Protein organization on the PHA inclusion cytoplasmic boundary

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Abstract

Polyhydroxyalkanoate (PHA) cellular inclusions consist of polyesters, phospholipids, and proteins. Both the polymerase and the depolymerase enzymes are active components of the structure. Recently, proteins associated with these inclusions have been described in a number of bacterial species. In order to further clarify the structure and function of these proteins in relation to polymer inclusions, ultrastructural studies of isolated polymer inclusions were initiated. The surface boundary characteristics of polymer inclusions, produced by several genera of bacteria, two different Pseudomonas putida deletion mutants and by Escherichia coli recombinants, were examined. The recombinant E. coli carried either the PHB biosynthesis operon (phaCAB) from Ralstonia eutropha alone, or both this operon and a gene encoding an inclusion surface protein of R. eutropha (phaP). The results support two suggestions: (i) specific genes in the PHA gene cluster code for the proteins forming the surface boundary arrays which characterize the polymer inclusion; and (ii) transfer of such a gene would result in subcellular compartmentalization of accumulating polymer. Although the proteins appear to serve a similar function among different genera, nevertheless, the different surface proteins are encoded by a variety of non-homologous genetic sequences. © 1998 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Bacterial polyhydroxyalkanoates (PHA) are sequestered in intracellular inclusions containing
2. Materials and methods

2.1. Organism cultivation

Pseudomonas oleovorans, P. putida BM01, a strain kindly provided by Dr S.C. Yoon (Gyeongsang National University, Korea) and R. eutropha were grown in Fernbach flasks on rotary shakers at 27°C. As described previously for P. oleovorans, wild type P. putida BM01 and P. putida deletion mutants, modified E* growth medium was used with octanoic acid, as the sole carbon source (Stuart et al., 1995); for R. eutropha, the same conditions were used for polymer generation except the sole carbon source was butyric acid. PHB containing Azotobacter vinelandii, was kindly provided by Dr William Page (Page and Cornish, 1993). Cultures of Nocardia corallina were grown to high density in Luria broth medium, then transferred to a nitrogen-free minimal medium containing 0.2% valeric acid as the sole carbon source (Schlegel et al., 1961). As previously described (Valentin et al., 1998), cultures of E. coli HMS 174 (pJM9238; Kidwell et al., 1995) and E. coli HMS174 (pJM9238, pBluescript KS::phaP) were grown in Luria broth at 30°C prior to induction. Selection for and maintenance of these two different strains was achieved by adding chloromphenicol (25 μg ml⁻¹) or ampicillin (100 μg ml⁻¹) and chloromphenicol (25 μg ml⁻¹), respectively to the culture medium. P. putida BM01 deletion mutants harboring the neo gene were grown in the presence of kanamycin (50 μg ml⁻¹). All chemicals were purchased from Aldrich or Sigma unless otherwise noted.

2.2. E. coli transformants and P. putida deletion mutants

The E. coli transformants and the P. putida deletion mutants utilized here were prepared as follows and as described in the accompanying paper (Valentin et al., 1998). Genes isolated from the PHA gene cluster of R. eutropha were transferred into E. coli HMS174 cells using plasmid constructs. E. coli HMS174 transformants were carrying plasmid pJM9238, constructed from pRA90 (Nycomed, Pharma, Denmark) a runaway replication vector; the construct harbored the genes for PHA biosynthesis (phaCAB) and for chloramphenicol resistance. After growth of transformants to density at 34°C, PHA gene induction was initiated by incubation at 41°C for 30 min. Cultures then were maintained at 37°C while PHB accumulated (2–4 h). Another strain, E. coli HMS174 carried two plasmids (pJM9238 and pBluescript KS::phaP); these were harboring, respectively, genes for PHA biosynthesis, phaCAB, and the gene, phaP, which codes for an inclusion associated protein in R. eutropha (Wieczorek et al., 1995, 1996). The plasmids also introduced resistance to chloramphenicol (25 μg ml⁻¹) and ampicillin (100 μg ml⁻¹), allowing the initial se-
lection and subsequent maintenance of the strain. 
P. putida BM01 deletion mutants were prepared by transposon mutagenesis using a gene cassette encoding kanamycin resistance. P. putida mutant 201 contained pJM9612 inserted into the GA-1 gene, which thus was inactivated; nevertheless mutant 201 retained a functional gene, GA-2, encoding a boundary lattice protein. A second mutant, P. putida 42, containing the pJM9611 insert, lacked the genes encoding both the GA-1 and GA-2 proteins. Both mutants were initially selected and subsequently maintained using medium with 50 μg ml⁻¹ kanamycin (Pharmacia Biochemicals, Piscataway, NJ).

2.3. Polymer inclusion isolation and analysis

After French press cell disruption, samples were centrifuged onto a 50–60% glycerol pad. Isolated proteins from the purified inclusions were examined using SDS-PAGE. Western blot analysis was carried out using antibodies elicited by immunization with an SDS-PAGE purified P. oleovorans inclusion protein with the observed molecular mass of 43 kDa. This protein is the product of the pseudomonad gene GA-2 described in the accompanying paper (Valentin et al., 1998). The techniques of protein fractionation, electrotransfer and Western blot analysis, were carried out as previously described (Stuart et al., 1995, 1996).

2.4. Electron microscopy

Inclusions were adsorbed onto carbon coated grids, then stained briefly with 1% uranyl acetate. Prior to absorption and uranyl acetate staining, some samples from P. putida mutant 201 were fixed with methanol (66% methanol, purified inclusions-v/v). As a control to verify that the adsorption method did not generate an artificial boundary, samples of P. oleovorans inclusions were also subjected to freeze fracture prior to examination. Polymer inclusions isolated from two different E. coli transformants and from the two different P. putida deletion mutants described above were studied using these same methods for adsorption and staining. All specimens were examined with a Phillips CM 10 electron microscope.

3. Results and discussion

3.1. Polymer inclusion morphology

Initial investigations of the surface boundary of PHO inclusions formed by P. oleovorans when grown on sodium octanoate clearly indicated the presence of a complex structural array. To demonstrate that this array was not an artifact generated by the staining treatment and associated procedures, isolated inclusions were examined as freeze fracture preparations. As clearly shown in Fig. 1, the surface of the isolated inclusions when prepared by freeze fracture is characterized by the ordered lattice pattern.

For all the other genera examined by electron microscopy, the results indicated that the subcellular compartmentalization of PHA inclusions was the result of discrete cytoplasmic inclusion boundary arrays. Fig. 2 compares isolated inclusions produced by two different pseudomonad species with inclusions produced by R. eutropha. It is clearly evident that the surface boundary morphology of isolated inclusions produced by P. oleovorans and P. putida (Fig. 2a, b) are essentially identical, but very different from the arrays exhibited by R. eutropha (Fig. 2c).

Fig. 1. Freeze fracture preparation of P. oleovorans inclusion showing geometric surface lattice pattern. Magnification 165000.
polymer inclusions produced by the pseudomonads characteristically exhibit highly organized networks at the cytosol/polymer interface. In contrast, inclusions purified from *R. eutropha* were normally enveloped in a lose boundary array which lacked a uniform, repetitive spacing. In addition, individual inclusions of *R. eutropha* appear to have only a single surface array, rather than the double geometric lattice characteristic of the pseudomonads. *N. corallina* and *A. 6* inlandii also exhibited defined boundary surface arrays but they differed from those displayed by inclusions from either of the pseudomonad species or *R. eutropha*.

Fig. 3 shows inclusions produced by *N. corallina* (Fig. 3a) and by *A. vinelandii* (Fig. 3b). Both characteristically exhibit an organized surface array which although appearing double in nature, nevertheless lacked the rigid geometric pattern characteristic of arrays produced both the pseudomonad species; in addition, the boundary array of *N. corallina* appeared sturdier than that of the pseudomonads.

Thus, polymer inclusions isolated from these four different microbial genera all displayed a characteristic protein boundary component resulting in a discrete subcellular compartment within the cytoplasm, but the precise patterning of the surface arrays differed among the different genera. These different types of ‘native’ polymer packaging may be useful to biotechnologists as the genes for polymer synthesis are increasingly transferred to eukaryotic systems. Within eukaryotes, development of effective and viable cells may be dependent upon separation of accumulating polymer from complex cellular organelles and biosynthetic machinery. Thus, including bacterial genes encoding the protein arrays described here could, perhaps, provide a simple means of ensuring polymer segregation and containment.

### 3.2. Diversity of inclusion associated proteins

To further characterize the PHA inclusions, the proteins associated with them were analyzed using several species. Fig. 4 (lanes 1–4) shows a typical SDS-PAGE profile of the polymer inclusion associated proteins. This analysis demonstrated that different genera characteristically exhibited different protein species. Subsequently, a Western blot, utilizing antibody raised against the protein from *P. oleovorans* inclusions, with an observed molecular mass of 43 kDa, (the gene GA-2 expression product), demonstrated that this protein from *P. oleovorans* has the same apparent molecular mass as that of *P. putida* and is at least antigenically related (Fig. 4, lanes 8, 9). As a control, similarly blotted inclusion proteins were incubated with pre-immune serum, and no binding occurred (Fig. 4, lanes 11, 12).

These results support the conclusion that although the GA-2 protein expressed in the two
different pseudomonads are antigenically related, the boundary proteins of the other genera are antigenically distinct. The outer surface layers of bacteria which are generally referred to as S-layers, provide precedence for such a protein diversity. These external bacterial boundary proteins, which display a variety of morphological lattice-like patterns and differing amino acid compositions, also exhibit significant diversity and the proteins involved are characteristic for the genera or the species from which the S-layers were obtained.

It has been previously shown that *P. oleovorans* grown on acetate does not produce PHA inclusions. However, guanidine hydrochloride extracts of such cells contain a protein with a molecular mass of 43 kDa (Stuart et al., 1995, 1996). This protein is recognized by the antibody elicited by the 43 kDa protein isolated from PHA inclusions produced by *P. oleovorans* during growth on octanoate. The precise function and significance of this external S-layer and of inclusion proteins are still unknown, and to date they are only related in terms of an organized structure of the network and the presence of a common antigenic epitope.

### 3.3. Ultrastructure of PHA inclusions from genetically altered bacteria

Structural boundary proteins in this study were examined using *E. coli* recombinants and *P. putida* deletion mutants. In order to further determine whether the observed boundary morphology was the result of non-specific contamination from cytoplasmic proteins that might adhere to isolated PHA inclusions, polymer was isolated from two different transformants; namely from: (i) *E. coli* HM174 transformed with plasmid pJM9238, which includes the polymerase gene (*phaC*) as well as the 3-ketothiolase and the acetoacetyl-CoA reductase genes from *R. eutropha* (Valentin et al., 1998); and (ii) from *E. coli* transformed with two plasmid: pJM9238 and pBluescript KS::phaP. This second transformant can produce the polymerase as well as a small (24 kDa) structural protein associated with *R. eutropha* PHA inclusions (Wieczorek et al., 1995). If this latter protein were important in establishing a native boundary array, then the PHA inclusions from these two transformants should exhibit two very different morphologies. As shown in Fig. 5, this is indeed the case. PHA inclusions isolated from transformants expressing only PHA biosynthesis pathway (*phaCAB*) exhibited relatively unorganized surface protein (Fig. 5a), perhaps similar to the biomimetic inclusion described previously (Horowitz and Saunders, 1994). In contrast, double transformants which expressed both the genes of the biosynthetic pathway and a gene for the
inclusion surface protein, produced an organized boundary array similar to that of the R. eutropha native inclusion bodies (Fig. 5b).

Importantly, these results clearly demonstrate that the boundary structures on the native inclusions result from the association of a very specific protein rather than from adherence by non-specific, cytoplasmic proteins. To further substantiate the above observations, additional experiments were carried out. Wild type P. putida, which accumulates PHA and surrounds the inclusion with a highly organized protein grid, was subjected to insertion mutagenesis resulting in the replacement of various regions of its PHA gene loci. One mutant, mutant 42, retains phaCAB, the polymer biosynthetic genes but it lacks the genes which encode the structural proteins: GA-1 and GA-2 (Valentin et al., 1998). As a result, although intracellular polymer was formed by mutant 42, no structurally intact inclusions could be isolated from these bacteria. In contrast, in another experiment, structurally intact polymer inclusions could be readily isolated from mutant 201, which contains both the genes of the PHA biosynthetic pathway and the GA-2 gene of P. putida; the latter gene codes for an outer inclusion associated lattice protein. Subsequently, Western blots showed that indeed the GA-2 gene was expressed by mutant 201 cells (Fig. 6, lanes 1, 2).

Ultrastructural study indicated the inclusions isolated from mutant 201 exhibited a discrete surface protein, although it appeared relatively unstable, and readily degenerated during isolation. In an attempt to further stabilize these inclusions and their protein arrays, they were treated with 33% methanol prior to adsorption on grids and staining with uranyl acetate. Examination of

Fig. 5. PHA inclusions isolated from: (a) recombinant E. coli containing only the R. eutropha genes for polymer biosynthesis (magnification: 136,800); and (b) a E. coli-double recombinant (magnification:136,700). The double recombinant contains the genes from R. eutropha necessary for polymer biosynthesis and the gene for the 24 kDa protein. Uranyl acetate stained.
4. Conclusions

Information obtained from these ultrastructural and molecular genetic studies supports the following conclusions: (i) *P. oleovorans* and *P. putida* display very similar, geometric protein arrays at the boundary of their PHA inclusions; (ii) during polymer synthesis by *P. putida*, in the absence of co-expressed gene(s) for inclusion associated protein(s), there is also an absence of discrete inclusions with organized boundary arrays, thus such protein(s) may normally take part in forming the subcellular compartment and the associated boundary array; (iii) as shown by results from the ultrastructural study of PHA inclusions in *E. coli* double transformants (containing both the polymerase and the Phasin protein), the presence of a low molecular weight protein (encoded by phaP in *R. eutropha*) is associated with a remarkably more ordered and defined inclusion boundary array which begins to effectively segregate polymer from the cytosol.

The results leading to these conclusions may be of especial interest to biotechnologists since they indicate a natural ‘packaging’ of polymer during biosynthesis. Thus, including the gene(s) for boundary proteins when transformations are carried out could ensure appropriate polymer compartmentalization within the cytosol. Indeed, transformations which include the gene(s) responsible for the boundary arrays may permit the utilization of eukaryotic systems without concomitantly requiring the targeting of biosynthetic gene expression to particular sites. In addition, the different types of boundary arrays, e.g. double lattice versus single lose array, suggests a choice of ‘packaging’ which could have distinct advantages in eukaryotic expression systems, both when polymer is harvested as well as when it is synthesized.

5. Summary statement

In conclusion, it is quite clear that PHA storage inclusions in a number of bacterial species consist of the PHA, the polymerase and depolymerase enzymes, and apparently a number of non-enzy-
matic structural proteins. Although clearly these proteins effectively segregate polymer from the general cytosol, their other functions are not yet known. At least in pseudomonads, however, they appear to be structurally similar to the proteins in the outer cell wall of many bacterial species. If further experiments demonstrate a similar function of the PHA inclusion-cytoplasm interface, these structures indeed will represent a most unusual prokaryotic intracellular compartment. Experiments aimed at fully defining the genes necessary for these surface arrays and the nature of their protein products in pseudomonads are currently underway in a collaboration with Mr. Gerhard Schoo and Professor Werner Lubitz at the University of Vienna.

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References
