are seen in the intact cells, which are short filaments. At elevated temperatures cells become sensitive to a wide range of inhibitors. Isolated inclusions consist almost entirely of protein and are digested by Pronase or trypsin. Electrophoresis of inclusions on sodium dodecyl sulphate-polyacrylamide gels shows them to contain a number of protein components. Ultrastructural studies show that granules are commonly found close to the internal surface of the cytoplasmic membrane. In section they are seen to be grainy in appearance but have no ordered structure. Dissociation of the granules by sodium deoxycholate or trypsin leads to the formation of spirally wound filaments with a subunit structure.

Introduction

Conditional lethal mutants are being increasingly used to investigate the biosynthesis of the bacterial cell envelope. To obtain mutants of this type we screened a collection of temperature-sensitive mutants of *Escherichia coli* K-12 for the ability to grow at 42°C on nutrient agar, the osmotic pressure of which had been increased by the addition of sodium chloride or sucrose. The growth characteristics of these mutants were then determined in liquid medium (nutrient broth) and we obtained a class of mutants which lysed at 42°C and which we anticipated would be defective in peptidoglycan synthesis. Cultures of these strains were examined by phase-contrast microscopy during lysis and one, strain JP1148, was seen to give rise largely to ghosts containing refractile inclusions. Addition of 12·5% w/v sucrose to the broth prevented lysis and short filamentous cells containing the bright inclusions were then observed.

This paper describes the growth properties of the mutant strain JP1148, the isolation and initial characterization of the inclusions and some ultrastructural studies on the isolated inclusions. Some of these results were presented at a meeting of the Australian Biochemical Society (Egan and Morton 1972).

Materials and Methods

*Materials*

Bovine serum albumin and lysozyme were obtained from Sigma Chemical Co., St Louis, Mo, U.S.A. All other enzymes were from Calbiochem, Los Angeles, Cal., U.S.A. Ampicillin was a generous gift from Beecham Research Laboratories, Moorabbin, Vic. Isotopes were obtained from the Radiochemical Centre, Amersham, England. The buffer used was 0·1m tris-HCl, pH 7·5.
Nutrient broth consisted of Oxoid nutrient broth No. 2, plus 0·3% Oxoid yeast extract, solidified for plates with 1% Oxoid agar No. 1.

Organisms and Growth Conditions

The inclusion-containing mutant strain, JP1148, was obtained from a collection of temperature-sensitive mutants kindly provided by Dr R. R. B. Russell. These were isolated by mutation of strain KA56 (HfrH, thi-*, galEPL5, rel-I) after mutagenesis with nitrosoguanidine as described by Russell and Pittard (1971).

Incubations were at 32, 37 and 42°C as indicated in the text. For the assessment of growth on solid media a suspension containing about 10¹⁰ cells/ml in nutrient broth was applied to plates as a streak about 25 mm long. For liquid medium studies, appropriate volumes of logarithmic-phase cultures of the strains were inoculated into 125-ml Erlenmeyer flasks equipped with side arms and containing 10 ml of medium. Flasks were shaken at about 150 rev/min in New Brunswick Gyrotory Shakers. The optical density of the cultures was followed using a Klett-Summerson photoelectric colorimeter fitted with a No. 66 filter. Samples were taken at various times to determine the number of cells able to form colonies on nutrient agar at 32°C. When NaCl or sucrose were required these were added at concentrations of 1 and 12·5% w/v respectively.

Measurement of DNA, RNA, Protein and Peptidoglycan Synthesis

Cells of strain JP1148 growing logarithmically in nutrient broth at 32°C were diluted in fresh broth to 5 Klett units. Flasks containing 10 ml of culture were prepared and these were incubated at 32°C until the turbidity was 10 Klett units. Macromolecular syntheses were followed by measuring the incorporation of specific radioactive precursors, which were added to pairs of flasks at the final concentrations and activities shown below. DNA synthesis was followed by measuring the incorporation of 0·1 µM [methyl-³H]thymidine (0·2 µCi/ml); RNA synthesis by the incorporation of 3·3 µM [2·14C]uracil (0·12 µCi/ml); protein synthesis by the incorporation of 0·6 µM [U-¹⁴C]leucine (0·2 µCi/ml); and peptidoglycan synthesis by the incorporation of 0·05 mM [U-³H]-2,6-diaminopimelic acid dihydrochloride (1 µCi/ml). Deoxyadenosine (200 µg/ml) was added in the experiments on DNA synthesis to increase the incorporation of thymidine (Yagil and Rosner 1970). The flasks were incubated at 32°C for a further 30 min then one flask of each pair was transferred to 42°C. At intervals after the addition of the precursors, 0·2-ml samples were taken from each flask and the incorporation of radioactivity into acid-precipitable material was determined. The incorporation of label was assayed by transferring each sample into 2 ml of ice-cold 10% trichloracetic acid. The precipitated samples were then left for at least 30 min before being collected on membrane filters (0·45 µm pore diameter; Millipore Corp., Bedford, Mass., U.S.A.). The filters were washed twice with 2 ml of 5% trichloracetic acid, air-dried overnight, placed in 5 ml of toluene scintillator and counted in a Packard Tri-Carb liquid scintillation counter.

Isolation of Inclusions

Strain JP1148 was inoculated into 1 litre of nutrient broth containing 12·5% sucrose in a 2-litre flask and grown by shaking at 32°C to a density of about 4·5 x 10⁷ cells/ml (40 Klett units). The temperature was raised to 42°C and incubation continued for about 6 h, by which time growth had ceased. All further steps were carried out at 2°C. Cells were collected by centrifugation at 10 000 g for 15 min, the pellet was resuspended and washed in buffer, and the cells were again centrifuged. The cells were then suspended at 1 g wet wt/3 ml of buffer and broken in an Amino–French pressure cell operated at 1378 x 10⁵ Pa (20 000 lb/in.²). Unbroken cells and large fragments were removed by centrifugation at 120 g for 15 min. The pellet was discarded and the supernatant centrifuged at 2500 g for 15 min. The pellet thus obtained contained the inclusions. The sequence of centrifuging first at 120 g and then at 2500 g was repeated. Finally the inclusions were washed once more in buffer and sedimented at 2500 g and the supernatant discarded.

Gel Electrophoresis

Disc electrophoresis in acrylamide gels containing sodium dodecyl sulphate and using siliconized tubes was carried out as described by Burgess (1972). This is a modification of the high-resolution method described by King and Laemmli (1971). The sample buffer used contained glycerol (10%), 2-mercaptoethanol (5%) and sodium dodecyl sulphate (3%) in 0·125M tris-HCl buffer, pH 6·8.
Isolated inclusions were solubilized by heating in this buffer for 2 min at 90°C. Samples (0.5–10 μg protein) were applied in 100 μl of sample buffer and run at 3 mA/tube.

**Electron Microscopy**

Suspensions of cells in buffer or growth media were fixed by the method of Kellenberger *et al.* (1958). The fixed cells were pelleted in agar, dehydrated in a graded ethanol series and embedded in Araldite. Sections were cut using a diamond knife and stained with uranyl acetate followed by lead citrate. Negative staining of preparations was carried out on a carbon-coated grid with 1% potassium silicotungstate (pH 7.0) in water. The preparations were examined with a Philips EM300 electron microscope at 80 kV.

**Results**

**Growth**

When suspensions of cells of strain JP1148 were applied as streaks to agar plates good growth was observed at 32°C, poor growth at 37°C and no visible growth at 42°C. Growth occurred at 42°C on plates supplemented with NaCl or sucrose. Growth as single colonies at 42°C was also possible only on plates containing added NaCl or sucrose.

![Fig. 1. Growth of strain JP1148 in nutrient broth. Cells in the early logarithmic phase of growth at 32°C were diluted into fresh broth and incubated at 32°C (■), 42°C (○), 42°C in broth containing 1% NaCl (○) or 42°C in broth containing 12.5% sucrose (▲).](image)

The growth of the mutant strain in nutrient broth was examined (Fig. 1). Cells growing actively at 32°C were diluted in fresh broth to a density of about 1–2 × 10⁷ cells/ml. At 32°C logarithmic growth at a rate corresponding to a mean generation time of about 60 min was seen. However, when the cell densities reached 3–6 × 10⁷ cells/ml (35–50 Klett units) the growth rate slowed.

At 42°C an initial increase in growth rate was observed for about 1 h. After this time lysis was observed. The addition of NaCl (1%) or sucrose (12.5%) to nutrient broth prevented lysis (Fig. 1). Table 1 shows the effect of temperature on the number of colony-forming units in cultures of cells of strain JP1148 incubated in broth at 32 and 42°C. (The turbidities of the cultures changed with time similarly to those shown in Fig. 1.) At 32°C division occurred normally but at 42°C the number of viable cells rapidly declined. When incubated at 42°C in broth supplemented with sucrose or NaCl, the ability to divide was largely restored.

**Phase-contrast Light Microscopy**

Cells of strain JP1148 grown in nutrient broth were examined by phase-contrast microscopy. Cells in the logarithmic phase of growth at 32°C appeared as rods or
short filaments varying in length up to about 10 times the length of cells of the parent strain growing under the same conditions. Cells grown at 42°C and allowed to lyse appeared as ghosts, usually of short filaments, and contained large refractile (often polar) inclusions. The inclusions were retained inside the lysed cells and seldom seen free in the medium. Occasional longer filaments had several inclusions distributed along their length. Figs 3 and 4 show phase-contrast micrographs of cells grown at 32 and 42°C in broth supplemented with 12.5% w/v sucrose to limit lysis. In each case the culture consisted of a mixture of rods and short filaments of varying lengths. At 42°C the highly refractile inclusions could be seen in both intact and lysed cells (Fig. 4).

Table 1. Viability of mutant strain JP1148 in nutrient broth at 32 and 42°C

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>32°C</th>
<th>42°C</th>
<th>42°C</th>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>+ NaCl</td>
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<td>2.0</td>
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</tr>
<tr>
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<td>6.5</td>
<td>7.4</td>
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Cytological staining tests for cell wall material, nucleic acid, lipid, carbohydrate and polyphosphate were carried out (Meynell and Meynell 1970). Both intact and lysed cells, with inclusions present in each case, were examined but results were all negative, the inclusions remaining unstained in all experiments.

Selected inhibitors were added to cultures of cells growing at 32°C in nutrient broth containing 12.5% sucrose. At the same time the cultures were shifted to 42°C. After 2 h incubation at 42°C cultures were examined microscopically. Observation of 1000 cells showed that inclusion formation was inhibited more than 90% by chloramphenicol (100 µg/ml) and about 50% by rifampicin (200 µg/ml). Nalidixic acid (100 µg/ml) and ampicillin (20 µg/ml) did not appear to inhibit granule formation. Growth was strongly inhibited by each compound, however.

Macromolecular Synthesis

The incorporation of radioactive precursors into macromolecules in cells being incubated in nutrient broth at 32°C and then shifted to 42°C is shown in Fig. 2. Results are expressed relative to the level of incorporation at the time of the temperature shift. There was an initial increase in the rate of incorporation of [3H]thymidine into DNA and [14C]uracil into RNA but these rates then progressively declined. The rates of incorporation of [14C]leucine into protein and [3H]-2,6-diaminopimelic acid into peptidoglycan declined progressively after the temperature shift. Incorporation of all precursors had essentially ceased after 90 min at 42°C, but there was no strongly
marked preferential inhibition of any one synthesis. The rate of incorporation of precursors into macromolecules in cells of control cultures left at 32°C continued to increase exponentially.

**Effect of Inhibitors on the Growth of Strain JP1148**

The effect of a range of dyes and antibiotics on the growth of strains KA56 and JP1148 (when streaked) on nutrient agar plates at 32 and 37°C and on nutrient agar plates supplemented with sucrose (12.5%) at 42°C was determined. The growth of strain JP1148 was inhibited at 37 and 42°C by methylene blue (10 μg/ml), sodium deoxycholate (10 μg/ml), bacitracin (0.5 μg/ml), rifampicin (1 μg/ml) and triphenyl tetrazolium chloride (0.5 μg/ml). No inhibition of the growth of the parent strain KA56 was observed.

![Fig. 2. Incorporation of the radioactive precursors (methyl-3H)thymidine into DNA (▲), [2-14C]uracil into RNA (■), [U-14C]leucine into protein (●) and [U-14C]-2,6-diaminopimelic acid into peptidoglycan (○) in cells of strain JP1148 at 32 and 42°C. Vertical arrow indicates time of temperature shift.](image)

**Electron Microscopy of Inclusion-containing Cells**

Non-lysed cells of the mutant strain were examined after growth at 32 and 42°C in broth containing sucrose. Negative staining revealed no detectable difference between the surfaces of cells of the mutant and parental strains. Sectioned material revealed the ultrastructural appearance of inclusion-containing cells. Fig. 5 shows a cell containing electron-dense polar granular inclusions. These inclusions are generally 0.5 μm in diameter and have a somewhat grainy appearance, but apart from this have no other ordered structure apparent within them. Some of the variations in size and distribution which may be encountered are illustrated in Fig. 6. Inclusions were commonly seen to be in contact at a number of points with the inner surface of the cytoplasmic membrane, but there was no evidence for the presence of a limiting membrane surrounding them.

**Properties of the Isolated Inclusions**

Phase-contrast microscopy indicated that the pellet obtained consisted of large granular inclusions (see Methods). Negatively stained preparations examined by electron microscopy showed that only a small percentage of the inclusions had membrane material associated with them. Occasional cell-wall fragments were also seen. The granular material could be stored as a frozen paste or could be lyophilized...
after which it was a greyish amorphous powder. Resuspension of lyophilized material in buffer gave a whitish suspension which, when viewed by phase-contrast microscopy, was seen to consist of brightly shining spherical granules.

Extraction of the lyophilized material with ether or acetone had no visible effect on the material. The solvent-extracted material suspended in buffer appeared

**Figs 3 and 4.** Phase-contrast micrographs of cells of strain JP1148 incubated at 32 (3) and 42°C (4). × 1600.

**Fig. 5.** Electron micrograph showing terminal inclusions in cells of strain JP1148 grown at 42°C. × 30 000.
unchanged when examined by light microscopy. Treatment of aqueous suspensions of the inclusions at 25°C with various reagents and enzymes was carried out. HCl solution (pH 1), EDTA, amylase, phospholipase, deoxyribonuclease, ribonuclease and lysozyme had no visible effect except for a tendency to cause clumping. Addition of 0·1 vol. of 1N NaOH solution caused almost immediate solubilization. Sodium dodecyl sulphate (1%) and sodium deoxycholate (2%) also solubilized the inclusions. The inclusions were also susceptible to rapid digestion with Pronase and slow digestion by trypsin. Protein estimation by the method of Lowry et al. (1951) gave a protein content in excess of 90% w/v. Total phosphorus estimated by the method of Allen (1940) was 0·23% w/w, which, if due to the presence of phospholipid, would be equivalent to a phospholipid content of about 4–5%.

Isolated inclusions were solubilized as described in the Methods section and subjected to electrophoresis on acrylamide gels. They contained one major component which was of high mobility together with about five other minor components. When known proteins were run as reference standards it was found that the major component had the same mobility as lysozyme, suggesting a molecular weight of approximately 14 000 for the polypeptide from the inclusion.

Electron Microscopy of Isolated Inclusions

Isolated inclusions layered onto a carbon film on a grid were extremely electron-dense and could be readily visualized without the need for negative staining before electron microscopy. They were present either as single inclusions or more frequently as groups of particles which appeared to be connected by material (Fig. 7). No internal structure was discernible at 100 kV accelerating voltage. After treatment of inclusions with 2% sodium deoxycholate for 30 min at 37°C strands or cables of varying size were seen (Fig. 8). These cables were invariably associated with dark-staining material which did not appear to be pools of excess stain and may consist of remnants of the inclusions. The cables could be seen to be composed of helically twisted filaments. After treatment of isolated inclusions with trypsin at 37°C for 30 min filaments 6–7 nm in diameter were seen (Fig. 9). These were formed of two strands wound together with a repeat distance of 170 nm. Higher magnification of the filaments (Fig. 10) showed evidence for a subunit structure. Arrows indicate the presence of a possible tetrad of subunits.

Discussion

Intracellular inclusions isolated from strain JP1148 consist almost entirely of protein. These inclusions retain their refractility and ultrastructural appearance, indicating that there has been no substantial structural change caused by the isolation procedures. These procedures probably result in an inclusion preparation enriched with larger inclusions. Ultrastructural studies show, however, that small inclusions (which may not be visible when viewed by light microscopy) have the same ultrastructural appearance as larger inclusions. No information concerning the development of the inclusion bodies has been obtained.

Inclusions commonly occur in close contact with the inner surface of cytoplasmic membrane but no structural attachment to the membrane has been observed. In section the inclusions have a grainy appearance, but no other ordered structure, and do not possess a limiting membrane. Ultrastructural studies have further shown
Fig. 6. Electron micrograph of transverse section of a cell grown at 42°C showing inclusions in close contact with cytoplasmic membrane. × 65 000.

Fig. 7. Electron micrograph of a group of whole unstained isolated inclusions placed on a grid as for negative staining. × 53 000.

Fig. 8. Negatively stained product of treatment of isolated inclusions with 2% sodium deoxycholate. × 46 000.

Figs 9 and 10. Negatively stained products of treatment of isolated inclusions with trypsin. 9, Spirally wound filaments are seen. × 95 000. 10, Higher magnification shows presence of a subunit structure (arrows). × 200 000.
that isolated inclusions can be dissociated and form spiral filaments composed of linear assemblies of small units. It is impossible to decide at this stage whether the filaments are formed by reaggregation of dissociated material or whether the inclusions consist of tightly wound filaments. It is suggested that the refractility of the inclusions is due to the dense packing of the protein.

Sensitivity to a wide range of inhibitors at 42°C shows that the cells have increased permeability indicative of an effect of the mutation(s) on the cell envelope. Lysis at 42°C suggests that this effect is on the cytoplasmic membrane or peptidoglycan components rather than on the lipopolysaccharide component. The synthesis of DNA, RNA, protein and peptidoglycan are all inhibited at 42°C. However, there is no rapid marked preferential inhibition of any one of these syntheses as is commonly seen in temperature-sensitive mutants with lesions in the synthesis of a particular type of macromolecule (see for example Russell and Pittard 1971). The presence of a number of polypeptide components as shown by electrophoresis on sodium dodecyl sulphate–polyacrylamide gels suggests that the isolated inclusions contain a number of protein components. The minor components are present in too high a concentration to be accounted for by the small amount of membrane present in the inclusion preparation.

Two reports have previously appeared of bacterial mutant strains that contain granules somewhat similar in ultrastructural appearance to the ones reported here. Hirota et al. (1968) described a mutant of E. coli K-12, strain 200 Ps t112, that contains a mutation preventing autonomous replication of the sex factor at the restrictive temperature. After 3 h incubation at 42°C half of the population of cells of this strain contain a large polar granule. These granules stain with Sudan black and seem rich in lipids rather than protein. Recently, Behme and Fitz-James (1972) have reported the accumulation of protein inclusions in a temperature-sensitive mutant of Bacillus subtilis. These latter inclusions are non-refractile when cells are viewed microscopically and thus appear to differ from the inclusions described in this paper. The B. subtilis mutant exhibits a pleiotropic phenotype indicative of a defect in lipid synthesis. The relationship of the inclusions reported here to those induced in E. coli during canavanine death (Schachtele et al. 1968) is also not clear. Both types consist predominantly of protein and appear similar in thin sections of cells, yet are different when viewed by phase-contrast microscopy. The mutant cells described in this paper show a normal distribution of nuclear material, whereas canavanine-treated cells do not.

Genetic studies with strain JP1148 have proved difficult and have shown it to contain several mutations. Results so far obtained suggest that inclusion formation may be due to two mutations. A mutant strain of E. coli K-12 with many properties similar to those of strain JP1148 has been isolated by Dr Henry C. Wu, Department of Microbiology, University of Connecticut Health Center, Farmington, Conn., U.S.A. (Wu, personal communication). A preliminary examination of this strain, E425, in our laboratory has confirmed the similarities.

Acknowledgments

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