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(54) Title: ACCUMULATION OF METABOLIC PRODUCTS IN BACTERIAL MICROCOMPARTMENTS

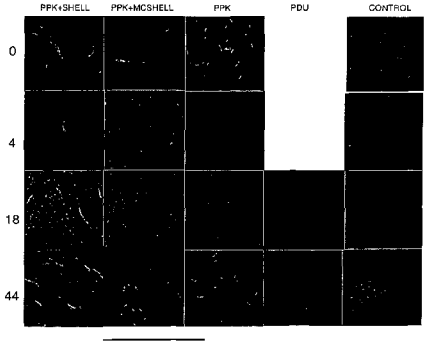

Figure 1A

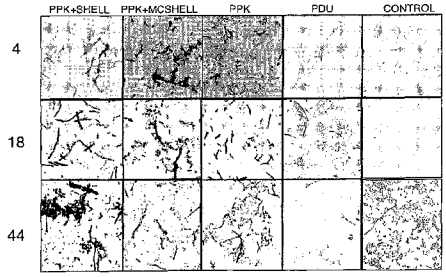

Figure 1B

(57) Abstract: A non-therapeutic method of accumulating a polymeric or high molecular weight molecular product within a bacterial microcompartment in bacterial cytoplasm, which method employs a recombinant bacteria which is transformed to express a microcompartment containing an enzyme capable of converting a low molecular weight substrate into a polymeric or high molecular weight product, the method comprising the steps of: incubating the recombinant bacteria with the low-molecular weight substrate, or a precursor of the low molecular weight substrate which is capable of being metabolised to the substrate within the recombinant bacteria, such that the substrate or precursor is taken up by the bacteria, wherein the substrate enters the microcompartment and the enzyme within the microcompartment converts the substrate to a polymeric or high molecular weight molecular product, and wherein the polymeric or high molecular weight molecular product is accumulated within the microcompartment due to its size.

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Title

Accumulation of metabolic products in bacterial microcompartments

Field of the Invention

The invention relates to a method for accumulation of compounds inside bacterial cells. In particular, the invention relates to a method for and uses of accumulating compounds within a recombinant bacterial compartment.

Background to the Invention

Bacterial microcompartments (metabolosomes) are closed polyhedral shells 100-150 nm diameter made of thin protein sheets (with pores less than 1 nm in diameter which can be positively or negatively charged), enclosing enzymes and cofactors for carbon fixation (carboxysomes) or various forms of fermentative metabolism. Although bacterial microcompartments were first seen over fifty years ago in photosynthetic cyanobacteria, their presence in the cytoplasm of heterotrophic bacteria was only confirmed in 1998, because they require induction by specific metabolites to form. In fact, twenty percent of bacterial genome sequences contain microcompartment structural genes, in many cases associated with enzymes of unknown function. A high percentage of bacteria therefore make a major investment in retaining and expressing large (20+ gene) operons encoding these structures and associated enzymes. It is believed the structures help bacterial metabolic efficiency by selective limitation of the shell pores on the passage of reactants, by metabolic channelling, or other mechanisms achieving temporary retention of small reaction intermediates within the structure, but these advantages have not been fully quantified.

Native microcompartment operons consist of a combination of genes specifying structural components making up the microcompartment shell, and genes specifying enzymes or cofactors, which are located within the microcompartment, or process products leaving the microcompartment. The diameter of identified pores in microcompartment shells of heterotrophic bacteria is sufficient to admit the typical primary substrates such as 1,2-propanediol, glycerol, or ethanolamine (Table 1). Most molecules consumed or produced by the natural enzyme-catalysed reactions within the microcompartment are less than 100 Daltons. The largest substrate molecules believed

to enter and leave native microcompartments (on the basis of the location of the enzyme which utilises them) are cofactors such as Coenzyme A (MW 767.54 Da) which circulate between the microcompartment interior and the cytoplasm. Although they are present in commensal bacteria, expression of different microcompartment operons occurs in enteric pathogens in the intestine and after phagocytosis.

Recombinant microcompartments can be expressed heterologously in *E. coli*, both with and without the associated interior enzyme (Parsons , J., S. Frank, D. Bhella, M. Liang, M.B. Prentice, D. Mulvihill, and M.J. Warren, *Synthesis of Empty Bacterial Microcompartments, Directed Organelle Protein Incorporation, and Evidence of Filament-Associated Organelle Movement.* Molecular Cell, 2010. **38**: p. 305–315). A clonable localisation signal comprising a short peptide enabling enzyme targeting to the microcompartment interior has been identified. Fusion of a 42 amino acid peptide (Parsons *et al.* 2010). or an 18 amino acid peptide (Fan, C., S. Cheng, Y. Liu, C.M. Escobar, C.S. Crowley, R.E. Jefferson, T.O. Yeates, and T.A. Bobik, *Short N-terminal sequences package proteins into bacterial microcompartments.* Proc Natl Acad Sci U S A, 2010. **107**(16): p. 7509-14) from the N-terminus of PduP enzymes localised green fluorescent protein within microcompartments. Multiple sequence alignment of microcompartment associated enzymes reveals conserved N-terminal extensions of approximately 18 amino acids compared with non-microcompartment associated homologues for diol dehydratase small and medium subunits (PduD,E) ethanolamine ammonia lyase small subunit Eut C, Eut G, and pyruvate formate lyase Pfl2 (Fan *et al* 2010). General features of these N-terminal extensions, incorporating conserved hydrophobic residues followed by a less conserved linker region have been described (Sutter, M., D. Boehringer, S. Gutmann, S. Gunther, D. Prangishvili, M.J. Loessner, K.O. Stetter, E. Weber-Ban, and N. Ban, *Structural basis of enzyme encapsulation into a bacterial nanocompartment.* Nat Struct Mol Biol, 2008. **15**(9): p. 939-947). Compartmentalisation of the cellular interior is a functionally transforming process which underlies such radical events as the emergence of eukaryotes. Nanotechnological applications of biological compartment systems have included the use of viral capsids for DNA delivery and lumazine synthase enclosure of HIV protease (Worsdorfer, B., K.J. Woycechowsky, and D. Hilvert, *Directed evolution of a protein container.* Science, 2011. **331**(6017): p. 589-92).

Bacteria contain certain polymeric compounds often used as energy or nutrient stores, which are subject to dynamic synthesis and breakdown by different enzymes, according to prevailing conditions, usually under global regulatory control. One example is cyanophycin, an amino acid polymer originally detected in cyanobacteria and formed by the action of cyanophycin synthetase CphA, an enzyme which can be produced in recombinant form in *E. coli* (Aboulmagd, E., F.B. Oppermann-Sanio, and A. Steinbüchel, *Molecular characterization of the cyanophycin synthetase from Synechocystis sp. strain PCC6308.* Archives of Microbiology, 2000. **174**(5): p. 297-306.)

Another example, polyphosphate kinase PPK1 (E.C. 2.7.4.1), forms inorganic polyphosphate polymers (metachromatic volutin granules) in bacterial cytoplasm by catalysing the reaction nATP ↔ $(polyphosphate)_n$ + nADP. This enzyme was the first enzyme (PPK) recognised to catalyse polyphosphate synthesis and is now usually termed PPK1 to differentiate it from another subsequently described group of enzymes (PPK2) which primarily catalyse GTP synthesis but also have limited phosphate polymerising capacity. Generally, any mentions of polyphosphate kinase or PPK without qualification in the literature and herein refer to PPK1. PPK1 is widely distributed in bacteria and some eukaryotes and is characterised by a highly conserved ATP-binding tunnel containing an autophosphorylating histidine residue (Zhu, Y., W. Huang, S.S. Lee, and W. Xu, *Crystal structure of a polyphosphate kinase and its implications for polyphosphate synthesis.* EMBO Rep, 2005. **6**(7): p. 681-7). Dimerization is crucial for the enzymatic activity of PPK1. A PPK1 monomer includes four structural domains, the N-terminal (N) domain, the head (H) domain, and two related C-terminal (C1 and C2) domains. The ATP-binding tunnel is formed by N, C1, and C2 domains, so conserved residues required for ATP-binding are distributed throughout the length of all known PPK1 proteins. Although nATP ↔ $(polyphosphate)_n$ + nADP is a reversible reaction, in *E.coli* this enzyme generally favours synthesis of polyphosphate over breakdown (V_{max} ratio of 4.1). However, the balance between net accumulation and breakdown changes dynamically with growth phase, and external stimuli, in part due to the action of other enzymes.

E.coli also contains two exopolyphosphatases which release orthophosphate from the termini of long chain polyphosphate: $(polyphosphate)_n \rightarrow (polyphosphate)_{n-1} + Pi$. These are PPX (E.C. 3.6.1.11, sometimes called PPX1) and its homologue guanosine pentaphosphate phosphohydrolase (GPPA or PPX2), both described by InterPro Accession IPR003695. GPPA (E.C. 3.6.1.40) also hydrolyses guanosine pentaphosphate (pppGpp) to guanosine tetraphosphate (ppGpp) with phosphate release, and pppGpp is a competitive inhibitor of PPX and the polyphosphate hydrolytic activity of GPPA. Amino acid starvation in *E.coli* leads to accumulation of large amounts of polyphosphate due to high levels of pppGpp produced in the stringent response inhibiting PPX.

Phosphate pollution in waterways and water treatment plants is a major problem. Removal of phosphate from wastewater is required to treat agricultural phosphate-containing discharges to reduce eutrophication, the algal blooms and "dead zones" in lakes and coastal marine ecosystems. The established biological method to remove inorganic phosphate from wastewater (Enhanced Biological Phosphate Removal, EBPR) relies on empirical selection by cyclical aerobic and anaerobic incubation of a community of uncultivated bacteria capable of temporary polyphosphate storage. There are various disadvantages to the process: the microbiological basis is not understood, it can take months of pumping to become established, and it is thereafter operationally unstable (prone to unexplained failure).

In medicine, oral phosphorus chelation therapy is required in the management of chronic renal failure subjects because of toxicity resulting from accumulation of dietary phosphate in the absence of urinary secretion (hyperphosphatemia). One method that was used for reducing phosphate in such subjects was the use of oral aluminum hydroxide. However, the use of aluminum hydroxide has severe side-effects due to build up of aluminum in the body. Calcium-based salts are an effective replacement for aluminum therapy and are currently the most widely used but there is a concern about their association with hypercalcaemia and vascular calcification. Newer oral phosphate binding medicaments with fewer side effects have recently been introduced in place of aluminum hydroxide and calcium-based salts, namely the anion exchange resin sevelamer hydrochloride, and lanthanum carbonate. However, these are both much more

expensive than calcium or aluminium salts and financial considerations may adversely affect their provision for all renal patients requiring phosphate binding therapy.

It is an object of the present invention to overcome some of the above-mentioned problems.

Summary of the Invention

The invention provides a means to accumulate polymeric metabolic products or metabolic products of high molecular weight within bacterial microcompartments as set out in the appended claims. The invention employs bacteria that are genetically modified or transformed to express a heterologous bacterial microcompartment, an enzyme that is capable of converting a low molecular weight substrate into a high molecular weight or polymeric product, and a bacterial microcompartment localisation signal that is capable of targeting the enzyme to the microcompartment. Two specific applications of this invention are given as an illustrative example of the utility of the invention. The first application is a method of removing or reducing the levels of inorganic phosphate from a system or environment by employing recombinant bacteria which are capable of non-reversibly accumulating phosphate in the form of the polymer polyphosphate. The second application is a method for accumulating an amino acid polymer (cyanophycin) with an increase in chain length.

In the first example, the bacteria are genetically transformed such that they express microcompartments containing polyphosphate kinase enzyme (ideally PPK1 enzyme), which form a sink for Pi in the system which after normal metabolic uptake by the bacteria is naturally incorporated into the essential coenzyme adenosine triphosphate (ATP) which passes into the microcompartments where it is converted into polyphosphate. As the polyphosphate polymer molecule is too large to pass out of the pores of the microcompartments, it accumulates as a localised phosphate store in the microcompartments. The microcompartment structure generates irreversibility in the process by excluding exopolyphosphatases, and thus preventing phosphate (Pi) liberation from polyphosphate to be lost from the bacterial cell and eventually returned to the system/environment. Data presented below shows that compartmentalisation of

PPK1 enzyme has resulted in two-fold uptake and retention of phosphate compared with PPK1 over-expression alone.

In the second example, the bacteria are genetically transformed such that they express microcompartments containing cyanophycin synthetase (CphA), an enzyme capable of assembling a long chain amino acid polymer (cyanophycin). It is well known that expression of cyanobacterial CphA alone in *E. coli* without the cyanobacterial peptidase cyanophycinase (CphB) results in cyanophycin accumulation. Data presented below shows that an increase in chain length of cyanophycin is obtained by localisation of CphA within a microcompartment compared with expression of CphA alone. Thus, a consistent feature of the intracompartment localising process with two different enzyme classes is the enhanced stabilisation of a polymeric molecule.

According to the invention, there is provided a method of accumulating a polymeric or high molecular weight molecular product within a bacterial microcompartment in bacterial cytoplasm, which method employs a recombinant bacteria which is transformed to express a heterologous microcompartment operon and an enzyme fused to a microcompartment localisation signal, the enzyme capable of converting a low molecular weight substrate into a polymeric or high molecular weight product, the method comprising the steps of:

incubating the recombinant bacteria with the low-molecular weight substrate, or a precursor of the low molecular weight substrate which is capable of being metabolised to the substrate within the recombinant bacteria, such that the substrate or precursor is taken up by the bacteria,

wherein the substrate enters the microcompartment and the enzyme within the microcompartment converts the substrate to a polymeric or high molecular weight molecular product, and

wherein the polymeric or high molecular weight molecular product is accumulated within the microcompartment. Accumulation is typically due to its size and the fact that it is shielded from native enzymatic metabolic processes otherwise capable acting on it to convert the product to other molecular products.

In one embodiment, the method is a method of removing inorganic phosphate (Pi) from a system such as for example a body of water, or a human or animal body. The method generally employs recombinant bacteria which are transformed to express a microcompartment (metabolosome) operon and a polyphosphate kinase (i.e. PPK1 or a variant thereof) enzyme fused to a microcompartment localisation sequence. The method suitably comprises the steps of incubating the recombinant bacteria with the system such that the recombinant bacteria comes into contact with the Pi (precursor) in the system, wherein Pi from the system is taken up the bacteria as part of normal metabolism for incorporation in the intracellular metabolite adenosine triphosphate. This passes into the bacteria and is metabolised to adenosine triphosphate (substrate) which enters the microcompartment and is converted to a high molecular weight polyphosphate polymer by the polyphosphate kinase (i.e. PPK1) enzyme located within the microcompartment, and wherein the polyphosphate is accumulated within the microcompartment due to its size. The bacterial microcompartment employed in the methods, bacteria, plasmids, and kits of the invention preferably includes pores which are typically dimensioned to allow passage of ATP adenosine triphosphate (substrate) but to prevent passage of PPK1-formed polyphosphate.

In a further embodiment of the invention, there is provided a method of rendering irreversible a metabolic pathway of the type involving enzymatic synthesis of a polymeric or high molecular weight compound, which pathway is normally reversible due to the action of one or more endogenous enzymes, the method comprising a step of:

accumulating the polymeric or high molecular weight compound within a bacterial microcompartment according to the method described above,

wherein the microcompartment prevents the or each endogenous enzymes coming into contact with the accumulated polymeric or high molecular weight compound.

In a separate, but linked aspect, the invention provides a recombinant bacteria as defined in the claims. Typically, the recombinant bacteria is transformed to express a bacterial microcompartment operon and an enzyme fused to a bacterial microcompartment localisation signal, the enzyme capable of converting a low molecular weight substrate into a polymeric or high molecular weight product, in which the microcompartment has

pores that are dimensioned to allow the low molecular weight substrate pass into the microcompartment and prevent the polymeric or high molecular weight molecular products pass out of the microcompartment.

Typically, the bacteria is transformed to express a microcompartment localisation signal peptide capable of targeting the enzyme to the microcompartment. Suitably, the microcompartment localisation signal is defined by the 18 N-terminal amino acids of NCBI locus CAM57296, GI:171854198, (SEQ ID NO: 4), or a variant thereof having the property of targeting the enzyme (i.e. a PPH enztme) to a bacterial microcompartment. A variant may be selected from another N-terminal region of native microcompartment-targeted enzymes capable of targeting the enzyme to the congruent microcompartment.

Suitably, the bacteria are transformed to express both the microcompartment and the enzyme.

In a preferred embodiment, the bacterial microcompartment is encoded by an empty microcompartment operon such as that described in Parsons *et al.* in reference to the Pdu operon structural genes expressed in the order *pduABJKNU* (hereafter "empty Pdu microcompartment"). Ideally the empty Pdu microcompartment is defined by NCBI loci CAM57283.1 (SEQ ID NO: 5), CAM57284.1 (SEQ ID NO: 6), CAM57290.1 (SEQ ID NO: 7), CAM57291.1 (SEQ ID NO: 8), CAM57294.1 (SEQ ID NO: 9), and CAM57300.1 (SEQ ID NO: 10), respectively, or a variant thereof having the property of being able to form a microcompartment when expressed by a cell.

In an alternative embodiment, the empty bacterial microcompartment is encoded by combining three microcompartment operon structural genes; one encoding a hexamer (PduA-like, CAM57283.1(SEQ ID NO: 5), CAM57290.1(SEQ ID NO: 7) above) containing a single microcompartment domain (InterPro domain IPR000249), one encoding a trimeric pseudohexameric molecule (PduB-like, CAM57284.1(SEQ ID NO: 6)) containing two such microcompartment domains, and one encoding a distinct microcompartment domain (EutN/CcmL-like InterPro domain IPR004992, CAM57294.1 (SEQ ID NO: 9) above).

In one example, the microcompartment is capable of accumulation of polyphosphate, *i.e.* having a pore size dimensioned to allow passage of ATP and prevent outward passage of PPK1-formed polyphosphate. As many different bacteria possess native microcompartment operons and native PPK1 enzymes, it is envisaged that gene rearrangement or self cloning of sufficient native microcompartment genes to make an empty microcompartment, with addition of the congruent N-terminal microcompartment targeting signal to the N-terminal of the native PPK1 enzyme, would achieve the same phenotype in many bacteria. C-terminal location of the microcompartment localisation signal relative to the enzyme to be targeted is also effective and may be useful in certain circumstances, depending on the location of the enzyme active site.

The nature of the targeting sequence, an 18 amino acid peptide, means that novel targeting sequences can readily be generated by one skilled in the art using well established functional techniques such as phage display to select random 18-mers capable of binding microcompartment shell proteins. A flexible linking peptide commonly used to separate attachment sites from functional regions of fusion proteins (Sengupta, A., C.K. Thai, M.S.R. Sastry, J.F. Matthaei, D.T. Schwartz, E.J. Davis, and F. Baneyx, *A Genetic Approach for Controlling the Binding and Orientation of Proteins on Nanoparticles.* Langmuir, 2008. **24**(5): p. 2000-2008). may be fused to the C-terminal part of the 18-mer between the enzyme and the targeting sequence to preserve function of the fused protein.

In one embodiment, the bacteria may be selected from *Escherichia coli*, *Lactobacillus, Bifidobacteria, Pseudomonas, Accumilibacter, Thermus thermophilus, Thermosynechococcus, Halomonas* or *Halobacterium* or other salt tolerant species or other bacteria already adapted to function in the system where phosphate uptake is desired.

Suitably, the body of water constituting the system where phosphate uptake is desired may be a river, a lake, a sea, or a reservoir. A waste liquid stream may be, for example, an effluent stream, an industrial waste stream.

The recombinant bacteria is generally transformed with a plasmid, typically a plasmid according to the claims. Suitably, the plasmid comprises a nucleotide sequence

encoding an empty Pdu microcompartment, ideally an empty Pdu microcompartment as defined by NCBI loci CAM57283.1 (SEQ ID NO: 5), CAM57284.1 (SEQ ID NO: 6), CAM57290.1 (SEQ ID NO: 7), CAM57291.1 (SEQ ID NO: 8), CAM57294.1 (SEQ ID NO: 9), and CAM57300.1 (SEQ ID NO: 10) or a variant thereof, or a combination of a minimum of three structural types of microcompartment operon structural genes encoding a microcompartment structure, and a nucleotide sequence encoding the PPK1 enzyme, for example an *E. coli* PPK1 enzyme as defined by NCBI locus NP_416996.1, GI:16130426, or SEQ ID NO: 3, or a variant thereof having the polyphosphate kinase activity of the PPK enzyme, fused to a microcompartment localisation signal sequence, for example as defined by SEQ ID NO: 4 or variants thereof.

Alternatively, the nucleotide sequence encoding the empty Pdu microcompartment (such as that defined by NCBI loci CAM57283.1 (SEQ ID NO: 5), CAM57284.1 (SEQ ID NO: 6), CAM57290.1 (SEQ ID NO: 7), CAM57291.1 (SEQ ID NO: 8), CAM57294.1 (SEQ ID NO: 9), and CAM57300.1 (SEQ ID NO: 10), or a variant thereof, or containing the three structural types of microcompartment shell proteins defined above, capable of accumulating polyphosphate), and the nucleotide sequence encoding the PPK1 enzyme (such as that defined by SEQ ID NO: 3), or variants thereof defined in SEQ ID NO:12-16, are encoded on separate plasmids. The nucleic acid sequence encoding the microcompartment localisation signal peptide is generally located on the same plasmid as the nucleic acid sequence encoding the enzyme so that a fusion protein of the two encoded peptides is produced. Gene insertion in the chromosome of the recombinant bacteria is also envisaged. Because many bacteria already possess genes encoding both microcompartment structural proteins and PPK1, rearrangement of these pre-existing chromosomal genes with the insertion of the cognate microcompartment localisation sequence N-terminal to the native *ppk1* would be possible.

Suitably, the bacteria is selected from *Escherichia coli*, *Lactobacillus, Pseudomonas, Acinetobacter, Accumilibacter,* and *Bacillus*. In an embodiment of the invention in which the recombinant bacteria is intended to be administered to a human, the bacteria will be a food grade bacteria, for example a probiotic bacteria such as a lactobacillus. Details of food-grade and probiotic bacteria will be well known to those skilled in the art.

Suitably, the bacteria is selected from from *Escherichia coli*, *Lactobacillus, (L. reuteri. L. acidophilus, L.casei , L. plantarum, L. johnsonii, L. rhamnosus) Lactococcus lactis, Bifidobacteria (B. infantis, B. breve, B. longum, Bifidobacterium animalis ssp. Lactis)*, *Enterococcus faecalis, E. faecium), Streptoccus thermophilus, Pseudomonas, Accumilibacter,* or other *Proteobacteria, Bacteroidetes, Planctomycetales Firmicutes* or *Actinobacteria* species.

In one embodiment, the recombinant bacteria is transformed to express a microcompartment (metabolosome) containing polyphosphate kinase (PPK1) enzyme, in which the microcompartment has pores that are dimensioned to allow inorganic phosphate pass into the microcompartment and prevent PPK1-formed polyphosphate pass out of the microcompartment. Generally, the PPK1 enzyme is the only metabolic enzyme contained within the microcompartment, normal cytoplasmic enzymes which breakdown polyphosphate (*e.g.* exopolyphosphatase, PPX), or other polymerising/depolymerising enzymes, are excluded.

Suitably, the bacteria is transformed to express a microcompartment localisation signal peptide which targets the enzyme to the microcompartment In one embodiment, the microcompartment localisation signal is defined by SEQ ID NO: 4, or a variant thereof capable of targeting the enzyme to the microcompartment.

In one embodiment, the invention also relates to a recombinant bacteria of the invention for use as a medicament.

The invention also relates to a recombinant bacteria of the invention, for use in treating a disease or condition associated with accumulation of dietary phosphate, wherein the recombinant bacteria is a food grade bacteria. Typically, the disease or condition is selected from the group consisting of chronic renal disease, acute vitamin D intoxication, hypoparathyroidism, rhabdomyolysis, tumor lysis syndrome, or excessive oral sodium phosphate administration for bowel preparation prior to colonoscopy or barium enema. In one embodiment, the renal disease is selected from the group comprising: Horseshoe kidney; Polycystic kidney disease; Renal dysplasia; Unilateral

small kidney; Diabetic nephropathy; Glomerulonephritis; Hydronephrosis; Interstitial nephritis; Lupus nephritis; nephrotic syndrome; and acute renal failure due to excessive phosphate ingestion; chronic renal failure.

The invention also relates to a pharmaceutical composition comprising a recombinant bacteria of the invention in combination with a suitable pharmaceutical excipient.

The invention also relates to a plasmid, or kit of parts, according to the claims. Typically, the plasmid comprises a nucleic acid sequence encoding an enzyme capable of converting a low molecular weight substrate into a polymeric or high molecular weight molecular product, and a nucleic acid sequence encoding a microcompartment localisation signal, the plasmid being capable of expressing a fusion protein comprising the enzyme and the microcompartment localisation signal. Typically, the enzyme is PPK1 and the signal is located at an N-terminal of the PPK1 enzyme.

Typically, the PPK1 gene is defined by SEQ ID NO: 3, or variants thereof having PPK1 activity, and the microcompartment localisation signal is defined by SEQ ID NO: 4, or variants thereof having the property of the microcompartment localisation signal encoded by SEQ ID NO: 4.

Suitably, the plasmid comprises a nucleic acid sequence expressing a microcompartment. Typically, the microcompartment is an empty Pdu microcompartment, such as that encoded by NCBI loci CAM57283.1 (SEQ ID NO: 5), CAM57284.1 (SEQ ID NO: 6), CAM57290.1 (SEQ ID NO: 7), CAM57291.1 (SEQ ID NO: 8), CAM57294.1 (SEQ ID NO: 9), and CAM57300.1 (SEQ ID NO: 10) or variants thereof encoding a microcompartment having the properties of a Pdu microcompartment.

The invention also provides the plasmid comprising a PPK1 gene and a nucleic acid encoding a microcompartment localisation signal, in combination with a separate plasmid expressing a microcompartment. Typically, the microcompartment is an empty Pdu microcompartment encoded by NCBI loci CAM57283.1 (SEQ ID NO: 5), CAM57284.1 (SEQ ID NO: 6), CAM57290.1 (SEQ ID NO: 7), CAM57291.1 (SEQ ID NO: 8), CAM57294.1 (SEQ ID NO: 9), and CAM57300.1 (SEQ ID NO: 10) or variants

thereof encoding a microcompartment having the properties of a Pdu microcompartment.

Definitions

As used herein, a variant is intended to mean a substantially similar sequence. For polynucleotides, a variant comprises a substitution of one or more nucleotides at one or more sites in the native polynucleotide, and/or a deletion and/or addition of one or more nucleotides at one or more internal sites within the native polynucleotide. One of skill in the art will recognise that variants of the nucleic acid of the invention will be constructed such that the open reading frame is maintained. Generally, variants of a particular polynucleotide of the invention will have at least about 30%, 35%, 40% 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular polynucleotide as determined by sequence alignment programmes. A variant of PPK1 is a protein having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% sequence identity with SEQ ID NO: 3 as determined by sequence alignment programmes, for example BLAST.

Variant protein is intended to mean proteins derived from the native protein by addition of deletion of one or more amino acids at one or more internal site in the native proteins and/or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins are biologically active, that is they continue to possess the desired biological activity of the native protein. Variants may result from genetic polymorphism or from human manipulation. Biologically active variants of a native microcompartment localisation sequence , or microcompartment shell protein, or PPK1 enzyme or CphA enzyme of the invention will have at least about 30%, 35%, 40% 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, or more preferably 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the amino acid sequence for the native proteins as determined by sequence alignment programmes (such as BLAST).

In the specification, the term “transformed” should be understood to mean bacterial transformation which is a stable genetic change brought about by the uptake of naked DNA (DNA without associated cells or proteins) and competence refers to the state of being able to take up exogenous DNA from the environment. Artificial competence can

be induced in laboratory procedures that involve making the cell passively permeable to DNA by exposing it to conditions that do not normally occur in nature. Such transformation procedures are known to the skilled person, such as, for example, calcium chloride transformation or electroporation.

Unless stated otherwise, the term "microcompartment" should be understood to mean "bacterial microcompartment" and the term "microcompartment localisation signal" should be understood to mean "bacterial microcompartment localisation signal".

Brief Description of the Drawings

The invention will be more clearly understood from the following description of an embodiment thereof, given by way of example only, with reference to the accompanying drawings, in which:-

Figure 1A illustrates a series of fluorescent microscopy image of bacteria in MOPS captured at 0, 4, 18 and 44 hours incubation in MOPS buffer; induction time of *ppk* is at -1 hours; and **Figure 1B** illustrates Neisser's stain (Toluidine blue with chrysoidine counterstain) of fixed films of *E.coli* clones captured at 4, 18 and 44 hours incubation in MOPS buffer; induction time of *ppk* is at -1 hours. Fluorescence microscopy (Figure 1A) demonstrated the presence of large yellow fluorescent granules in tetracycline-stained *E. coli* cells containing the cloned *pdu* structural operon and *ppk* gene and localisation signal (*lppk/pdu*). Smaller granules were seen in *E. coli* containing the *ppk* gene alone. Intracellular polyphosphate was detected in clones over-expressing *ppk* by light microscopy with Neisser's stain (Figure 1B – PPK). Blue black granules were also apparent with Neisser's stain in a proportion of all cells containing a cloned *ppk* gene, but not the *E. coli* BL21 insert-free control or the *pdu*-only clone (Figure 1B). These appearances are consistent with the accumulation of intracellular polyphosphate in cells with increased *ppk* activity. The *lppk/pdu* clones retained the polyphosphate staining at 44 hours (Figure 1A and 1B) whereas the *ppk* only clone showed reduced staining at 44 hours (Figure 1B). More intracellular polyphosphate was observed at 44 hours in the *lppk/pdu ppk* clone (Figure 1B – PPK1+SHELL). All clones containing *ppk* showed a heterogeneous granule phenotype, with a proportion of non-fluorescing or non-toluidine blue staining cells in all fields.

Figure 2 illustrates Fluorescent Microscopy Localisation (i). 18 hours post *ppk* induction and (ii) 44 hours post induction. 'A' represents Tetracycline stain (yellow-green polyphosphate), 'B' represents MCherry Fluorescence, while 'C' represents an overlay of 'A' and 'B'. All *pdu* operon containing clones had a proportion of cells which were greatly elongated. Granules of the PPK1 metabolic product polyphosphate lie within MCherry labelled microcompartment protein (Figure 2), and microcompartment extractions show the presence of PPK1. Some of the cells in all

clones forming multiple polyphosphate granules tended to be larger than the non-granulate cells, presumably because of distension by the granules. However, the largest cells were seen with the combination of *pdu* and *ppk*. MCherry labelled PduA was observed to enclose polyphosphate visualised with tetracycline fluorescence (Figure 2, Panels A and B). At earlier incubations (Figure 2, Panels A) the MCherry labelled protein was in excess (the *pdu* operon is constitutively expressed from the plasmid before *ppk* induction occurs), but at 48 hours (Figure 2, Panels B) the co-location of MCherry and polyphosphate was more obvious. Intracellular polyphosphate was detected in clones over-expressing *ppk* by fluorescent microscopy with tetracycline staining (Figure 2 – Panel B). There was heterogeneity in MCherry labelling and the presence of polyphosphate granules in the MCherry and *lppk/pdu* clones. The *lppk/pdu* clones retained the polyphosphate staining at 44 hours (Figure 2, Panels A and B).

Figure 3 illustrates (a) Phosphate removal and (b) polyphosphate accumulation. Referring now to Figures 3A and 3B, increased phosphate uptake from culture medium compared with the host *E. coli* control was displayed by both the *ppk* clone (square symbol) and the clone with both a *pdu* structural operon and a *ppk* gene with a localisation signal (*lppk/pdu*) (triangle symbol), reaching a maximal uptake of approximately 0.2 mM at 20 hours for both constructs. However, the *ppk* clone returned approximately 50% of this phosphate to the supernatant after 44 hours, while the *lppk/pdu* clone retained approximately 100% of the phosphate taken up for at least 48 hours. Correspondingly, the cell-associated polyphosphate levels of the *ppk* clone were maximal at 20 hours and declined thereafter, while the *lppk/pdu* clone retained the same level of cell-associated polyphosphate at 48 hours as at 20 hours.

Figure 4 illustrates the size distribution of extracted polyphosphate. A DAPI PAGE gel of polyphosphate extractions quantified in Figure 2. Polyphosphate associated with DAPI is bleached by prior UV exposure and appears as a dark band. High molecular weight shadows below the inoculation well correspond to lipid (extracts treated with micrococcal nuclease to remove DNA). Lanes 1 and 5: Sodium phosphate glass Type 45 (Sigma). Lanes 2, 3, 4: Time zero extractions. Lane 6, 7, 8: time 24 hours. Lanes 9, 10, 11: time 48 hours. Lanes 2, 6, 9: *E. coli* BL21. Lanes 3, 7, 10: *E. coli* BL21 *ppk*. Lanes 4, 8, 11: *E. coli lppk/pdu*. DAPI negative staining of polyphosphate extracts size-

separated on a PAGE gel (Figure 4) showed that the polyphosphate detected in both *ppk* and *lppk/pdu* clones exceeded the length of the sodium phosphate glass Type 45 control and was therefore in long chains; that is as long-chain polyphosphate molecules. No qualitative difference in chain length was detected between *ppk* and *lppk/pdu* clones. No long chain polyphosphate was detected in the *E. coli* control.

Figure 5 illustrates the shape of presumed polyphosphate granules in the *E. coli* clone containing both a *pdu* structural operon and a *ppk* gene with a localisation signal (*lppk/pdu*). The light microscopy appearance of this clone in the same incubation conditions (shown in Figure 1A and 1B as PPK + Shell) shows extensive polyphosphate granules. This section of a tomogram constructed using images from a cryoelectron microscope shows electron dense material resembling polyphosphate granules (white asterisks) in polygonal clusters of approximately uniform size 120-150 nm in diameter. Although obvious shell structures surrounding entire polyphosphate granules are not apparent with the resolution of this tomogram, in this tomographic section there is a clear linear separation of adjacent clusters by less electron dense material (white arrowheads), representative of planes of separation visible in the tomographic reconstruction. This suggests enclosure of polyphosphate by a distinct, less electron dense material. These appearances are unlike previously published cryoelectron microscopy tomographic appearances of intracellular polyphosphate granules, which were isolated, often spherical and much more variable in size. The restriction of polyphosphate granules to uniform polygonal shapes with clear planar demarcation between adjacent bodies suggests polyphosphate is being formed inside the containment of a polygonal microcompartment shell.

Figure 6 illustrates the effect of cloning the *pdu* structural operon and expressing *cphA* cyanophycin synthetase in *E. coli.* A Western Blot is provided where Lane 1 contains an extract from cells expressing *cphA*, and lane 2 CphA with a localisation signal and a *pdu* structural operon (empty microcompartment). An increase in molecular weight of the predominant band is noted from 16-18 kD to 17-23 kDa.

The results presented herein illustrate that the enclosure of PPK within a bacterial microcompartment still allows access of the small molecule substrate ATP to the

enzyme, but effectively stabilises the large polymer polyphosphate product by retaining it in the interior of the microcompartment, presumably by preventing access of PPX, GPPA or other cytoplasmic phosphatases. In this way, a metabolic pathway which is reversible *in vivo* by the action of competing enzymes has been converted to an irreversible pathway by removing access of the competing enzyme to the metabolic product. Because of the apparent instability of cytoplasmic polyphosphate accumulation even in *ppk* containing organisms, presumably due to competing enzymes breaking down polyphosphate, wastewater treatment plants currently using enhanced biological phosphorus removal (EBPR) employ a cycling process. In EBPR, a community of microorganisms, usually including a dominant phosphate-retaining uncultivated organism, *Accumilibacter phosphatis*, incubated in aerobic conditions takes up phosphate for storage as polyphosphate, and then during anaerobic incubation releases phosphate from polyphosphate stores. Incremental net phosphate uptake occurs due to a slight excess of aerobic uptake over anaerobic release. A requirement for cycling environmental conditions places a restriction on the application of EBPR, for example during transit through the intestine it would be difficult to arrange, even if acceptable bacteria for oral administration could be identified.

It has been shown here that localisation of an enzyme with a short N-terminal sequence to the interior of a bacterial microcompartment can affect turnover of the product of the reaction catalysed by the enzyme, presumably due to restriction of access for competing enzymes acting on the metabolic product. Toxic metabolic products could also be similarly compartmentalised.

Detailed Description of the Drawings

The use of compartmentalisation by microcompartments to manipulate metabolic pathways rendering reversible cellular reactions irreversible, and engineer novel metabolites is described herein.

Table 1 Molecular weights of native microcompartment enzyme substrates and products, and polymeric products of recombinant enzymes targeted to microcompartments.

Process	Enzyme	Substrate (MW, g/mol)	Product (MW, g/mol)
Native Propanediol utilisation (1)			

	Diol dehydratase PduCDE	1,2-propanediol (76.09)	Propionaldehyde (58.08)
	Diol dehydratase PduCDE	Glycerol (92.09)	3-hydroxy-propionaldehyde (74.078)
	Propionaldehyde dehydrogenase PduP	Propionaldehyde (58.08) Co-enzyme A (767.54)	Propionyl-CoA (823.6)
Native Ethanolamine utilisation (2)			
	Ethanolamine deaminase EutBC	Ethanolamine (61.08)	Acetaldehyde (44.05)
	Acetaldehyde dehydrogenase EutE	Acetaldehyde (44.05) Co-enzyme A (767.54)	Acetyl-CoA (809.57)
	Alcohol dehydrogenase EutG	Acetaldehyde (44.05)	Ethanol (46.068)
Recombinant polyphosphate formation			
	Polyphosphate kinase PPK1	ATP (507.18)	Polyphosphate polymer 50,000-80,000 (500-800 phosphate monomers 94.97) (Figure 4)
Recombinant cyanophycin synthesis			
	Cyanophycin synthetase CphA	Aspartic acid (133.11) Arginine (174.2)	Cyanophycin polymer (20-30,000) (Figure 5)

1. Havemann, G.D. and T.A. Bobik, *Protein content of polyhedral organelles involved in coenzyme B12-dependent degradation of 1,2-propanediol in Salmonella enterica serovar Typhimurium LT2.* J Bacteriol, 2003. **185**(17): p. 5086-95.

2. Brinsmade, S.R., T. Paldon, and J.C. Escalante-Semerena, *Minimal functions and physiological conditions required for growth of salmonella enterica on ethanolamine in the absence of the metabolosome.* J Bacteriol, 2005. **187**(23): p. 8039-46.

Methods

Strains and plasmids

Table 2: Plasmids, Strains and their sources used

Plasmids	**Source**
pET23b	Novagen
pET23b-GFPpduP18	Prof. Martin Warren, University of Kent
pET23bpduP18-ppk (pYY001)	This study
pLysSPduABJKNY	Parsons J. *et al.**
pLysSmcherryPduABJKNU	(*ibid*)
Strains	**Source**
E.coli JM109	Promega

E.coli Top 10	Invitrogen
E.coli BL21 (DE3)	Stratagene

* Parsons , J., S. Frank, D. Bhella, M. Liang, M.B. Prentice , D. Mulvihill, and M.J. Warren, Synthesis of Empty Bacterial Microcompartments, Directed Organelle Protein Incorporation, and Evidence of Filament-Associated Organelle Movement. Molecular Cell, 2010. **38**: p. 305–315.

Media

MOPS medium was as described (Neidhardt, F.C., P.L. Bloch, and D.F. Smith, *Culture Medium for Enterobacteria.* J. Bacteriol., 1974. **119**(3): p. 736-747) with either K_2HPO_4 0.5 mM or 1.0 mM.

The *ppk* gene coding for polyphosphate kinase (PPK1) was PCR-amplified with *Taq* using genomic DNA from *E. coli* JM109 as template, using the forward primer (5-AGT GAG CTC ATG GGT CAG GAA AAG CTA TAC ATC GAA AAA GAA CTC- 3 – SEQ ID NO: 1) and a reverse primer (5- AAT AAA GCT TTT ATT CAG GTT GTT CGA G-3 – SEQ ID NO. 2). The PCR product was digested with *Sac* I and *Hind* III (Fermentas) followed by ligation to pET23b-GFP-pduP18 digested with *Sac* I and *Hind* III. The *gfp* gene was thus replaced by *ppk* with retention of the *pdu* localization sequence.

SEQ ID NO: 3 *ppk* sequence:

ATGGGTCAGGAAAAGCTATACATCGAAAAAGAACTCAGTTGGTTATCGTTC
AATGAACGCGTGCTTCAGGAAGCGGCGGACAAATCTAACCCGCTGATTGAA
AGGATGCGTTTCCTGGGGATCTATTCCAATAACCTTGATGAGTTCTATAAAG
TCCGCTTCGCTGAACTGAAGCGACGCATCATTATTAGCGAAGAACAAGGCT
CCAACTCTCATTCCCGCCATTTACTGGGCAAAATTCAGTCCCGGGTGCTGAA
AGCCGATCAGGAATTCGACGGCCTCTACAACGAGCTATTGCTGGAGATGGC
GCGCAACCAGATCTTCCTGATTAATGAACGCCAGCTCTCCGTCAATCAACAA
AACTGGCTGCGTCATTATTTTAAGCAGTATCTGCGTCAGCACATTACGCCGA
TTTTAATCAATCCTGACACTGACTTAGTGCAGTTCCTGAAAGATGATTACAC
CTATCTGGCGGTGGAAATTATCCGTGGCGATACCATCCGTTACGCGCTGCTG
GAGATCCCATCAGATAAAGTGCCGCGCTTTGTGAATTTACCGCCAGAAGCG
CCGCGTCGACGCAAGCCGATGATTCTTCTGGATAACATTCTGCGTTACTGCC
TTGATGATATTTTCAAAGGCTTCTTTGATTATGACGCGCTGAATGCCTATTCA
ATGAAGATGACCCGCGATGCCGAATACGATTTAGTGCATGAGATGGAAGCC

AGCCTGATGGAGTTGATGTCTTCCAGTCTCAAGCAGCGTTTAACTGCTGAGC
CGGTGCGTTTTGTTTATCAGCGCGATATGCCCAATGCGCTGGTTGAAGTGTT
ACGCGAAAAACTGACTATTTCCCGCTACGACTCCATCGTCCCCGGCGGTCGT
TATCATAATTTTAAAGACTTTATTAATTTCCCCAATGTCGGCAAAGCCAATC
TGGTGAACAAACCACTGCCGCGTTTACGCCATATTTGGTTTGATAAAGCCCA
GTTCCGCAATGGTTTTGATGCCATTCGCGAACGCGATGTGTTGCTCTATTAT
CCTTATCACACCTTTGAGCATGTGCTGGAACTGCTGCGTCAGGCTTCGTTCG
ACCCGAGCGTACTGGCGATTAAAATTAACATTTACCGCGTGGCGAAAGATT
CACGCATCATCGACTCGATGATCCACGCCGCACATAACGGTAAGAAAGTCA
CCGTGGTGGTTGAGTTACAGGCGCGTTTCGACGAAGAAGCCAACATTCACT
GGGCGAAGCGCCTGACCGAAGCAGGCGTGCACGTTATCTTCTCTGCGCCGG
GGCTGAAAATTCACGCCAAACTGTTCCTGATTTCACGTAAAGAAAACGGTG
AAGTGGTGCGTTATGCACACATCGGGACCGGGAACTTTAACGAAAAAACCG
CGCGTCTTTATACTGACTATTCGTTGCTGACCGCCGATGCGCGCATCACCAA
CGAAGTACGGCGGGTATTTAACTTTATTGAAAACCCATACCGTCCGGTGACA
TTTGATTATTTAATGGTATCGCCGCAAAACTCCCGCCGCCTATTGTATGAAA
TGGTGGACCGCGAGATCGCCAACGCGCAGCAAGGGCTGCCCAGTGGTATCA
CCCTGAAGCTAAATAACCTTGTCGATAAAGGCCTGGTTGATCGTCTGTATGC
GGCCTCCAGCTCCGGCGTACCGGTTAATCTGCTGGTTCGCGGAATGTGTTCG
CTGATCCCCAATCTGGAAGGCATTAGCGACAACATTCGTGCCATCAGTATTG
TTGACCGTTACCTTGAACATGACCGGGTTTATATTTTTGAAAATGGCGGCGA
TAAAAAGGTCTACCTTTCTTCCGCCGACTGGATGACGCGCAATATTGATTAT
CGTATTGAAGTGGCGACGCCGCTGCTCGATCCGCGCCTGAAGCAGCGGGTA
CTGGACATCATCGACATATTGTTCAGCGATACGGTCAAAGCACGTTATATCG
ATAAAGAACTCAGTAATCGCTACGTTCCCCGCGGCAATCGCCGCAAAGTAC
GGGCGCAGTTGGCGATTTATGACTACATCAAATCACTCGAACAACCTGAAT
AAAAGCTTG

SEQ ID NO: 4 *pdu* localisation sequence
M N T S E L E T L I R N I L S E Q L A

SEQ ID NO: 11 Cyanophycin synthase CphA from <u>*Geminocystis herdmanii*</u> (*Synechocystis* sp) strain PCC6308 (UniProt P56947, EMBL-CDS: AAF43647.2):

MKILKTQTLRGPNYWSIRRQKLIQMRLDLEDVAEKPSNLIPGFYEGLVKILPSLV
EHFCSRDHRGGFLERVQEGTYMGHIVEHIALELQELAGMPVGFGRTRETSTPGI
YNVVFEYVYEEAGRYAGRVAVRLCNSIITTGAYGLDELAQDLSDLKDLRANSA
LGPSTETIIKEAEARQIPWMLLSARAMVQLGYGANQQRIQATLSNKTGILGVEL
ACDKEGTKTTLAEAGIPVPRGTVIYYADELADAIADVGGYPIVLKPLDGNHGRG
ITIDINSQQEAEEAYDLASAASKTRSVIVERYYKGNDHRVLVINGKLVAVSERIP
AHVTGNGSSTIEELIQETNEHPDRGDGHDNVLTRISIDRTSLGVLKRQGFEMDTV
LKKGEVAYLRATANLSTGGIAIDRTDEIHPQNIWIAERVAKIIGLDIAGIDVVTPD
ITKPLTEVDGVIVEVNAAPGFRMHVAPSQGLPRNVAAPVIDMLFPDNHPSRIPIL
AVTGTNGKTTTTRLLAHIYRQTGKVVGYTSTDGIYLGDYMVEKGDNTGPVSA
GVILRDPTVEVAVLECARGGILRSGLAFESCDVGVVLNVAEDHLGLGDIDTIEQ
MAKVKGVIAESVNADGYAVLNADDPLVAQMAKNVKGKIAYFSMSKDNPIIID
HLRRNGMAAVYENGYLSIFEGEWTLRIEKAENIPVTMKAMAPFMIANALAASL
AAFVHGIDIELIRQGVRSFNPGANQTPGRMNLFDMKDFSVLIDYAHNPAGYLAV
GSFVKNWKGDRLGVIGGPGDRRDEDLMLLGKIASQIFDHIIIKEDDDNRGRDRG
TVADLIAKGIVAENPNASYDDILDETEAIETGLKKVDKGGLVVIFPESVTGSIEMI
EKYHLSSE

SEQ ID NO: 12 >gi|13878615|sp|O25654.1|PPK_HELPY
MNRFFNRELSWLAFNTRVLNEAKDESLPLLERLKFLAIYDTNLDEFYMIRVAGL
KQLYEHKIASKGIDGASPEEQLEKIKHYLAHEIEERELEFQKIQALLFKKGLCITP
YNELNLEQKAKAKTYFKEQLYALVLPFKLDSSHTFPPLANLTFALFARIKDKET
QIISYALIKLPSFIFRFVELEKGLFVLAEEIVEAHLEELFLEHEILDCMAFRVTCDA
DIAITEDEAHDYADLMSKSLRKRNQGEIVRLQTQKGSQELLKTLLASLRSFQTH
SYKKHKLTGMHIYKSAIMLNLGDLWELVNHSDFKALKSPNFTPKIHPHFNEND
LFKSIEKQDLLLFHPYESFEPVIDLIEQAASDPATLSIKMTLYRVGKHSPIVKALIE
AASKIQVSVLVELKARFDEESNLHWAKALERAGALVVYGVFKLKVHAKMLLI
TKKTDNQLRHFTHLSTGNYNPLSAKVYTDVSFFSAKNEIANDIIKLFHSLLTSSA
TNSALETLFMAPKQIKPKIIELIQNEMNHQQEGYIILKANALVDSEIIEWLYQASQ
KGVKIDLIIRGICCLKPQVKGLSENIRVYSIVGKYLEHARIYYFKHENIYFSSADL
MPRNLERRVELLIPATNPKIAHKLLHILEIQLKDTLKRYELNSKGRYIKVSNPND
PLNSQDYFEKQALKTF

SEQ ID NO: 13 >gi|157157220|ref|YP_001463823.1| polyphosphate kinase [Escherichia coli E24377A]

MGQEKLYIEKELSWLSFNERVLQEAADKSNPLIERMRFLGIYSNNLDEFYKVRF
AELKRRIIISEEQGSNSHSRHLLGKIQSRVLKADQEFDGLYNELLLEMARNQIFLI
NERQLSVNQQNWLRHYFKQYLRQHITPILINPDTDLVQFLKDDYTYLAVEIIRG
DTIRYALLEIPSDKVPRFVNLPPEAPRRRKPMILLDNILRYCLDDIFKGFFDYDAL
NAYSMKMTRDAEYDLVHEMEASLMELMSSSLKQRLTAEPVRFVYQRDMPNA
LVEVLREKLTISRYDSIVPGGRYHNFKDFINFPNVGKANLVNKPLPRLRHIWFDK
AQFRNGFDAIRERDVLLYYPYHTFEHVLELLRQASFDPSVLAIKINIYRVAKDSR
IIDSMIHAAHNGKKVTVVVELQARFDEEANIHWAKRLTEAGVHVIFSAPGLKIH
AKLFLISRKENGEVVRYAHIGTGNFNEKTARLYTDYSLLTADARITNEVRRVFN
FIENPYRPVTFDYLMVSPQNSRRLLYEMVDREIANAQQGLPSGITLKLNNLVDK
GLVDRLYAASSSGVPVNLLVRGMCSLIPNLEGISDNIRAISIVDRYLEHDRVYIFE
NGGDKKVYLSSADWMTRNIDYRIEVATPLLDPRLKQRVLDIIDILFSDTVKARYI
DKELSNRYVPRGNRRKVRAQLAIYDYIKSLEQPE

SEQ ID NO: 14 >gi|3452465|gb|AAC32883.1| polyphosphate kinase [Vibrio cholerae]

MSADKLYIDKELSWLSFNERVLQEAADKTVPLIERIRFLGIFSNNLDEFYKVRFA
DVKRQILINRERGGNDISKHLLSRMQSKALKLNQDFDNLYNELILEMARRRIFL
VNETQLDEIQLKWVKKYFHKVMLPHVTPIMLRDDIDVMQFLKDEYAYIAVEM
RSGDEFKYALIEIPTDQLPRFVMLPEQKGKRRKTIILLDNIIRLCLDEIFRGFYDYD
TLNGYAMKMTRDAEYDLRHEVEYSLLEQMSEGLSQRLTALPVRFVYEREMPE
AMLKFLCYKLKISHYDSLIPGGRYHNFKDFISFPNVGRDYLENKPLPPMTCADF
EGYANAFDAIRAQDILLHYPYHSFEHMTELVRQASFDPKVVSIKINIYRVAKDS
KLMNSLVDAVHNGKRVVVVVELQARFDEEANIEWSRILTDAGVHVIFGVPGM
KIHAKLLLITRKEGDEFVRYAHIGTGNFHERTARIYTDFALLTANQELAAEVRA
VFGYIENPFRPVKFNHLIVSPRNSRTQIYRLLDSEIANAKAGKKAAITLKVNNLV
DKGLINKLYGASAAGVKIRMIIRGMCSLVPGVEGVSDNIEIISIIDRFLEHPRVLV
VHNDGNPQVFISSADWMERNIDHRIEVMAPIRDERLKQRIIDILNIQFIDTVKARR
IDKEMSNQYVERGNRRKVRSQIAIYDYLKNVEKQTRKAKGQQETNDNSSQ

SEQ ID NO: 15 >gi|15600435|ref|NP_253929.1| ppk gene product [Pseudomonas aeruginosa PAO1]

MNTQQGLDEIERIAADETVVANVESEAEVKMAETIPVETPPAVVPSVDDSSLYI
HRELSQLQFNIRVLEQALDESYPLLERLKFLLIFSSNLDEFFEIRIAGLKKQITFAR
EQAGADGLLPHQALARISELVHEQVSRQYRILNETLLPELAKHQIRFIRRRHWTL
KIKTWVRRFFRDEIAPIITPIGLDPTHPFPLLVNKSLNFIVELEGMDAFGRDSGLAI
IPAPRLLPRIIRLPEDVGGEGDNYVFLSSMIHAHADDLFPGMKVKGCYQFRLTR
NADLSVDTEDVEDLARALRGELFSRRYGDAVRLEVVDTCPQNLTNYLLKQFGL
SESELYKVSGPVNLTRLFSVTGLESHPELQYPPFTPAIPRLLQKKENLFNVLSKLD
VLLMHPFESFTPVIDLLRQAAKDPNVLAIKQTLYRSGANSEIVDALVEAARNGK
EVTAVIELRARFDEESNLQLASRLQQAGAVVIYGVVGFKTHAKMMLILRREDG
ELRRYAHLGTGNYHAGNARLYTDYSLLTADVALCEDLHKLFNQLIGMGKTLR
MKKLLHAPFTLKKNLLEMINREAAQAALGQPAHIMAKVNSLTDPKVIRALYKA
SQAGVRIDLVVRGMCCLRPGIPGVSHNIHVRSIIGRFLEHSRIYYFLNGGDEKLY
LSSADWMERNLDMRVETCFPVEGKKLVQRVKKELETYLTDNTQAWVLQADG
SYQRLSPTGNQNPRNTQATLLEKLAAPVLTAR

SEQ ID NO: 16 >gi|30264066|ref|NP_846443.1| ppk gene product [Bacillus anthracis str. Ames]
MELSKGNIVNLNDTAYYNNRELSWLAFNERVLQEAQDETNPLLERLKFISIFSS
NLDEFFMVRVAGLKDQVSAGFNQPENKAGLTPKKQLNKIAIKAHELMTVQYG
TFKNYVLPALELEGIERLTFHDLTKEQREFIEEYFDEQIFPVLTPVAIDAYRPFPM
LLNKSLNLATLLYDEKQVEEENRTKLGIVQVPSLLERFIFLPSEGQKHKFILLED
VISSFTHKLFTGYKVSSVTRFRITRNADLTIHEEGARDLLKVIEKELKKRKWGAA
VRLEVGKEHIDERVLALLYEVLEVKDEDVYIMDGPLDLTCLFSLYKKLAPLYEH
LVYPALIPQRPQDLGDAEDVFEKAIEHDILLHHPFESFQPVVDFVRDAADDPNV
LAIKQTLYRVSGDSPIIQALKIAAEKGKQVTVLVELKARFDEENNVHWAKELEQ
AGCHVIYGVSHLKTHSKITLVVRRKNGKIERFVHLGTGNYNDATAKLYTDFGYI
TSRKDFGVDATNFFNYLSGYTTKPHFHHLSVAPFDIREQFMDLIDEEIRYHRQY
GNGYIIAKMNSLTDKPLIKKMYEASQAGVKVELIVRGTCCLRPGIPNVSENIRVV
SVVGRYLEHSRIYYFHHNGEEKIYLSSADWMTRNMEKRVEISFPILDIEMKARIK
AILQLTLADNVKTREQNKDGDYYYVINSGAEEIDSQVKLFKMAYQNTDAE

Table 3 Polyphosphate kinase multiple alignment of SEQ ID NOs: 12 to 16 showing essential conserved residues which bind AMPPNP (β–γ-imidoadenosine 5-phosphate, a nonhydrolysable ATP analogue)

```
 gi|13878615/1-675  ---------------------------------------- 0
gi|157157220/1-688  ---------------------------------------- 0
  gi|3452465/1-701  ---------------------------------------- 0
 gi|15600435/1-736  MNTQQGLDEIERIAADETVVANVESEAEVKMAETIPVETP 40
 gi|30264066/1-702  ------------------------------------MELS 4

 gi|13878615/1-675  ----------MNRFFNRELSWLAFNTRVLNEAKDESLPLLE 31
gi|157157220/1-688  -------MGQEKLYIEKELSWLSFNERVLQEAADKSNPLIE 34
  gi|3452465/1-701  -------MSADKLYIDKELSWLSFNERVLQEAADKTVPLIE 34
 gi|15600435/1-736  PAVVPSVDDSSLYIHRELSQLQFNIRVLEQALDESYPLLE 80
 gi|30264066/1-702  KGNIVNLNDTAYYNNRELSWLAFNERVLQEAQDETNPLLE 44
                                           ^    ^

 gi|13878615/1-675  RLKFLAIYDTNLDEFYMIRVAGLKQLYEHKIASKGID-GA 70
gi|157157220/1-688  RMRFLGIYSNNLDEFYKVRFAELKRRIIISEEQGSNS--- 71
  gi|3452465/1-701  RIRFLGIFSNNLDEFYKVRFADVKRQILINRERGGND--- 71
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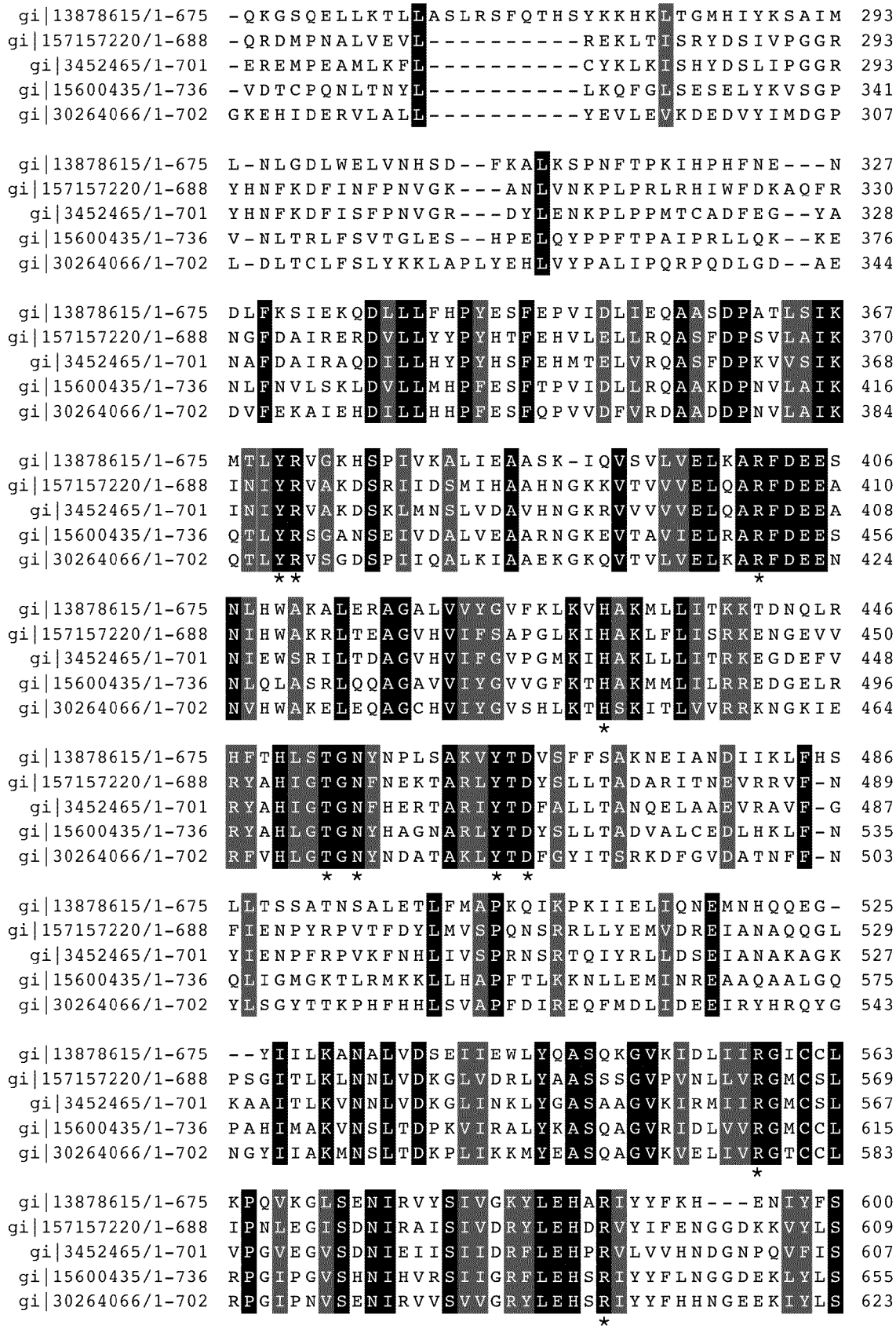
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^ indicates conserved residues involved in side-chain contacts with the adenine ring of the AMPPNP, * indicates residues involved in side-chain contacts with the phosphate groups of AMPPNP. Based on Zhu, Y., W. Huang, S.S. Lee, and W. Xu, *Crystal structure of a polyphosphate kinase and its implications for polyphosphate synthesis.* EMBO Rep, 2005. **6**(7): p. 681-7.). PPK1 cds from GI:13878615 *Helicobacter pylori*, GI:157157220 *Escherichia coli*, GI:3452465 *Vibrio cholerae*, GI:15600435 *Pseudomonas aeruginosa*, GI:30264066 *Bacillus anthracis.*

The ligation product was transformed into *E.coli* Top 10 electrocompetent cells (Invitrogen) by electroporation. The new vector, named pET23bpduP18-ppk, was extracted and the *ppk* insert was sequenced (GATC-Biotech) to confirm no mutation had occurred. Two constructs, pET23bpduP18-ppk and pSF37 (pLysS-pduABJKNU, expressing an empty *pdu* microcompartment), were co-transferred into *E.coli* BL21 (DE3) by electroporation.

Polyphosphate extraction from pelleted *E. coli* cells was carried out using a GENECLEAN™ kit (MP Biomedicals Europe) as described (Ault-Riche, D., C.D. Fraley, C.-M. Tzeng, and A. Kornberg, *Novel Assay Reveals Multiple Pathways Regulating Stress-Induced Accumulations of Inorganic Polyphosphate in Escherichia coli.* J. Bacteriol., 1998. **180**(7): p. 1841-1847). Briefly, 4 M guanidine isothiocyanate (GITC)–50 mM Tris-HCl, pH 7 (GITC lysis buffer), pre-warmed to 95°C was used to

lyse pelleted cells, then 10% sodium dodecyl sulphate (SDS), 95% ethanol, and Glassmilk was added to adsorb polyphosphate, washing with New Wash buffer. Polyphosphate was eluted from the pellet by adding 50 µl of 50 mM Tris-HCl (pH 8.0) at 95°C for 2 min, recovery of polyphosphate was completed with two additional elutions.

Molar quantification of phosphate as polyphosphate in cell extracts was performed metachromatically using the 530/630nm absorbance ratio of 10µl of sample added to 1ml of toluidine dye solution (6 mg/L toluidine blue in 40mM acetic acid) as described (Mullan, A., J.P. Quinn, and J.W. McGrath, *A nonradioactive method for the assay of polyphosphate kinase activity and its application in the study of polyphosphate metabolism in Burkholderia cepacia.* Analytical Biochemistry, 2002. **308**(2): p. 294-299). A polyphosphate standard curve was prepared using sodium phosphate glass Type 45 (S4379 Aldrich) and sodium hexametaphosphate (SX0583). Protein concentration of cell extracts was measured using 10-µl sample, with Coomassie Plus Protein Assay Reagent (Pierce) with bovine serum albumin as the standard, resuspended in the same buffer as the sample. Phosphate uptake was determined as follows. Overnight cultures of bacteria were induced by 0.5 mM IPTG in Luria broth for 1 hr before transfer to pH 5.5 MOPS medium containing 0.01 mM $FeSO_4.7H_2O$ and 0.5 mM potassium phosphate, at an optical density of 600 nm (OD600) of 0.2. Incubation was continued at 37°C with intermittent sampling of 0.2 ml up to 48 hours. Samples were centrifuged and supernatant used for phosphate assay, and the pellet used for polyphosphate and protein assays. Phosphate was assayed using a molybdovanadate colorimetric method (Eaton, A., L. Clesceri, E. Rice, A. Greenberg, and M. Franson, eds. *Standard Methods for the Examination of Water and Wastewater.* 21st ed. 2005, American Public Health Association). 0.2 ml of molybdovanadate solution (Reagecon) was added to 5 ml of culture supernatant, mixed and incubated at room temperature for 5 minutes. Optical density of 1ml at 430nm was measured against a blank of 4% molybdovanadate in distilled water and a calibration curve of potassium phosphate in MOPS.

Polyphosphate was visualised in polyacrylamide gels by 4'6-diamidino-2-phenylindol (DAPI) negative staining. Gels were agitated for 30 min in 2 mg/mL DAPI in fixative

at room temperature. Gels were then exposed to 365 nm light via a UV transilluminator for 2–20 min to induce specific photobleaching of polyphosphate bound to DAPI.

Microscopy

Fluorescent microscopy visualisation of intracellular polyphosphate with tetracycline staining was carried out as described (Smith, S.A. and J.H. Morrissey, *Sensitive fluorescence detection of polyphosphate in polyacrylamide gels using 4',6-diamidino-2-phenylindol.* Electrophoresis, 2007. 28(19): p. 3461-3465). A 2-mg ml^{-1} stock solution of tetracycline hydrochoride (Sigma) in double-distilled water was added to cell suspensions to give a final concentration of 0.225 mM. Fresh stock solutions were prepared before each experiment because of rapid deterioration in fluorescence with solution storage. Microscopy was carried out with an Olympus BX51 microscope excitation G 365, BS 395, emission LP 420 for green fluorescence. Polyphosphate granules were also visualised in fixed films by Neisser's stain using Chrysoidin counterstain.

For cryo-electron microscopy (TEM) (Figure 5) a single colony was inoculated in LB medium with 30 μg/ml chloramphenicol and 100 μg/ml ampicillin, and grown with shaking at 37°C for about 16 hrs. 1 ml of overnight culture was inoculated in 50 ml fresh LB medium containing the same antibiotics and grown for about 2-3 hrs until OD600 reaches 0.4. 0.2 mM IPTG was added to the culture and bacterium were grown for another 1 hrs. Cells were then harvested by centrifugation and washed twice with MOPS medium without added K_2HPO_4. The washed cells were resuspended in 50 ml of MOPS medium containing 1 mM K_2HPO_4, 30 μg/ml chloramphenicol, 100 μg/ml ampicillin, 0.2 mM IPTG and 30 μg/ml of cefsulodin and incubated at 37°C for between 4 and 18 hours. Bacterial cell cultures of OD600 of 0.5 were used neat for microscopy, cultures less than OD600 0.5 were concentrated by centrifugation at 12,000 rpm for 2 minutes with resuspension of deposit in MOPS to achieve OD600 of 0.5.

100 μl of 10 nm colloidal gold was mixed with 25 μl of 5 % BSA and centrifuged at 12,000 x g for 15 minutes. Supernatant was removed and pellet of colloidal gold was mixed with 20 μl of cells at appropriate density (OD600=0.5). 300 mesh quantifoil grids were glow discharged for 3 minutes. 4 μl of sample was added to the carbon side of a

grid in a Vitrobot at 95 % RH, 21 C. Blotting was performed automatically with a Vitrobot with two 1 second blots and 1 second dry time before plunging into liquid ethane. Grid was transferred under liquid nitrogen for imaging. Imaging was performed on a Technai Polara G2 at 300 keV under cryo-conditions. All tomograms were collected using SerialEM on a 4K GATAN CCD binned at 2K x 2K resolution.

In this specification, the term "system" should be taken to include, but not limited to, a "biological system", for example a cell, a cell line, a tissue sample, an organ, a unicellular or multicellular organism, or a lower or higher life form; a "water body" or "body of water", for example a lake, a reservoir, a pond, a swimming pool, a canal, settlement ponds/pools, water treatment tanks, domestic water storage containers, rivers, streams, an aquarium, and the like; and "waste streams", for example water treatment tanks, agricultural run-off treatment tanks, and the like.

When the "system" is a human or animal body, various delivery methods are known and can be used to administer a therapeutic of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules.

Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, and oral routes. The compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents and dietary supplements. Administration can be systemic or local. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

It may be desirable to administer the compositions of the invention locally to the area in need of treatment; this may be achieved, for example and not by way of limitation, by topical application, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a therapeutic, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like.

Yoghurt or soy yoghurt can also be used to administer the recombinant bacteria of the present invention. The recombinant bacteria are preferably probiotic bacteria or bacteria suitable for human consumption, for example, lactic acid bacteria (selected from, but not limited to, *Lactobacillus, (L. reuteri. L. acidophilus, L.casei , L. plantarum, L. johnsonii, L. rhamnosus), Lactococcus lactis, Bifidobacteria* (*B. infantis, B. breve, B. longum, Bifidobacterium animalis ssp.* Lactis), *Enterococcus faecalis, E. faecium*), *Streptoccus thermophilus*, and *Escherichia coli* strains. Yeast, such as *Saccharomyces*, may also be used to express and deliver the microcompartment and PPK enzyme to an animal or human body in a probiotic formulation.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like.

The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, *etc*. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. Such compositions will contain a therapeutically effective amount of the therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In the specification, the expression “low molecular weight substrate” should be understood to mean a substrate which is sufficiently small to pass into the microcompartment from the bacterial cytoplasm through the pores of the microcompartment.

In the specification, the expression “polymeric” or “high molecular weight molecular product(s)” should be understood to mean products of a size that is too large to pass out of the microcompartment through the pores of the microcompartment.

In the specification the terms "comprise, comprises, comprised and comprising" or any variation thereof and the terms “include, includes, included and including" or any variation thereof are considered to be totally interchangeable and they should all be afforded the widest possible interpretation and vice versa.

The invention is not limited to the embodiments hereinbefore described but may be varied in both construction and detail.

Claims

1. A non-therapeutic method of accumulating a polymeric or high molecular weight molecular product within a bacterial microcompartment in bacterial cytoplasm, which method employs a recombinant bacteria which is genetically transformed to express a heterologous bacterial microcompartment operon and an enzyme fused to a bacterial microcompartment localisation signal, wherein the enzyme is capable of converting a low molecular weight substrate into a polymeric or high molecular weight product, and wherein microcompartment localisation signal peptide is capable of targeting the enzyme to the microcompartment, the method comprising the steps of:

incubating the recombinant bacteria with the low-molecular weight substrate, or a precursor of the low molecular weight substrate which is capable of being metabolised to the substrate within the recombinant bacteria, such that the substrate or precursor is taken up by the bacteria,

wherein the substrate enters the microcompartment and the enzyme within the microcompartment converts the substrate to a polymeric or high molecular weight molecular product, and

wherein the polymeric or high molecular weight molecular product is accumulated within the microcompartment.

2. A method of Claim 1 which is a method of removing inorganic phosphate (Pi) from a system, in which the recombinant bacteria is transformed to express a bacterial microcompartment and a polyphosphate kinase enzyme fused to a microcompartment localisation signal, the method comprising the steps of:

incubating the recombinant bacteria with the system such that the recombinant bacteria comes into contact with the Pi (precursor) in the system,

wherein Pi in the system is taken up by the bacteria and metabolised to adenosine triphosphate (substrate) which enters the microcompartment and is converted to a high molecular weight polyphosphate polymer by the polyphosphate kinase enzyme located within the microcompartment, and

wherein the polyphosphate is accumulated within the microcompartment due to its size.

3. A method as claimed in Claim 1 or Claim 2 in which the microcompartment localisation signal is defined by SEQ ID NO: 4.

4. A method as claimed in Claim 2 or 3 in which the polyphosphate kinase enzyme is PPK1.

5. A method as claimed in Claim 4 in which PPK1 is encoded by SEQ ID NO: 3.

6. A method as claimed in any preceding claim wherein the microcompartment is a Pdu microcompartment or a microcompartment encoded by three microcompartment operon structural genes.

7. A method as claimed in any preceding claim wherein the bacteria is selected from *Escherichia coli*, *Lactobacillus, Bifidobacteria, Pseudomonas, Accumilibacter, Thermus thermophilus, Thermosynechococcus, Halomonas* or *Halobacterium* or other salt tolerant species or other bacteria already adapted to function in the system where phosphate uptake is desired.

8. A method as claimed in any preceding claim wherein the recombinant bacteria is genetically transformed with: a plasmid comprising a nucleotide sequence encoding a *pdu* microcompartment operon, and a nucleotide sequence encoding a PPK1 enzyme; or separate plasmids one of which encodes the Pdu microcompartment, and another of which encodes the nucleotide sequence encoding the PPK1 enzyme,

in which the nucleic acid sequence encoding the microcompartment localisation signal peptide is located on the same plasmid as the nucleic acid sequence encoding the enzyme.

9. A method of rendering irreversible a metabolic pathway of the type involving enzymatic synthesis of a polymeric or high molecular weight compound, which pathway is normally reversible due to the action of one or more endogenous enzymes, the method comprising a step of accumulating the polymeric or high molecular weight compound within a bacterial microcompartment according to a method of any of Claims 1 to 7, wherein the microcompartment prevents the or each endogenous enzymes coming into contact with the accumulated polymeric or high molecular weight compound.

10. A recombinant bacteria which is genetically transformed to express a heterologous bacterial microcompartment operon and an enzyme fused to a bacterial microcompartment localisation signal, in which the enzyme is capable of converting a low molecular weight substrate into a polymeric or high molecular weight product, in which the bacteria is transformed to express a microcompartment localisation signal peptide which targets the enzyme to the microcompartment, and in which the microcompartment has pores that are dimensioned to allow the low molecular weight substrate pass into the microcompartment and prevent the polymeric or high molecular weight molecular products pass out of the microcompartment.

11. A recombinant bacteria of Claim 10 in which the enzyme is polyphosphate kinase enzyme, and the heterologous bacterial microcompartment operon encodes a Pdu microcompartment or a microcompartment encoded by three microcompartment operon structural genes.

12. A recombinant bacteria as claimed in Claim 10 or Claim 11 wherein the bacteria is selected from *Escherichia coli*, *Lactobacillus, (L. reuteri. L. acidophilus, L. casei, L. plantarum, L. johnsonii, L. rhamnosus) Lactococcus lactis, Bifidobacteria (B. infantis, B. breve, B. longum, Bifidobacterium animalis ssp. Lactis)*, *Enterococcus faecalis, E. faecium), Streptoccus thermophilus, Pseudomonas, Accumilibacter, or other Proteobacteria, Bacteroidetes, Planctomycetales Firmicutes or Actinobacteria species.*

13. A recombinant bacteria as claimed in any of Claims 10 to 12 in which the bacterial microcompartment localisation signal is defined by SEQ ID NO: 4, or a variant thereof capable of targeting the enzyme to the microcompartment.

14. A recombinant bacteria as claimed in any of Claims 10 to 13 in which the polyphosphate kinase enzyme is PPK1.

15. A recombinant bacteria as claimed in Claim 14 in which the polyphosphate kinase enzyme is encoded by SEQ ID NO: 3.

16. A recombinant bacteria as claimed in Claim 10 in which the enzyme is a polyphosphate kinase enzyme PPK1; and the bacterial microcompartment is a Pdu microcompartment or a microcompartment encoded by three microcompartment operon structural genes, and wherein the bacterial microcompartment localisation signal is capable of targeting the PPK1 enzyme to the microcompartment.

17. A recombinant bacteria of any of Claims 10 to 16 for use as a medicament.

18. A recombinant bacteria of any of Claims 10 to 16 for use in treating a disease or condition associated with accumulation of dietary phosphate, wherein the recombinant bacteria is a food grade bacteria.

19. A use of Claim 18 in which the disease or condition is selected from the group consisting of chronic renal disease, acute vitamin D intoxication, hypoparathyroidism, rhabdomyolysis, tumor lysis syndrome, or excessive oral sodium phosphate administration for bowel preparation prior to colonoscopy or barium enema.

20. A use as claimed in Claim 19 in which the chronic renal disease is selected from the group comprising: Duplicated ureter; Horseshoe kidney; Polycystic kidney disease; Renal dysplasia; Unilateral small kidney; Diabetic nephropathy; Glomerulonephritis; Hydronephrosis; Interstitial nephritis; Lupus nephritis; nephrotic syndrome; and acute renal failure due to excessive phosphate ingestion; chronic renal failure.

21. A pharmaceutical composition comprising a recombinant bacteria of any of Claims 10 to16 in combination with a suitable pharmaceutical excipient.

22. A plasmid comprising a nucleic acid sequence encoding: an enzyme capable of converting a low molecular weight substrate into a polymeric or high molecular weight molecular weight product; a bacterial microcompartment localisation signal; and an empty bacterial microcompartment, wherein the plasmid is capable of expressing within a bacterial host an empty bacterial microcompartment and a fusion protein comprising the enzyme and the microcompartment localisation

signal, and wherein the microcompartment localisation signal peptide is capable of targeting the fusion protein to the microcompartment within the bacterial host.

23. A plasmid as claimed in Claim 22 in which the enzyme is a polyphosphate kinase.

24. A plasmid as claimed in Claim 22 or 23 in which the polyphosphate kinase is PPK1.

25. A plasmid as claimed in any of Claims 22 to 24 in which the PPK1 is encoded by SEQ ID NO: 3.

26. A plasmid as claimed in any of Claims 22 to 25 in which the microcompartment localisation signal peptide has a sequence of SEQ ID NO: 4.

27. A plasmid as claimed in any of Claims 22 to 26 in which the empty microcompartment is a Pdu microcompartment or a microcompartment encoded by three microcompartment operon structural genes.

28. A plasmid as claimed in Claim 27 in which the Pdu microcompartment is encoded by SEQ ID NO: 3.

29. A plasmid as claimed in Claim 22 and comprising a nucleic acid sequence encoding: a polyphosphate kinase enzyme PPK1; a bacterial microcompartment localisation signal; and a Pdu microcompartment or a bacterial microcompartment encoded by three microcompartment operon structural genes, wherein the plasmid is capable of expressing a fusion protein comprising the enzyme and the microcompartment localisation signal, and wherein the microcompartment localisation signal is capable of targeting the enzyme to the microcompartment in a bacteria transformed with the plasmid.

30. A kit of parts comprising:
a first plasmid comprising a nucleic acid sequence encoding: an enzyme capable of converting a low molecular weight substrate into a polymeric or high molecular weight molecular weight product; and a bacterial microcompartment localisation signal; and

a second plasmid comprising a nucleic acid sequence encoding a bacterial microcompartment,

in which the microcompartment localisation signal peptide is capable of targeting the enzyme to the bacterial microcompartment within a host bacterium.

31. A kit of parts as claimed in Claim 30 in which the enzyme is a polyphosphate kinase.

32. A kit of parts as claimed in Claim 30 or 31 in which the polyphosphate kinase is PPK1.

33. A kit of parts as claimed in any of Claims 30 to 32 in which the PPK1 is encoded by SEQ ID NO: 3.

34. A kit of parts as claimed in any of Claims 30 to 33 in which the microcompartment localisation signal peptide has a sequence of SEQ ID NO: 4.

35. A kit of parts as claimed in any of Claims 30 to 34 in which the empty microcompartment is a Pdu microcompartment or a microcompartment encoded by three microcompartment operon structural genes.

36. A kit of parts as claimed in Claim 35 in which the Pdu microcompartment is encoded by SEQ ID NO: 3.

Drawings

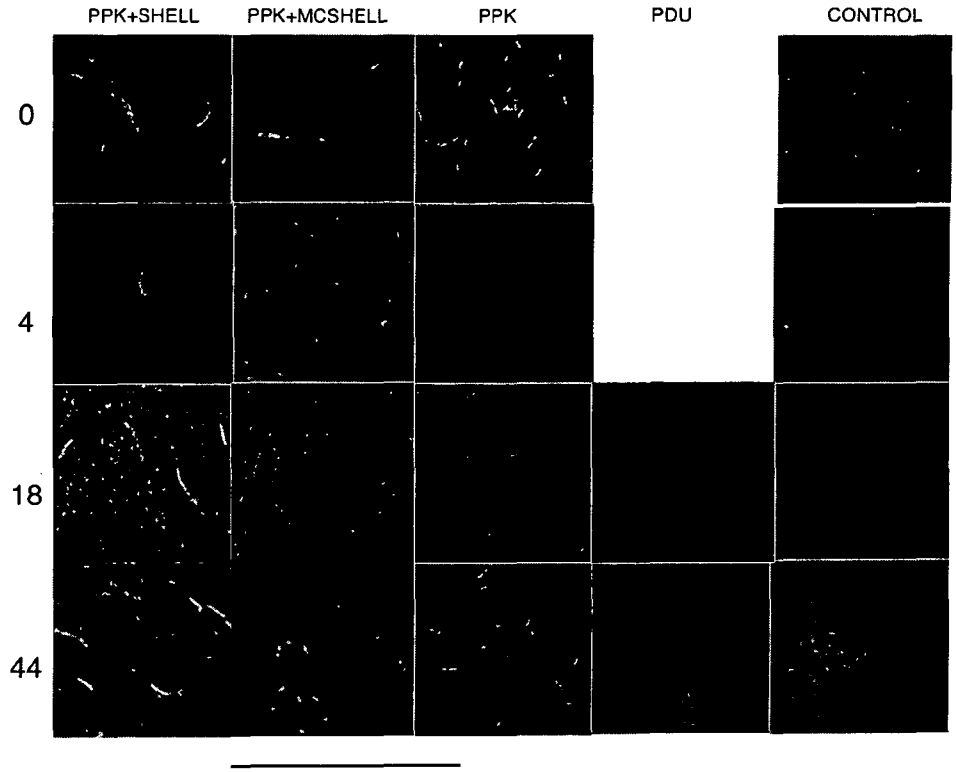

Figure 1A

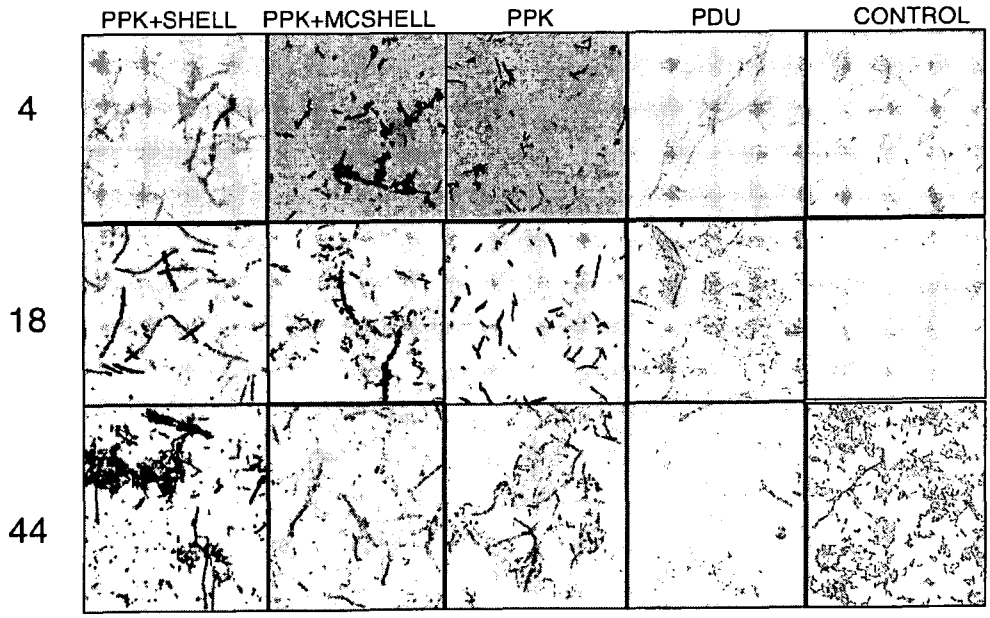

Figure 1B

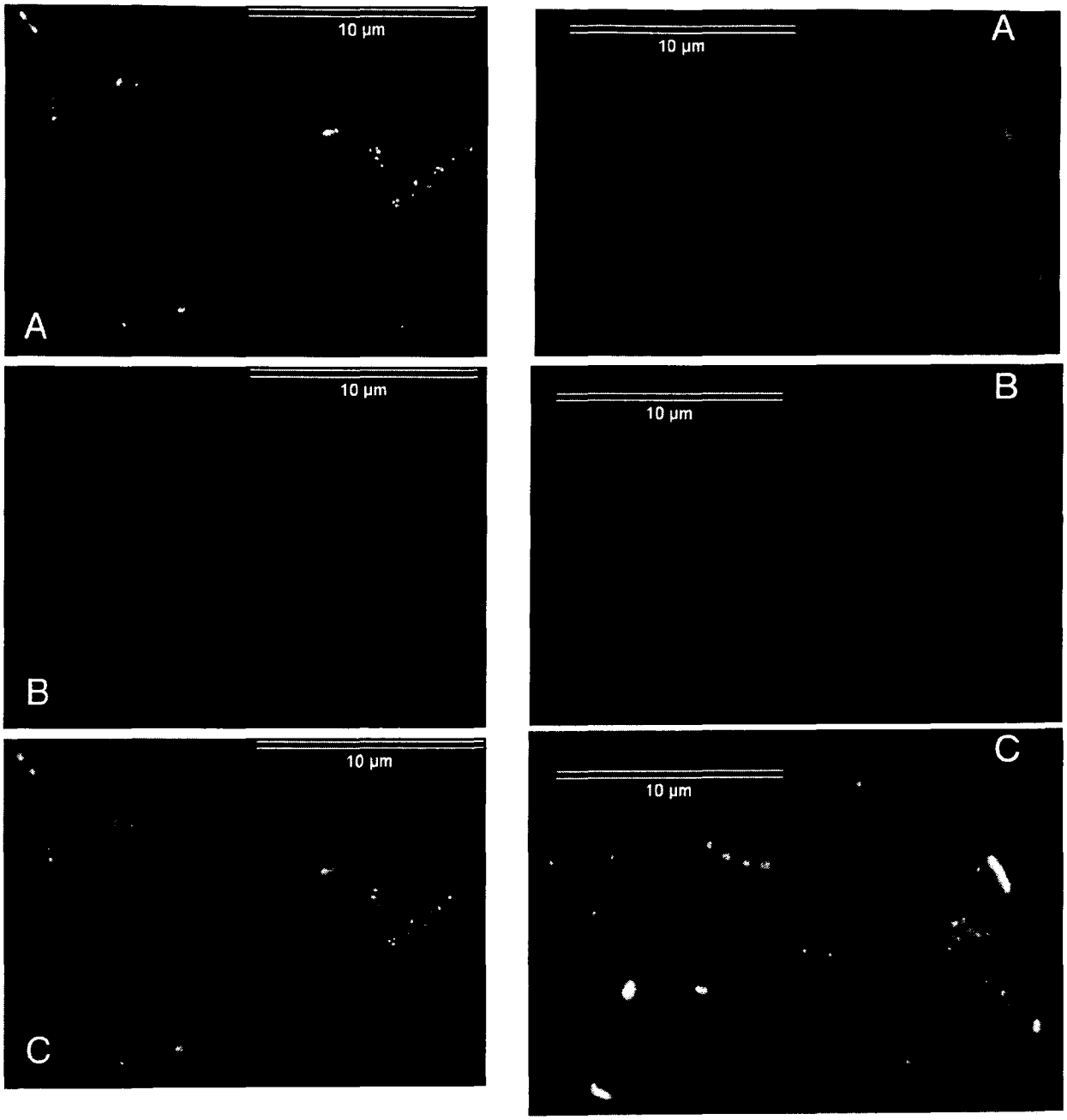

Figure 2

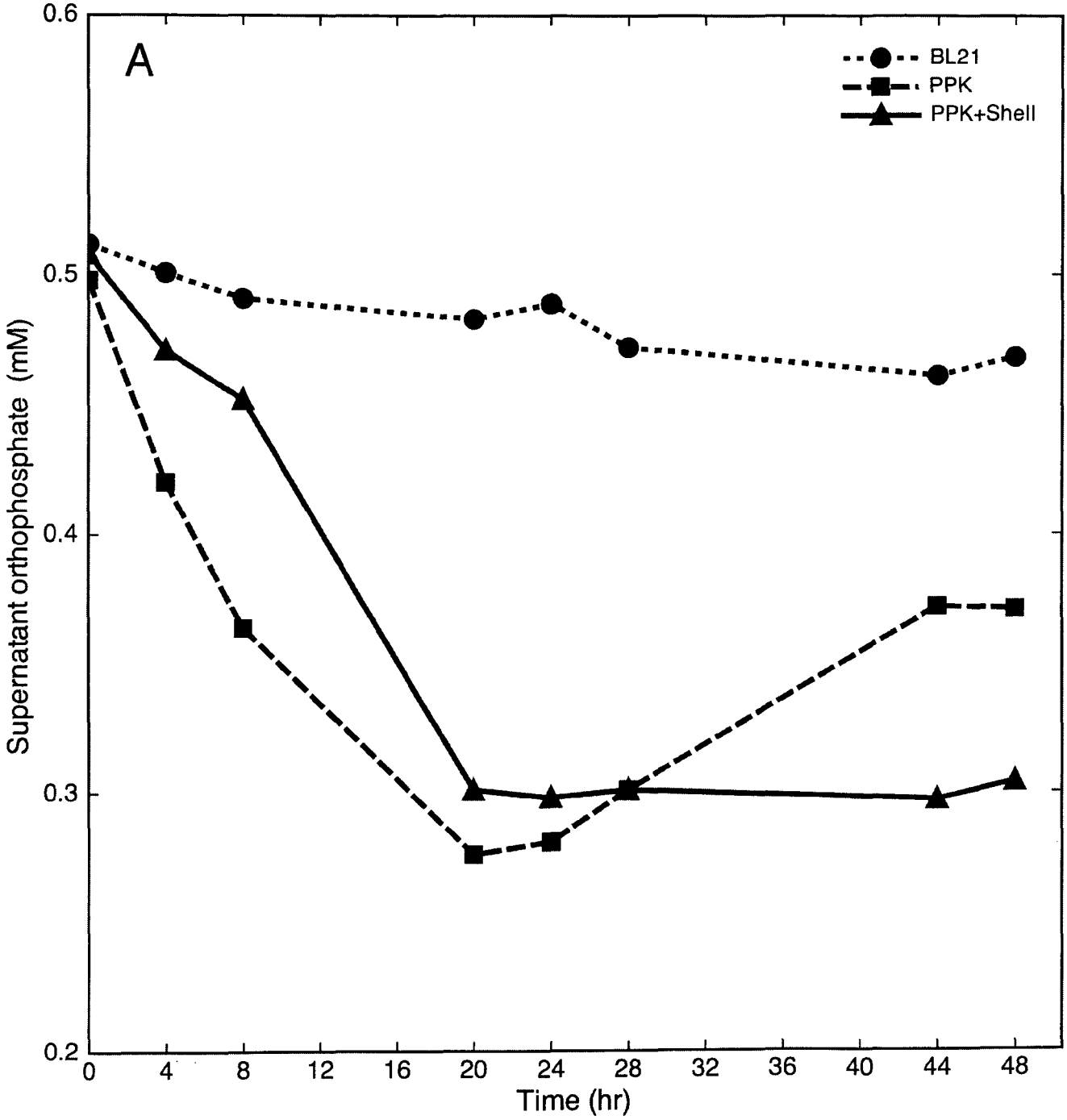
A
BL21
PPK
PPK+Shell
Supernatant orthophosphate (mM)
0.6
0.5
0.4
0.3
0.2
0
4
8
12
16
20
24
28
32
36
40
44
48
Time (hr)

Figure 3A

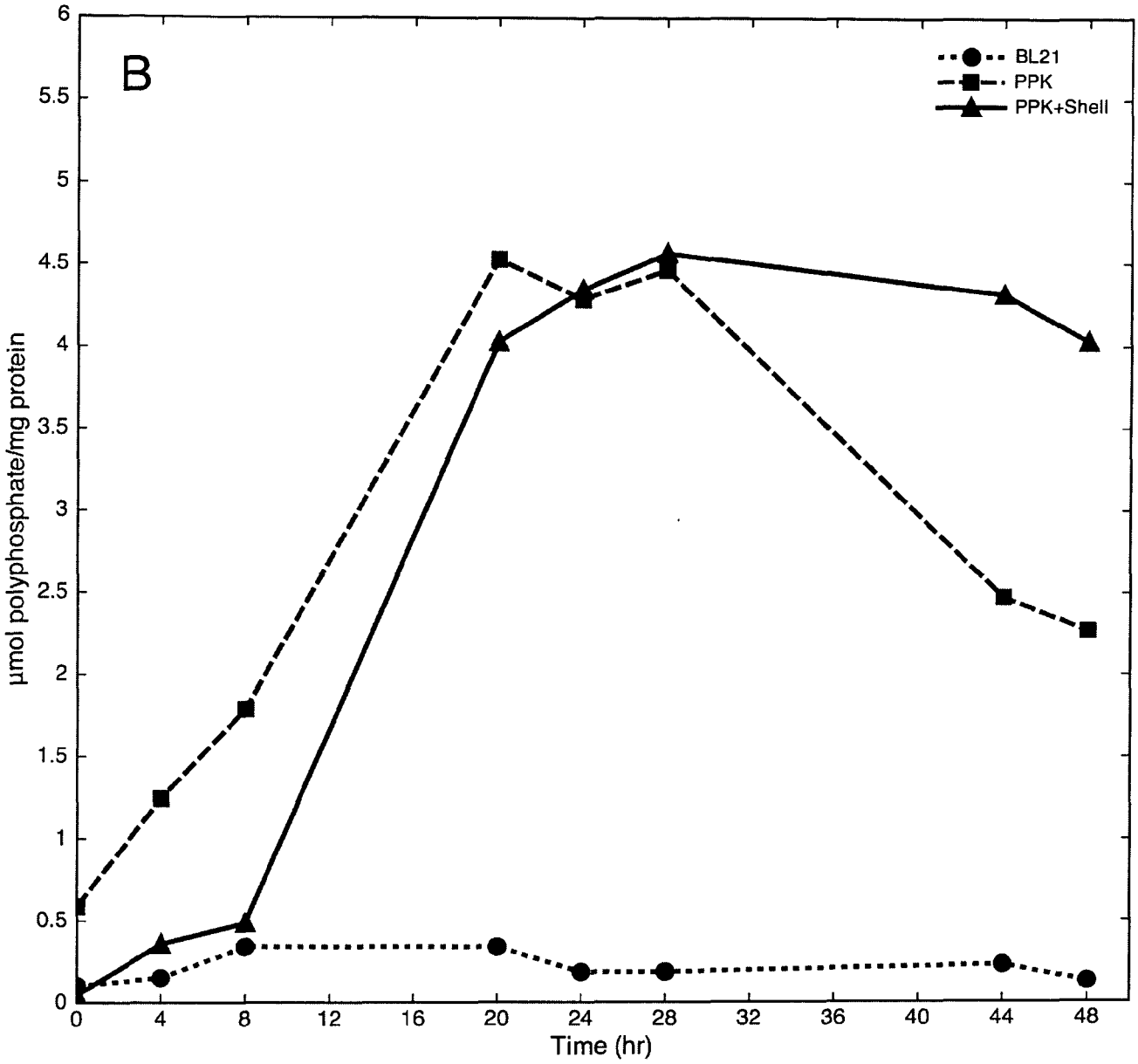
B
BL21
PPK
PPK+Shell
µmol polyphosphate/mg protein
Time (hr)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
0
4
8
12
16
20
24
28
32
36
40
44
48

Figure 3B

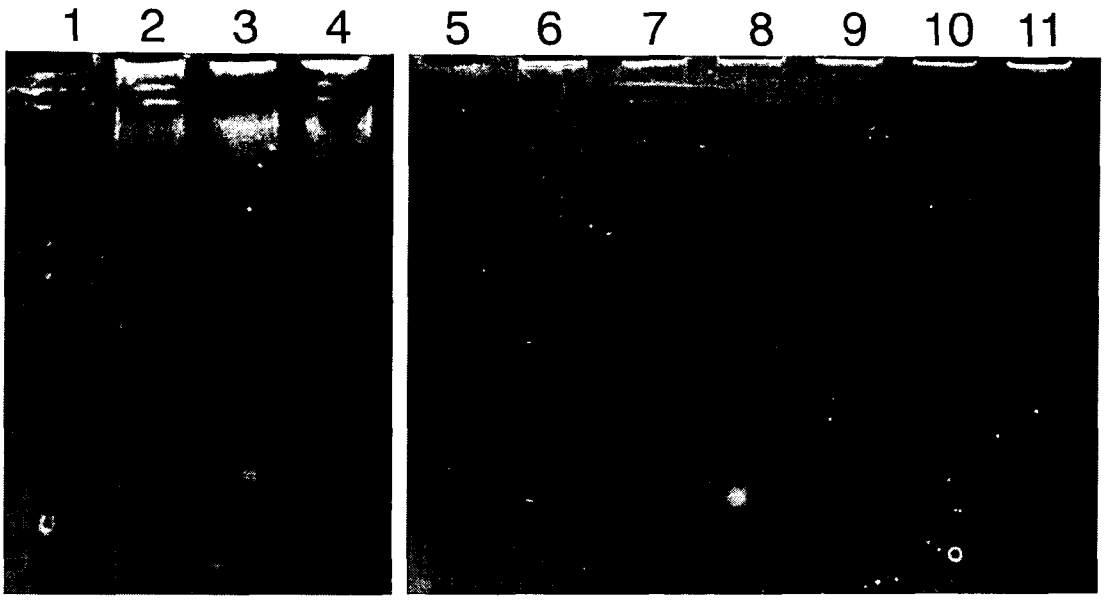

Figure 4

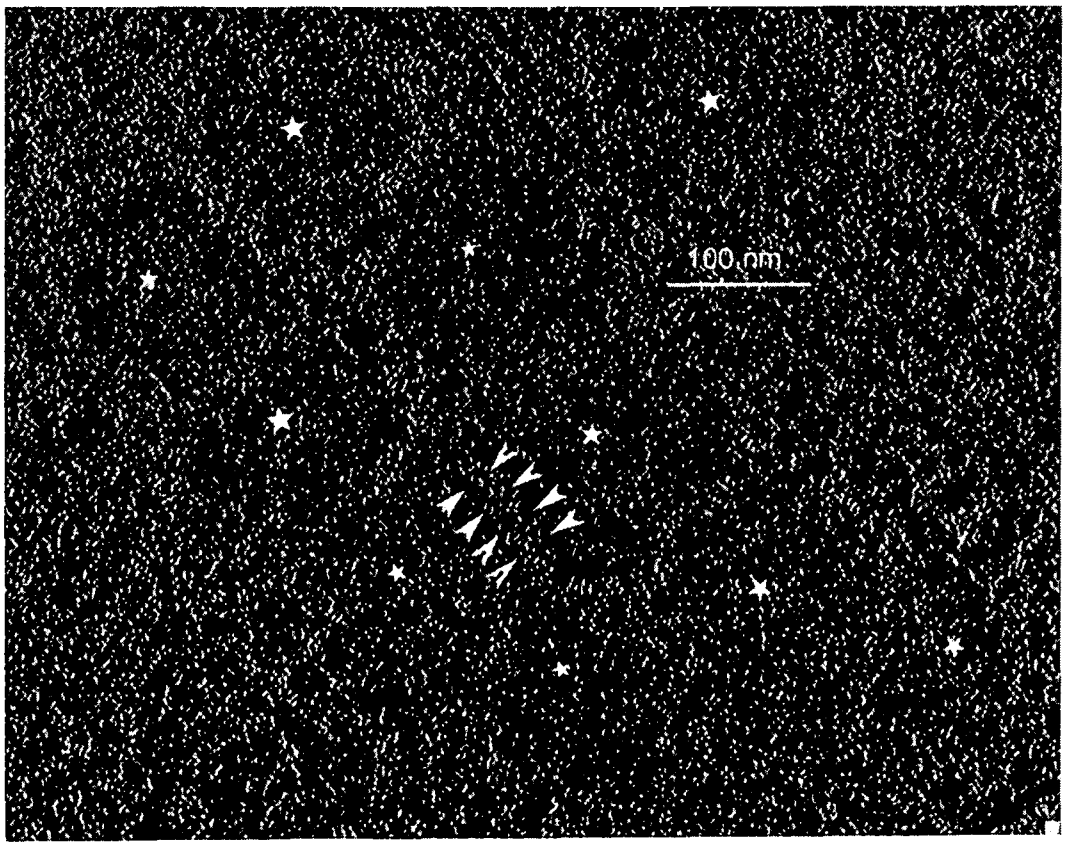

Figure 5

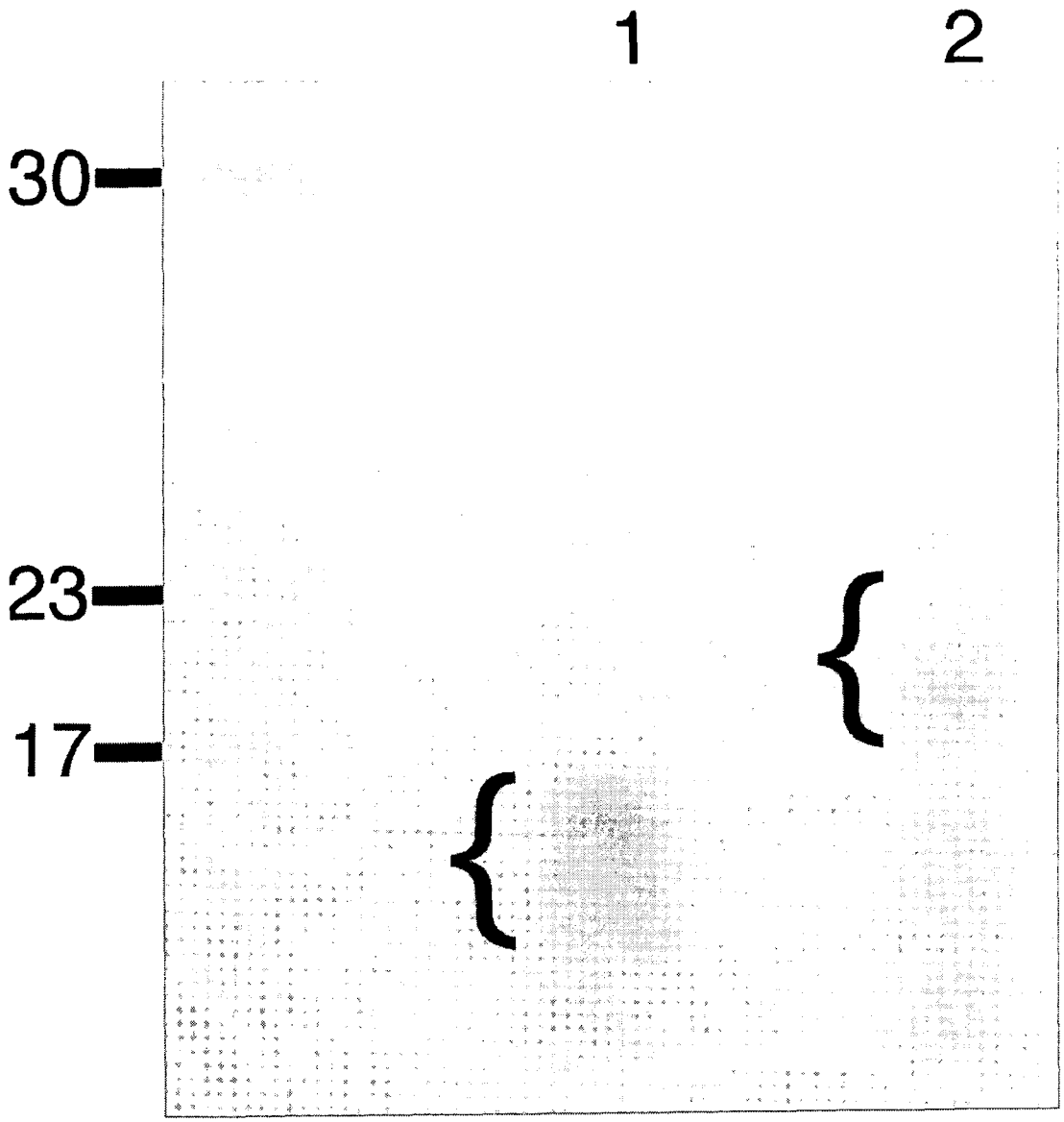

Figure 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/069106

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/195 C12N1/20 A23L1/30 A61K35/74 C02F3/30
C12N9/12
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N A23L A61K C02F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOSHUA B. PARSONS ET AL: "Synthesis of Empty Bacterial Microcompartments, Directed Organelle Protein Incorporation, and Evidence of Filament-Associated Organelle Movement", MOLECULAR CELL, vol. 38, no. 2, 1 April 2010 (2010-04-01), pages 305-315, XP055016073, ISSN: 1097-2765, DOI: 10.1016/j.molcel.2010.04.008 cited in the application the whole document ----- WO 2011/094765 A2 (UNIV CALIFORNIA [US]; KERFELD CHERYL A [US]; KINNEY JAMES N [US]) 4 August 2011 (2011-08-04) the whole document sequence 205 ----- -/--	1-36 1-36

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 2013	06/02/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Giebeler, Katharina

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2011/017458 A1 (UNIV CALIFORNIA [US]; KERFELD CHERYL A [US]; LOQUE DOMINIQUE [US]) 10 February 2011 (2011-02-10) the whole document	1-36
Y	M. LIBERTON ET AL: "Unique Thylakoid Membrane Architecture of a Unicellular N2-Fixing Cyanobacterium Revealed by Electron Tomography", PLANT PHYSIOLOGY, vol. 155, no. 4, 1 April 2011 (2011-04-01) , pages 1656-1666, XP055051494, ISSN: 0032-0889, DOI: 10.1104/pp.110.165332 the whole document introduction	1-36
Y	HONG LI ET AL: "Pattern of cyanophycin accumulation in nitrogen-fixing and non-nitrogen-fixing cyanobacteria", ARCHIVES OF MICROBIOLOGY, vol. 176, no. 1-2, 1 July 2001 (2001-07-01), pages 9-18, XP055051495, ISSN: 0302-8933, DOI: 10.1007/s002030100281 the whole document introduction	1-36
Y	MCGRATH JOHN W ET AL: "Microbial phosphate removal and polyphosphate production from wastewaters", ADVANCES IN APPLIED MICROBIOLOGY, ACADEMIC PRESS, UNITED STATES, vol. 52, 1 January 2003 (2003-01-01), pages 75-100, XP009155376, ISSN: 0065-2164 the whole document	1-36
Y	KATO ET AL: "Genetic improvement of Escherichia coli for enhanced biological removal of phosphate from wastewater.", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 59, no. 11, 1 November 1993 (1993-11-01), pages 3744-3749, XP055016536, ISSN: 0099-2240 the whole document	1-36

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	M. SEUFFERHELD ET AL: "Identification of Organelles in Bacteria Similar to Acidocalcisomes of Unicellular Eukaryotes", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 278, no. 32, 1 August 2003 (2003-08-01), pages 29971-29978, XP055016538, ISSN: 0021-9258, DOI: 10.1074/jbc.M304548200 the whole document -----	1-36
Y	ROBERTO DOCAMPO ET AL: "Acidocalcisomes", CELL CALCIUM, vol. 50, no. 2, 1 August 2011 (2011-08-01) , pages 113-119, XP055016539, ISSN: 0143-4160, DOI: 10.1016/j.ceca.2011.05.012 the whole document page 116, column 1, paragraph 2 -----	1-36
A	J. B. PARSONS ET AL: "Biochemical and Structural Insights into Bacterial Organelle Form and Biogenesis", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 283, no. 21, 1 May 2008 (2008-05-01), pages 14366-14375, XP055016541, ISSN: 0021-9258, DOI: 10.1074/jbc.M709214200 cited in the application the whole document -----	1-36

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/069106

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011094765 A2	04-08-2011	NONE	
WO 2011017458 A1	10-02-2011	US 2012210459 A1	16-08-2012
		WO 2011017458 A1	10-02-2011