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Deciphering the Genome of Polyphosphate Accumulating Actinobacterium *Microlunatus phosphovorus*

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Abstract

Polyphosphate accumulating organisms (PAOs) belong mostly to *Proteobacteria* and *Actinobacteria* and are quite divergent. Under aerobic conditions, they accumulate intracellular polyphosphate (polyP), while they typically synthesize polyhydroxyalkanoates (PHAs) under anaerobic conditions. Many ecological, physiological, and genomic analyses have been performed with proteobacterial PAOs, but few with actinobacterial PAOs. In this study, the whole genome sequence of an actinobacterial PAO, *Microlunatus phosphovorus* NM-1^T (NBRC 101784^T), was determined. The number of genes for polyP metabolism was greater in *M. phosphovorus* than in other actinobacteria; it possesses genes for four polyP kinases (*ppk*s), two polyP-dependent glucokinases (*ppgk*s), and three phosphate transporters (*pit*s). In contrast, it harbours only a single *ppx* gene for exopolyphosphatase, although two copies of *ppx* are generally present in other actinobacteria. Furthermore, *M. phosphovorus* lacks the *phaABC* genes for PHA synthesis and the *actP* gene encoding an acetate/H^+ symporter, both of which play crucial roles in anaerobic PHA accumulation in proteobacterial PAOs. Thus, while the general features of *M. phosphovorus* regarding aerobic polyP accumulation are similar to those of proteobacterial PAOs, its anaerobic polyP use and PHA synthesis appear to be different.

Key words: *Microlunatus phosphovorus*; whole genome sequence; polyphosphate accumulating organism; polyphosphate; polyhydroxyalkanoate

1. Introduction

Economically exploitable phosphate rock, the major source of industrial phosphorus, is estimated to be depleted in 50–100 years.[1,2] Nevertheless, wasted phosphorus is rarely reused and, to make matters worse, can induce eutrophication in the surrounding water. Polyphosphate accumulating organisms (PAOs) are expected to help solve these problems. PAOs are frequently found in activated sludges in the enhanced biological phosphate removal (EBPR) process, where they are believed to play a pivotal role in phosphorus removal from the wastewater.[3] The EBPR process is also attracting interest for its potential use in phosphorus recycling.[4,5] In the EBPR process, PAOs take up phosphate into the cells and accumulate it as polyphosphate (polyP) under aerobic conditions. In addition, under subsequent anaerobic conditions, PAOs in such sludges are thought to accumulate polyhydroxyalkanoates (PHAs), at the expense of

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polyP hydrolysis,[3] using volatile fatty acids such as acetate as substrates.

To date, the features of PAOs have been studied mainly in bacterial communities,[6–8] because few such microorganisms have been successfully isolated from EBPR sludge. Proteobacteria and/or actinobacteria are frequently observed in activated EBPR sludges, and several proteobacteria, e.g. *Acinetobacter* spp. and *Lampropedia* spp., have been isolated, although their metabolic or morphological characteristics differ from those typically observed in activated sludges.[3] Besides these bacterial isolates, an unculturable proteobacterium, '*Candidatus* Accumulibacter phosphatis', is regarded as a typical PAO based on its PolyP and PHA accumulation properties.[7] Because '*Ca*. Accumulibacter phosphatis' can predominate in an EBPR community fed with acetate or propionate, such sludges have been used for metagenomic and metaproteomic analyses.[9–11] In this way, the molecular information of polyP accumulating proteobacteria has gradually been accumulating.

Less is known about the cellular and molecular features of actinobacterial PAOs. Two species, *Microlunatus phosphovorus* and *Tetrasphaera elongata*, have been isolated from EBPR-activated sludges as candidate PAOs.[12–15] These actinobacteria aerobically accumulate polyP in their cells, as do proteobacterial PAOs. *Microlunatus phosphovorus*, in particular, accumulates substantially more polyP (>10% of cell mass as phosphorus on a dry weight basis) than *T. elongata* (<1%) and other proteobacterial PAOs. Unlike proteobacterial PAOs, these candidates do not release phosphate when fed with acetate.[12,15,16] Instead, glucose and mixed substrates (acetate, casamino acids, and yeast extract) induce phosphate release in *M. phosphovorus* and *T. elongata*, respectively. Although PHA synthesis in *M. phosphovorus* had not been observed for over a decade from its first isolation, Aker *et al.*[17] recently demonstrated the presence of PHA in *M. phosphovorus* cells using PHA staining and gas chromatography, suggesting the existence of some metabolic systems for PHA production.

In the present study, we determined the complete nucleotide sequence of the *M. phosphovorus* NM-1^T (NBRC 101784^T) genome. We put particular focus on (i) polyP synthesis and degradation, (ii) polyP transport, (iii) retention of polyP granules (volutin granules), and (iv) PHA synthesis, which are all considered to be essential traits of a typical PAO. Very recently, the whole genome sequence of '*Ca.* Accumulibacter phosphatis' was made available (INSD accession number: CP001715). We discuss similarities and differences between these two genome-sequenced organisms. This report is the first detailed analysis of the whole genome sequences of PAOs.

2. Materials and Methods

2.1. Genome sequencing, assembly, and gap closure

The complete genome sequence of *M. phosphovorus* NM-1^T (NBRC 101784^T) was determined using a conventional whole genome shotgun strategy, as described previously.[18] Shotgun libraries with average insert sizes of 1.5 and 6.0 kb were constructed in pUC118 vector (TaKaRa, Kyoto, Japan), and a fosmid library with an average insert size of 35 kb was constructed in pCC1FOS vector. Plasmid and fosmid clones were end-sequenced using dye-terminator chemistry on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Raw sequence data corresponding to the 8.6-fold coverage were assembled using PHRED/PHRAP/CONSED software.[19] Gaps between assembled sequences were closed either by primer walking on gap-spanning library clones or by the transposon-mediated random insertion method on bridging fosmid clones with a Template Generation System II Kit (Finnzymes, Vantaa, Finland).

2.2. Gene identification and annotation

Putative non-translated genes were identified using Rfam,[20] tRNAscan-SE,[21] and ARAGORN[22] programs. To predict protein-coding genes, GLIMMER3[23] was used. The initial set of open reading frames (ORFs) was manually selected from the predictions in combination with the similarity search results. Similarity searches against the Uniprot,[24] Interpro,[25] and HAMAP[26] databases were used for the functional prediction of ORFs. The KEGG[27] database was used for pathway reconstruction.

2.3. Data availability

The nucleotide sequence of *M. phosphovorus* NM-1^T (NBRC 101784^T) has been deposited in the INSD database with an accession number AP012204. The annotated genome sequence is also available in the genome database DOGAN (http://www.bio.nite.go.jp/dogan/project/view/MP1).

3. Results and discussion

3.1. General information

3.1.1. Genome overview The genome consisted of a single circular chromosome of 5 683 123 bp with an average G + C content of 67.3% (Fig. 1). No plasmid DNA sequence was detected. The chromosome was predicted to encode 5360 protein-coding genes, 46 transfer RNA genes, and a set of ribosomal RNA genes (16S, 23S, and 5S). Of the 5360 predicted protein-coding genes, 4887 (91%) were orthologous

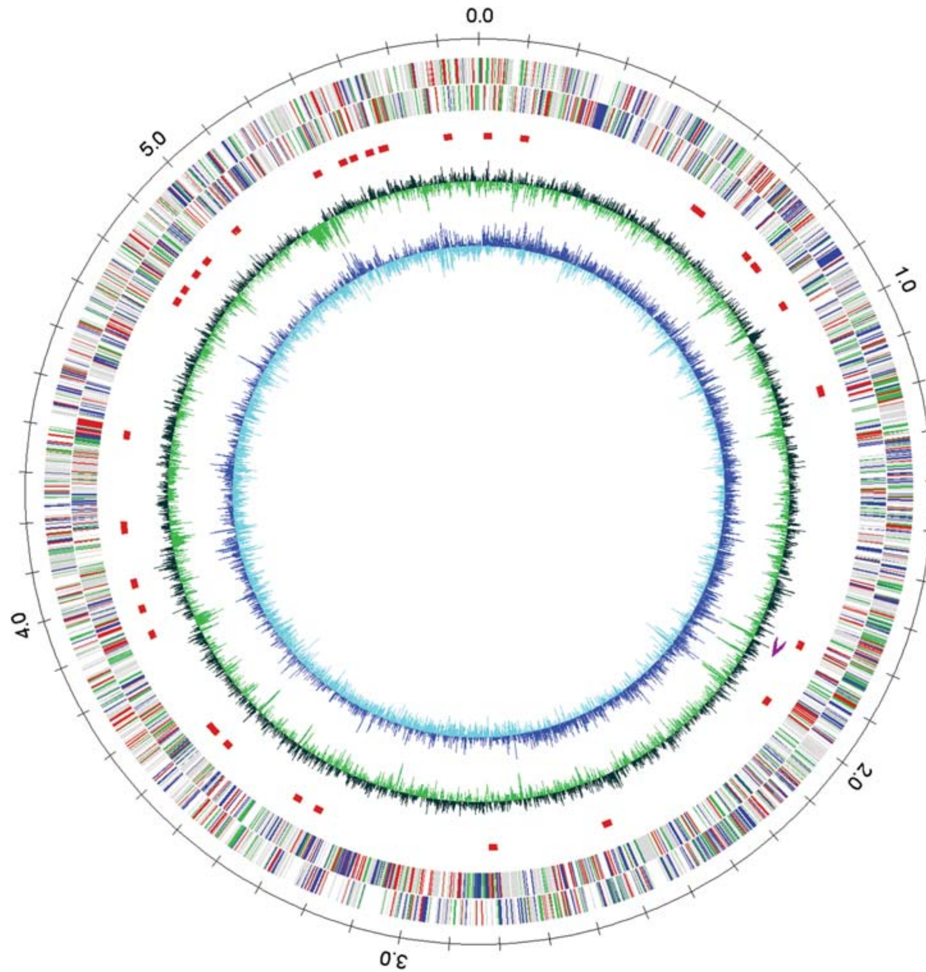

Figure 1. Schematic representation of the circular chromosome of *M. phosphovorus*. From the periphery toward the centre, circles indicate the scale in Mbp, ORFs predicted on the forward and reverse strands, tRNA genes, rRNA operon, G + C contents, and GC skew.

or had similarity to genes of known function or to hypothetical genes (*E*-value of <0.001), and the remaining 473 (9%) showed no significant similarity to any registered genes. After manual curation, 1999 (37%) genes could be assigned to known biological roles. *Microlunatus phosphovorus* harboured only a single rRNA operon, a significant finding given its relatively large genome. The general features of the *M. phosphovorus* genome are compared with those of 14 other actinobacterial species in Supplementary Table S1. In this comparison, target genomes were selected to represent each taxonomic rank from the genus *Microlunatus* stepwise to the class *Actinobacteria*. Supplementary Table S2 shows the distribution of ORFs in COG functional categories. No significant overrepresentation or underrepresentation of any particular functional category in *M. phosphovorus* was observed except that a higher percentage of ORFs was categorised as 'function unknown' in *M. phosphovorus* than in other actinobacteria.

3.1.2. Adaptation in anaerobic conditions *Microlunatus phosphovorus* NM-1^T is an obligately aerobic chemoorganotroph but can grow anaerobically if nitrate is added to the medium as an electron acceptor.[13] Consistent with this observation, we found a gene cluster that putatively encodes subunits of the membrane-bound respiratory nitrate reductase, NarG (MLP_46640), NarH (MLP_46650), NarJ (MLP_46660), and NarI (MLP_46670), linked to a NarK-type nitrate/nitrite transporter gene (MLP_46680). In addition, we found a gene that encodes another NarK-type nitrate/nitrite transporter (MLP_35250) located the downstream of putative assimilatory nitrite reductase genes (MLP_35260 and MLP_35270). These components together may constitute a system for nitrate respiration in anaerobic conditions as well as for nitrogen assimilation. Under anaerobic conditions, *M. phosphovorus* NM-1^T was reported to generate acetate from glucose, suggesting the presence of an acetate fermentation system.[28] In support of previous experimental data,[28] we found

putative genes, *pta-ackA* (MLP_01330 and MLP_01320), encoding phosphate acetyltransferase and acetate kinase, which usually ferment acetate in bacteria. Furthermore, putative *pflBA* genes (MLP_33410 and MLP_33420), which are necessary for the synthesis and activation of pyruvate formate lyase, a central enzyme in anaerobic glucose metabolism, were found in the *M. phosphovorus* genome. Because *pflBA* genes are present in few lineages of actinobacteria, and because an IS-like sequence, a putative transposase gene flanked by 17-bp inverted repeat sequences, was present just upstream of the *pflBA* genes, *pflBA* could have been acquired by horizontal gene transfer.

3.1.3. Substrates for growth Metabolic pathways reconstructed in this study were roughly in accordance with previous studies on nutrient use in *M. phosphovorus* NM-1^{T} (Supplementary Table S3).[13,29–31] However, while lactose and malate were predicted to be used as nutrients based on the genome information, the growth of *M. phosphovorus* NM-1^{T} on these nutrients has not been observed. On the other hand, while growth has been experimentally shown on mannose, galactose, N-acetyl-D-glucosamine, sorbose, salicin, p-arbutin, dulcitol, and adonitol, metabolic pathways for the use of these compounds were either missing or incomplete. Other substrates that have not yet been experimentally investigated, such as formate and butyrate, might also be used based on the predicted pathways. Butyrate metabolism may be advantageous for PAOs because butyrate, as well as acetate, could be a major fatty acid available in sewage water, and butyrate was consumed when nitrate is added under anaerobic conditions in an activated sludge sample.[32]

3.2. Predicted features as a PAO

3.2.1. PolyP metabolism As described above, *M. phosphovorus* NM-1^{T} aerobically accumulates polyP in its cells, and phosphorus can exceed 10% of the cell mass on a dry weight basis.[13] In addition, the rate of phosphorus release under anaerobic conditions is significantly higher in *M. phosphovorus* NM-1^{T} than in any other isolated PAO candidate.[12,28,33–35] The following sections describe the gene products that were implicated in the accumulation, degradation, and high turnover rate of polyP in *M. phosphovorus*.

3.2.1.1. PolyP kinase PolyP kinase (PPK) catalyses the transfer of phosphate between nucleoside phosphates and polyP. There are three main subtypes of PPK, PPK1, PPK2, and polyP-dependent AMP phosphotransferase (PAP), and they are present in a wide variety of bacterial species.[36] Even though the reaction catalysed by PPK is reversible, PPK1 favours polyP synthesis with nucleoside triphosphates as phosphate donors, and thus PPK1 is recognized to play a principal role in polyP accumulation. On the other hand, PAP favours polyP hydrolysis. The PPK2 subtype also catalyses polyP hydrolysis, but the dominance of either kinase or phosphatase activity varies in different actinobacterial species; *Corynebacterium glutamicum* PPK2 is a polyP kinase and *Mycobacterium tuberculosis* PPK2 is a polyP phosphatase.[37–39]

Four putative PPK genes were identified in the *M. phosphovorus* genome; a single *ppk1* (MLP_47700) and three *ppk2* (MLP_05750, MLP_50300, and MLP_23310) homologues. The number of *ppk* homologues in *M. phosphovorus* was relatively large; usually 1–4 *ppk* homologues exist in an actinobacterial genome (Table 1). Based on the similarity of deduced amino acid sequences, one of the *M. phosphovorus* PPK2s (MLP_05750) was of the *C. glutamicum* type (63% identity), whereas the other (MLP_50300) was of the *Myc. tuberculosis* type (78% identity; Fig. 2a). The third *ppk2* homologue (MLP_23310) was relatively similar to both *ppk2* and *pap*, but was located in a distinct cluster of undetermined function (Fig. 2a).

The presence of multiple PPK genes in *M. phosphovorus* may have favoured the high polyP turnover rate and accumulating ability, although which of the four PPK homologues are polyP-synthetic or degradative has yet to be clearly distinguished. The possible importance of multiple PPK genes was supported by the genome information of '*Ca.* Accumulibacter phosphatis'. '*Ca.* Accumulibacter phosphatis' harbours multiple putative PPK genes, a *ppk1*, four *ppk2*, and a *pap*, while proteobacterial species usually contain only one or two *ppk* subtypes.

3.2.1.2. Exopolyphosphatase

Exopolyphosphatase (PPX) mediates the hydrolysis of the terminal phosphate of polyP.[36] Two types of PPX, PPX1 and PPX2, are present in a wide variety of bacteria and archaea.[36] Although most actinobacterial species harbour both types of PPX, only the *ppx2* homologue (MLP_44770) was found in the *M. phosphovorus* genome (Fig. 2b). *Propionibacterium acnes*, which also belongs to the family Propionibacteriaceae, does not harbour *ppx1* either. In *C. glutamicum*, which was recently shown to form volutin granules, mutational analysis showed that the amount of polyP in the cells increased when one of these genes was mutated.[40] In contrast to *M. phosphovorus*, '*Ca.* Accumulibacter phosphatis', the proteobacterial PAO, has two *ppx* homologues in its genome.

Table 1. Number of putative genes related to polyP metabolism in actinobacteria

	ppk	ppx	ppgK	pit	pstSCAB
Propionibacteriaceae					
Microlunatus phosphovorus	4	1	2	3	1
Propionibacterium acnes	3	1	1	1	1
Propionibacterineae					
Nocardioides sp. JS614	3	2	1	1	2
Actinomycetales					
Catenulispora acidiphila	3	2	0	2	2
Corynebacterium glutamicum	3	2	1	1	1
Frankia alni	3	2	1	1	1
Kineococcus radiotolerans	3	2	1	2	1
Kocuria rhizophila	2	2	1	2	1
Mycobacterium smegmatis	3	2	1	1	1
Rhodococcus erythropolis	4	2	1	2	2
Streptomyces avermitilis	2	3	1	2	1
Actinobacteridae					
Bifidobacterium longum	2	2	1	1	1
Actinobacteria (class)					
Acidimicrobium ferrooxidans	2	1	0	0	0
Cryptobacterium curtum	1	1	0	1	0
Rubrobacter xylanophilus	1	0	1	0	1

Accession numbers of genomic sequences in INSD: *Propionibacterium acnes*, AE017283; *Nocardioides* sp. JS614, CP000509; *Catenulispora acidiphila*, CP001700; *Corynebacterium glutamicum*, BX927147; *Frankia alni*, CT573213; *Kineococcus radiotolerans*, CP000750; *Kocuria rhizophila*, AP009152; *Mycobacterium smegmatis*, CP000480; *Rhodococcus erythropolis*, AP008957; *Streptomyces avermitilis*, BA000030; *Bifidobacterium longum*, CP000605; *Acidimicrobium ferrooxidans*, CP001631; *Cryptobacterium curtum*, CP001682; *Rubrobacter xylanophilus*, CP000386.

3.2.1.3. PolyP-dependent glucokinase Glucokinase is a key enzyme that catalyses phosphorylation of glucose, the first reaction of glycolysis. Glucokinase is widely present in both prokaryotes and eukaryotes.[41] In almost all organisms, glucokinase uses ATP as the sole phosphoryl donor. In some actinobacterial species, however, a paralogue of glucokinase, polyP-dependent glucokinase (PPGK), has been identified which uses polyP as the phosphoryl donor as well as ATP.[42–46] In *C. glutamicum*, western blot analysis showed that putative PPGK was localized in the volutin granules, suggesting that PPGK uses polyP from those granules.[47] A PPGK has already been identified in *M. phosphovorus* NM-1^{T}.[48] That report showed that the PPGK was strictly polyP-specific, unlike other actinobacterial PPGKs, suggesting the importance of polyP as an energy source for *M. phosphovorus*.

In the present study, three genes putatively encoding glucokinase were identified in the *M. phosphovorus* genome; one was putative ATP-dependent *glk* (MLP_41670) and another (MLP_05430) was the *ppgK* reported previously.[48] The third gene (MLP_26610) was inferred to also be *ppgK* by molecular phylogenetic analysis (Fig. 2c). Internal deletions characteristic of PPGKs, as well as other functional motifs including the possible polyP-binding site, were observed in the deduced amino acid sequence of MLP_26610 (Supplementary Fig. S1), suggesting that MLP_26610 is another *ppgK* orthologue (hereafter MLP_05430 and MLP_26610 are designated *ppgK1* and *ppgK2*, respectively). This is the first report of two *ppgK* orthologues being present in a single organism (Table 1). In the presence of glucose, *M. phosphovorus* may use the dual *ppgK*s for efficient glycolysis by consuming intracellular polyP, which is then followed by acetate fermentation (see the 'Adaptation in anaerobic conditions' section) to adapt to anaerobic environments. This notion agrees well with the observation that adding glucose to a pure culture of *M. phosphovorus* NM-1^{T} under anaerobic conditions resulted in a rapid release of Pi from the cells.[12,16]

3.2.2. Phosphate transport systems For rapid polyP metabolism, *M. phosphovorus* must have efficient phosphate uptake and release. In bacteria, phosphate uptake commonly occurs via the phosphate-specific transport (Pst) and phosphate inorganic transport (Pit) systems.[49] The Pst is an ATP-binding cassette (ABC) transporter, encoded by the gene cluster *pstSCAB*, for active Pit, while the Pit is a symporter of divalent metal-chelated phosphate ($MeHPO_4$) and H^+. In some Gram-negative bacteria, another ABC transporter, PhnCDE, has been identified.[50–52] Recently, the PhnCDE transport system was also identified in an actinobacterium, *Mycobacterium smegmatis*,[53] although the system is not commonly present in this phylum.

A putative *pstSCAB* (MLP_47720, MLP_47730, MLP_47740, and MLP_47750) and three *pit*s (MLP_00530, MLP_29830, and MLP_51060) were identified in the *M. phosphovorus* genome. Notably, the number of *pit* genes in *M. phosphovorus* was among the largest known in an actinobacterial species (Table 1). Based on the molecular phylogenetic analysis, MLP_29830 and MLP_51060 were most homologous to those in actinobacteria (Fig. 2d). On the other hand, MLP_00530 seemed not to be an actinobacterial gene but rather was similar to proteobacterial genes (Fig. 2d) with a maximum amino acid identity of 48%. This might suggest that MLP_00530

Figure 2. Molecular phylogenetic analyses of the (A) polyP kinase family (PPK1, PPK2 and PAP) in bacteria, (B) exopolyphosphatase family (PPX1 and PPX2) in actinobacteria, (C) glucokinase family (PPGK and GK) in prokaryotes, with some eukaryotes, and (D) phosphate transporters (Pit) in actinobacteria and proteobacteria. Trees were constructed using the distance matrix method in ClustalX. Amino acid sequences deduced from nucleotide sequences were used as operational taxonomic units (OTUs) for the analyses. All topologies agreed with those of maximum parsimony analyses in PHYLIP. The numbers on interior nodes are bootstrap percentages. Single and double asterisks indicate OTUs in which the polyP-synthetic or degradative reaction is dominant, respectively, and the abbreviation 'U. C.' indicates a cluster for which the function has yet to be characterised.

was obtained from another phylum, such as *Proteobacteria*, although an upstream gene with a putative regulatory function (Pfam: PF01865), which is commonly associated with proteobacterial Pit genes, could not be found in the case of MLP_00530.

Thus far, the Pit system has not been much studied in Gram-positive bacteria. Rather, it appears not to be mandatory in the group; *pit* in *Myc. smegmatis* was dispensable, with the Pst and Phn systems suggested to compensate for the function of Pit,[54] and some actinobacteria do not harbour *pit* in their genomes (Table 1). The reversible Pi transport mediated by Pit without consuming ATP may be favourable to cope with drastic changes in intracellular Pi concentration

due to rapid polyP synthesis and degradation in PAO. The possible importance for PAOs of multiple *pit* genes is supported by the fact that the genome of '*Ca.* Accumulibacter phosphatis' harbours four *pit* homologues, more than other proteobacterial species.

3.2.3. Retention of volutin granules To accumulate a large amount of intracellular polyP, polyP must be stably maintained as volutin granules. Although molecular mechanisms of volutin granule formation have not been clarified in detail, polyamines were suggested to play a role in polyP accumulation in *Escherichia coli*.[55] Polyamines mainly consist of putrescine, spermidine, and spermine in eukaryotic cells, whereas spermine is contained in only a few prokaryotic species, including some actinobacteria.[56] Polyamines are aliphatic amines that are highly-charged cations under physiological conditions. They are generally recognized as essential for the maintenance of cell growth and macromolecular biosynthesis by interacting with nucleic acids, proteins, and membranes. In addition, Motomura *et al.*[55] demonstrated that intracellular polyP increased after adding spermidine and putrescine to *E. coli* cells in which polyamine synthesis genes had been disrupted. They further demonstrated *in vitro* that adding polyamines to volutin granule-containing solutions increased the retention time of the granules and that spermidine was the best polyamine for stabilizing the granules.

Polyamines are synthesized from L-arginine or L-ornithine via reactions catalysed by arginine decarboxylase (SpeA) and agmatine ureohydrolase (SpeB) or ornithine decarboxylase (SpeC) (Fig. 3). The synthesized putrescine is then converted to spermidine and spermine in the reactions catalysed by spermidine synthase (SpeE) and spermine synthase, respectively, although spermine synthase has not been found in prokaryotes thus far.[56] In the *M. phosphovorus* genome, putative *speA* (MLP_07520) and *speB* (MLP_15750) were identified but did not form a gene cluster. Although *speB* is conserved among actinobacteria, *speA* or its homologues are rarely identified in actinobacteria. In addition, similarities of

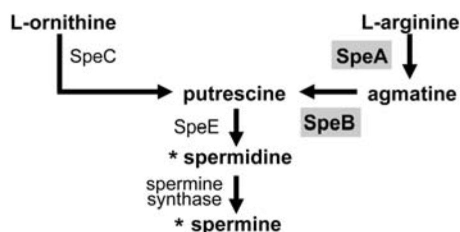

Figure 3. Polyamine metabolism and related enzymes. Shadows indicate enzymes for which putative genes were predicted in the *M. phosphovorus* genome. Polyamine species that were mainly detected in *M. phosphovorus* cells[57] are indicated by asterisks.

M. phosphovorus speA to non-actinobacterial genes are modest, with amino acid identities less than 33%, obscuring the exact origin of this gene. In contrast, the homologue of *speE*, which is usually present in actinobacteria, was not identified in the *M. phosphovorus* genome.

Busse and Schumann[57] reported that spermidine and spermine were the major polyamines in the cells of *M. phosphovorus* NM-1^T, while only a trace amount of putrescine was found. This result contradicts the prediction from the genomic data, i.e. *M. phosphovorus* can synthesize only putrescine among the three polyamines, as far as the currently recognized metabolic pathway (Fig. 3) is taken into account. Perhaps spermidine and/or spermine are synthesized via unknown synthases or taken up actively by polyamine transporters. *M. phosphovorus* harbours eight putative genes whose deduced amino acid sequences contain the amino acid/polyamine transporter I motif (InterPro ID: IPR002293). These transporters may be related to the stabilization of volutin granules, although their substrate specificities could not be clearly assigned.

3.2.4. PHA synthesis In addition to aerobic polyP accumulation, anaerobic PHA production is a recognized feature of PAOs.[3] Recently, Aker *et al.*[17] reported the detection in *M. phosphovorus* NM-1^T cells of two PHA species, polyhydroxybutyrate and polyhydroxyvalerate, by Sudan Black B- and Safranin O-staining and gas chromatography methods. However, the system of PHA production in *M. phosphovorus* appears to be different from that proposed in proteobacterial PAOs; *M. phosphovorus* apparently produces PHA under aerobic conditions, whereas proteobacterial PAOs are believed to synthesize PHAs anaerobically. In proteobacterial PAOs, PHA is produced using glycogen/glucose or volatile fatty acids such as acetate as substrates, and the uptake of the acetate is conducted via ActP, a proteobacteria-specific acetate/H^+ symporter. Acetate uptake through ActP is hypothesized to be counterbalanced by Pi release via Pit using the proton motive force.[58,59] The intermediate acetyl-CoA is then converted to PHAs by acetyl-CoA acetyltransferase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHA-synthase (PhaC) (Fig. 4).[60] This model was further supported in a proteobacterial PAO, '*Ca.* Accumulibacter phosphatis', by metagenomic and metaproteomic analyses.[9,11] In contrast, neither *actP* nor *phaABC* exists in most actinobacteria, and these genes were not found in the *M. phosphovorus* genome either.

In addition to the Pha system, other pathways derived from the β-oxidation pathway proposed in *E. coli* and *Pseudomonas putida* could synthesize PHA (Fig. 4).[61–63] In *E. coli*, the gene cluster *yfcYX* is

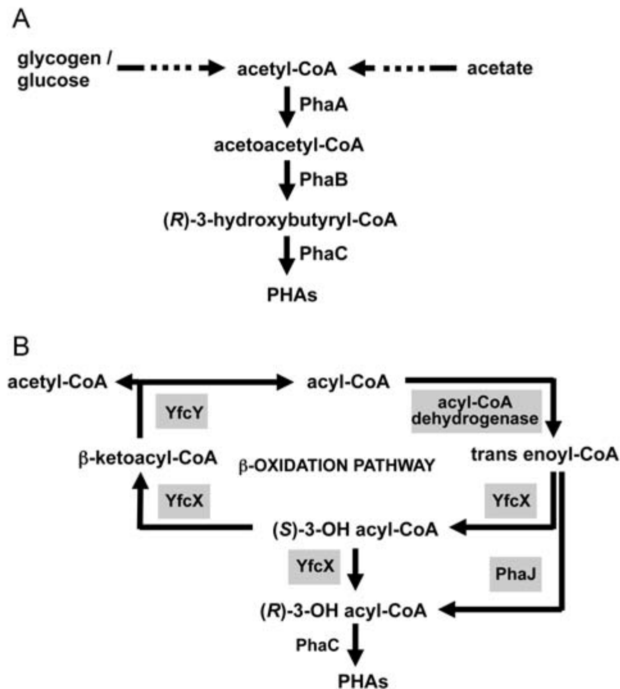

Figure 4. Two independent pathways for PHA synthesis through (A) the PhaABC system, and (B) the β-oxidation pathway. Enzymes for which putative genes were predicted in the *M. phosphovorus* genome are shadowed.

related to the pathway; the encoded YfcX was demonstrated to be a multifunctional protein with enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and putative 3-hydroxyacyl-CoA epimerase activities, and YfcY to be a β-ketothiolase. In *P. putida*, (*R*)-specific enoyl-CoA hydrolase (PhaJ) is involved in the system. Both YfcX and PhaJ result in the synthesis of (*R*)-3-hydroxyacyl-CoA, the monomer unit of PHAs. In the *M. phosphovorus* genome, homologues of *yfcYX* (MLP_23080 and MLP_23090) and *phaJ* (MLP_12780) were identified, suggesting that these genes might produce PHA in *M. phosphovorus* rather than the PhaABC system proposed in proteobacterial PAOs. There remains an unresolved issue; both pathways require PhaC for the final polymerization reaction, but the gene is not present in the *M. phosphovorus* genome. Novel unidentified PHA synthase(s) might exist in *M. phosphovorus* that enable it to synthesize PHAs via a pathway independent of polyP degradation and distinct from that conventionally proposed in proteobacterial PAOs.

3.2.5. A possible model for an actinobacterial PAO Based on the genome analysis as described in this report and previous experimental observations, possible pathways of *M. phosphovorus* that may represent the features of an actinobacterial PAO were summarized in Fig. 5 (upper panels) together with a model proposed in proteobacterial species (lower panels).[4,9,11] Under aerobic conditions, *M. phosphovorus* takes up Pi through a PstSCAB and multiple Pits. PhnCDE rarely exists in non-proteobacteria and is absent in *M. phosphovorus*. For Pi uptake via Pits, a proton motive force is required. In '*Ca.* Accumulibacter phosphatis', the electron transport chain of aerobic respiration was proposed for that function[11] and may be the primary source of the proton motive force in aerobic *M. phosphovorus*, as well. The ingested intracellular Pi is then polymerized into polyP by some of the multiple PPK(s) and stored as volutin granules. In *M. phosphovorus*, PHA may be synthesized through the β-oxidation pathway, whereas proteobacteria degrade PHA and utilize it as an energy source under the aerobic conditions.

Under anaerobic conditions, polyP is degraded by other PPK(s), a PPX2 and two PPGKs. Glucose-6-phosphate generated by PPGKs is possibly fed into glycolysis followed by acetate fermentation, and the NADH produced in the process can be used for nitrate respiration and nitrogen assimilation. Because PPGK has been identified only in actinobacteria thus far, this glycolytic pathway coupled with polyP consumption would be unique within this phylum. The release of Pi through Pit symporters generates a proton motive force. While the proton motive force is thought to be used for acetate uptake via ActP in proteobacterial PAOs, this model cannot be applied to non-proteobacteria, because ActP is present almost exclusively in proteobacteria. Instead, the proton motive force may be used, at least in part, for the symport of nitrate by NarK-type transporters in *M. phosphovorus*.

The mechanism of anaerobic polyP utilization in *M. phosphovorus* thus appears to be substantially different from that in proteobacterial PAOs, in contrast to the general similarities seen in aerobic polyP accumulation. In proteobacteria, the polyP degradation system is thought to be tightly linked to acetate uptake and the synthesis of PHA as an energy reservoir. The system in *M. phosphovorus*, however, does not appear to be directly linked to the synthesis of PHA, but may couple with the carbon and energy metabolism necessary for a minimal level of growth under anaerobic conditions. This might be another reason why *M. phosphovorus* can accumulate much larger amounts of polyP under the alternating aerobic/anaerobic conditions in the EBPR process.

3.3. Future perspectives

In the present study, we found genetic features in the *M. phosphovorus* genome that could allow it to express phenotypic characteristics of a PAO. Some of these features, such as the high multiplicity of *ppk* and *pit* genes, were commonly seen in the genome of a proteobacterial PAO candidate, '*Ca.*

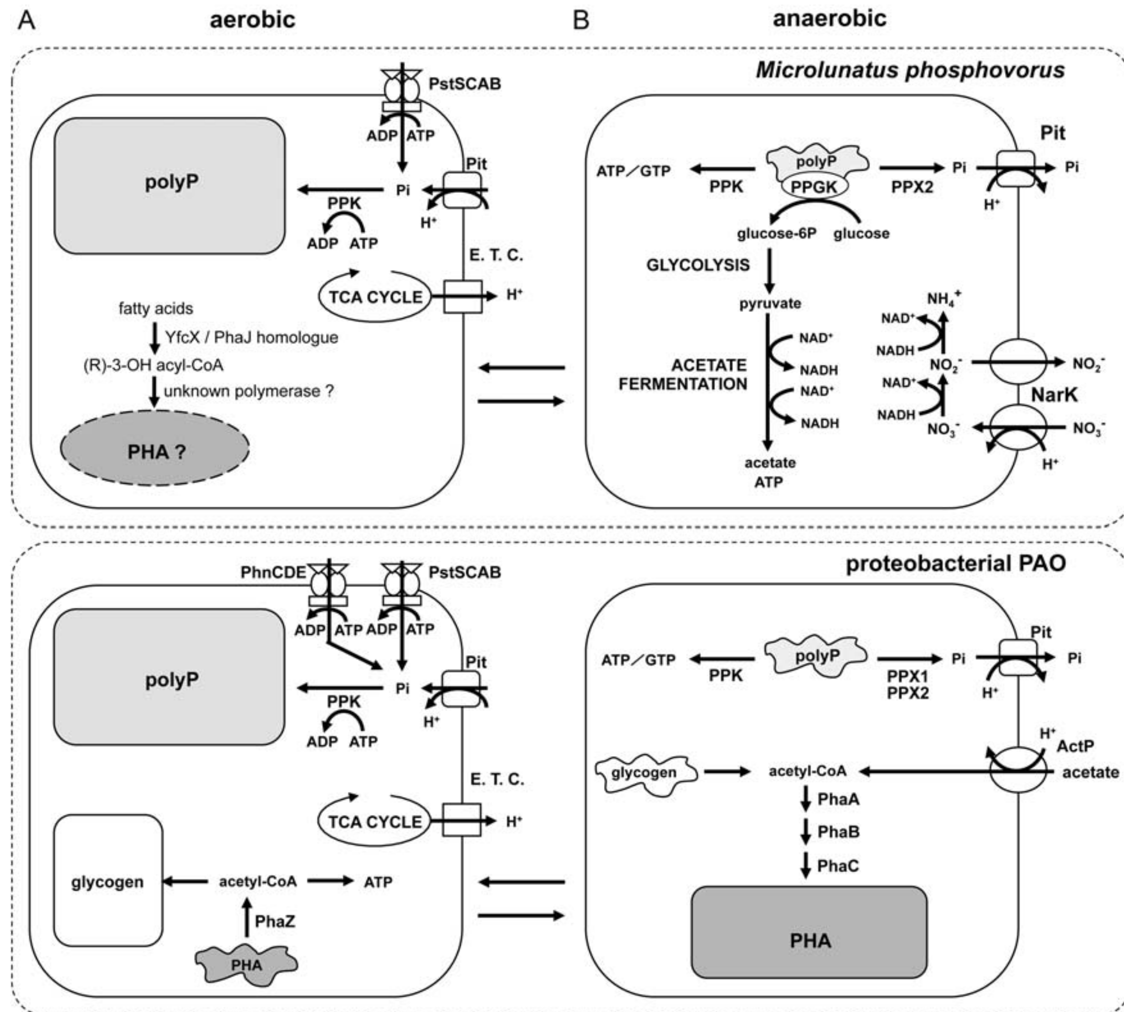

Figure 5. Schematic representation of possible pathways for polyP and PHA metabolisms under (A) aerobic and (B) anaerobic conditions. The upper panels in each figure represent models proposed in this study for *M. phosphovorus*, while the lower ones represent models recognized in proteobacterial species. Not all intermediate reactions are shown in detail.

Accumulibacter phosphatis', supporting the importance of these genes for polyP metabolisms. On the other hand, some other features, such as the presence of duplicated *ppgk* genes and the unique absence of one type of *ppx* gene, were specific to the *M. phosphovorus* genome. Yet to be elucidated, however, is whether other factors, such as expression levels and activities of these gene products or their differential regulation, would also affect the PAO phenotype.

Genes possibly related to polyP accumulation were widely dispersed across the genome of *M. phosphovorus*, and molecular phylogenetic analyses suggested that some had exogenous origins. This tendency was also seen in the '*Ca*. Accumulibacter phosphatis' genome (data not shown). In support of the high plasticity of *M. phosphovorus* genome, the number of genes whose predicted protein product had at least one Pfam domain related to transposase, integrase, or recombinase was highest in *M. phosphovorus* (99 genes) among the actinobacterial species listed in Table 1. From the aspect of genome evolution, a possible ancestral genetic locus for phosphate metabolism can be seen in the genome of *Gemmatimonas aurantiaca* (DOGAN database: http://www.bio.nite.go.jp/dogan/project/view/GA1, accession number in INSD: AP009153) which was also isolated from an EBPR-activated sludge.[64] In this organism, all predicted genes related to phosphate metabolism (a *pstSCAB*, a *ppx*, a *ppk*, a *pit*, and five regulator genes) occur as a single gene cluster. Provided that the gene cluster represents an ancestral trait, PAOs may have evolved convergently in a variety of taxa via complex recombinational events including duplication, deletion, and horizontal gene transfer that occurred independently in each lineage.

Recently, five species in the genus *Microlunatus* have newly been isolated; none accumulates polyP.[29–31,65,66] Genome data for these related species and comparative genomics approaches

would provide a clearer picture of the genetic background characteristic of PAOs.

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References

1. Steen, I. 1998, Phosphorus availability in the 21st Century: management of a nonrenewable resource, *Phosphorus and Potassium*, **217**, 25–31.
2. Smil, V. 2000, Phosphorus in the environment: natural flows and human interferences, *Annu. Rev. Energ. Environ.*, **25**, 53–88.
3. Mino, T. 2000, Microbial selection of polyphosphate-accumulating bacteria in activated sludge wastewater treatment processes for enhanced biological phosphate removal, *Biochemistry*, **65**, 341–8.
4. Hirota, R., Kuroda, A., Kato, J. and Ohtake, H. 2010, Bacterial phosphate metabolism and its application to phosphorus recovery and industrial bioprocesses, *J. Biosci. Bioeng.*, **109**, 423–32.
5. Kuroda, A., Takiguchi, N., Gotanda, T., et al. 2002, A simple method to release polyphosphate from activated sludge for phosphorus reuse and recycling, *Biotechnol. Bioeng.*, **78**, 333–8.
6. Kawaharasaki, M., Tanaka, H., Kanagawa, T. and Nakamura, K. 1999, *In situ* identification of polyphosphate-accumulating bacteria in activated sludge by dual staining with rRNA-targeted oligonucleotide probes and 4′,6-diamidino-2-phenylindol (DAPI) at a polyphosphate-probing concentration, *Water Res.*, **33**, 257–65.
7. Hesselmann, R.P., Werlen, C., Hahn, D., van der Meer, J.R. and Zehnder, A.J. 1999, Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphate removal in activated sludge, *Syst. Appl. Microbiol.*, **22**, 454–65.
8. Eschenhagen, M., Schuppler, M. and Röske, I. 2003, Molecular characterization of the microbial community structure in two activated sludge systems for the advanced treatment of domestic effluents, *Water Res.*, **37**, 3224–32.
9. Wilmes, P., Wexler, M. and Bond, P.L. 2008, Metaproteomics provides functional insight into activated sludge wastewater treatment, *PLoS One*, **3**, e1778.
10. Wexler, M., Richardson, D.J. and Bond, P.L. 2009, Radiolabelled proteomics to determine differential functioning of *Accumulibacter* during the anaerobic and aerobic phases of a bioreactor operating for enhanced biological phosphorus removal, *Environ. Microbiol.*, **11**, 3029–44.
11. García-Martín, H., Ivanova, N., Kunin, V., et al. 2006, Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities, *Nat. Biotechnol.*, **24**, 1263–9.
12. Nakamura, K., Masuda, K. and Mikami, E. 1991, Isolation of a new type of polyphosphate accumulating bacterium and its phosphate removal characteristics, *J. Ferment. Bioeng.*, **4**, 258–63.
13. Nakamura, K., Hiraishi, A., Yoshimi, Y., Kawaharasaki, M., Masuda, K. and Kamagata, Y. 1995, *Microlunatus phosphovorus gen*. nov., sp. nov., a new gram-positive polyphosphate-accumulating bacterium isolated from activated sludge, *Int. J. Syst. Bacteriol.*, **45**, 17–22.
14. Hanada, S., Liu, W.T., Shintani, T., Kamagata, Y. and Nakamura, K. 2002, *Tetrasphaera elongata* sp. nov., a polyphosphate-accumulating bacterium isolated from activated sludge, *Int. J. Syst. Evol. Microbiol.*, **52**, 883–7.
15. Onda, S. and Takii, S. 2002, Isolation and characterization of a Gram-positive polyphosphate-accumulating bacterium, *J. Gen. Appl. Microbiol.*, **48**, 125–33.
16. Nakamura, K., Ishikawa, S. and Kawaharasaki, M. 1995, Phosphate uptake and release activity in immobilized polyphosphate-accumulating bacterium *Microlunatus phosphovorus* strain NM-1, *J. Ferment. Bioeng.*, **80**, 377–82.
17. Aker, A., Akkaya, E.U., Yesiladali, S.K., et al. 2006, Accumulation of polyhydroxyalkanoates by *Microlunatus phosphovorus* under various growth conditions, *J. Ind. Microbiol. Biotechnol.*, **33**, 215–320.
18. Takarada, H., Sekine, M., Kosugi, H., et al. 2008, Complete genome sequence of the soil actinomycete *Kocuria rhizophila*, *J. Bacteriol.*, **190**, 4139–46.
19. Ewing, B., Hillier, L., Wendl, M.C. and Green, P. 1998, Base-calling of automated sequencer traces using phred. I. Accuracy assessment, *Genome Res.*, **8**, 175–85.
20. Griffiths-Jones, S., Bateman, A., Marshall, M., Khanna, A. and Eddy, S.R. 2003, Rfam: an RNA family database, *Nucleic Acids Res.*, **31**, 439–41.
21. Schattner, P., Brooks, A.N. and Lowe, T.M. 2005, The tRNAscan-SE, snoscan and snoGPS web servers for the detection of tRNAs and snoRNAs, *Nucleic Acids Res.*, **33**, W686–9.
22. Laslett, D. and Canback, B. 2004, ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences, *Nucleic Acids Res.*, **32**, 11–6.
23. Delcher, A.L., Bratke, K.A., Powers, E.C. and Salzberg, S.L. 2007, Identifying bacterial genes and endosymbiont DNA with Glimmer, *Bioinformatics*, **23**, 673–9.
24. UniProt Consortium. 2008, The universal protein resource (UniProt), *Nucleic Acids Res.*, **36**, D190–5.
25. Mulder, N.J., Apweiler, R., Attwood, T.K., et al. 2007, New developments in the InterPro database, *Nucleic Acids Res.*, **35**, D224–8.
26. Lima, T., Auchincloss, A.H., Coudert, E., et al. 2009, HAMAP: a database of completely sequenced microbial proteome sets and manually curated microbial protein families in UniProtKB/Swiss-Prot, *Nucleic Acids Res.*, **37**, D471–8.

27. Kanehisa, M., Araki, M., Goto, S., et al. 2008, KEGG for linking genomes to life and the environment, *Nucleic Acids Res.*, **36**, D480–4.
28. Santos, M.M., Lemos, P.C., Reis, M.A. and Santos, H. 1999, Glucose metabolism and kinetics of phosphorus removal by the fermentative bacterium *Microlunatus phosphovorus*, *Appl. Environ. Microbiol.*, **65**, 3920–8.
29. Wang, Y.X., Cai, M., Zhi, X.Y., et al. 2008, *Microlunatus aurantiacus* sp. nov., a novel actinobacterium isolated from a rhizosphere soil sample, *Int. J. Syst. Evol. Microbiol.*, **58**, 1873–7.
30. Kämpfer, P., Young, C.C., Busse, H.J., et al. 2009, *Microlunatus soli* sp. nov., isolated from soil, *Int. J. Syst. Evol. Microbiol.*, **60**, 824–7.
31. Kämpfer, P., Schäfer, J., Lodders, N. and Martin, K. 2010, *Microlunatus parietis* sp. nov., isolated from an indoor wall, *Int. J. Syst. Evol.*, **60**, 2420–3.
32. Lee, N., Nielsen, P.H., Andreasen, K.H., et al. 1999, Combination of fluorescent in situ hybridization and microautoradiography—a new tool for structure-function analyses in microbial ecology, *Appl. Environ. Microbiol.*, **65**, 1289–97.
33. Hiraishi, A. and Morishima, Y. 1990, Capacity for polyphosphate accumulation of predominant bacteria in activated sludge showing enhanced biological phosphate removal, *J. Ferment. Bioeng.*, **69**, 368–71.
34. Stante, L., Cellamare, C., Malaspina, F., Bortone, G. and Tilche, A. 1997, Biological phosphorus removal by pure culture *Lampropedia* spp., *Water Res.*, **31**, 1317–24.
35. Tandoi, V., Majone, M., May, J. and Ramadori, R. 1998, The behaviour of phosphate accumulating *Acinetobacter* isolates in an anaerobic-aerobic chemostat, *Water Res.*, **32**, 2903–12.
36. Rao, N.N., Gómez-García, M.R. and Kornberg, A. 2009, Inorganic polyphosphate: essential for growth and survival, *Annu. Rev. Biochem.*, **78**, 605–47.
37. Zhang, H., Ishige, K. and Kornberg, A. 2002, A polyphosphate kinase (PPK2) widely conserved in bacteria, *Proc. Natl Acad. Sci. USA*, **99**, 16678–83.
38. Lindner, S.N., Vidaurre, D., Willbold, S., Schoberth, S.M. and Wendisch, V.F. 2007, NCgl2620 encodes a class II polyphosphate kinase in *Corynebacterium glutamicum*, *Appl. Environ. Microbiol.*, **73**, 5026–33.
39. Sureka, K., Sanyal, S., Basu, J. and Kundu, M. 2009, Polyphosphate kinase 2: a modulator of nucleoside diphosphate kinase activity in mycobacteria, *Mol. Microbiol.*, **74**, 1187–97.
40. Lindner, S.N., Knebel, S., Wesseling, H., Schoberth, S.M. and Wendisch, V.F. 2009, Exopolyphosphatases PPX1 and PPX2 from *Corynebacterium glutamicum*, *Appl. Environ. Microbiol.*, **75**, 3161–70.
41. Bork, P., Sander, C. and Valencia, A. 1993, Convergent evolution of similar enzymatic function on different protein folds: the hexokinase, ribokinase, and galactokinase families of sugar kinases, *Protein Sci.*, **2**, 31–40.
42. Szymona, M. 1957, Utilization of inorganic polyphosphates for phosphorylation of glucose in *Mycobacterium phlei*, *Bull. Acad. Pol. Sci. Ser. Sci. Biol.*, **5**, 379–81.
43. Szymona, M. and Szymona, O. 1961, Participation of volutin in the hexokinase reaction of *Corynebacterium diphtheriae*, *Bull. Acad. Pol. Sci. Ser. Sci. Biol.*, **9**, 371.
44. Szymona, M. and Widomski, J. 1974, A kinetic study on inorganic polyphosphate glucokinase from *Mycobacterium tuberculosis* H37Ra, *Physiol. Chem. Phys.*, **6**, 393–404.
45. Wood, H.G. and Goss, N.H. 1985, Phosphorylation enzymes of the propionic acid bacteria and the roles of ATP, inorganic pyrophosphate, and polyphosphates, *Proc. Natl Acad. Sci. USA*, **82**, 312–5.
46. Hsieh, P.C., Shenoy, B.C., Samols, D. and Phillips, N.F.B. 1996, Cloning, expression, and characterization of polyphosphate glucokinase from *Mycobacterium tuberculosis*, *J. Biol. Chem.*, **271**, 4909–15.
47. Pallerla, S.R., Knebel, S., Polen, T., et al. 2005, Formation of volutin granules in *Corynebacterium glutamicum*, *FEMS Lett.*, **243**, 133–40.
48. Tanaka, S., Lee, S.O., Hamaoka, K., et al. 2003, Strictly polyphosphate-dependent glucokinase in a polyphosphate-accumulating bacterium, *Microlunatus phosphovorus*, *J. Bacteriol.*, **185**, 5654–6.
49. van Veen, H.W. 1997, Phosphate transport in prokaryotes: molecules, mediators and mechanisms, *Antonie van Leeuwenhoek*, **72**, 299–315.
50. Metcalf, W.W. and Wanner, B.L. 1991, Involvement of the *Escherichia coli phn* (*psiD*) gene cluster in assimilation of phosphorus in the form of phosphonates, phosphite, Pi esters, and Pi, *J. Bacteriol.*, **173**, 587–600.
51. Voegele, R.T., Bardin, S. and Finan, T.M. 1997, Characterization of the *Rhizobium* (*Sinorhizobium*) *meliloti* high- and low-affinity phosphate uptake systems, *J. Bacteriol.*, **179**, 7226–32.
52. Imazu, K., Tanaka, S., Kuroda, A., Anbe, Y. and Kato, J. 1998, Enhanced utilization of phosphonate and phosphite by *Klebsiella aerogenes*, *Appl. Environ. Microbiol.*, **64**, 3754–8.
53. Gebhard, S., Tran, S.L. and Cook, G.M. 2006, The Phn system of *Mycobacterium smegmatis*: a second high-affinity ABC-transporter for phosphate, *Microbiology*, **152**, 3453–65.
54. Gebhard, S., Ekanayaka, N. and Cook, G.M. 2009, The low-affinity phosphate transporter PitA is dispensable for in vitro growth of *Mycobacterium smegmatis*, *BMC Microbiol.*, **9**, 254.
55. Motomura, K., Takiguchi, N., Ohtake, H. and Kuroda, A. 2006, Polyamines affect polyphosphate accumulation in *Escherichia coli*, *J. Environ. Biotechnol.*, **6**, 41–6.
56. Pegg, A.E. and Michael, A.J. 2010, Spermine synthase, *Cell. Mol. Life Sci.*, **67**, 113–21.
57. Busse, H.J. and Schumann, P. 1999, Polyamine profiles within genera of the class *Actinobacteria* with LL-diaminopimelic acid in the peptidoglycan, *Int. J. Syst. Bacteriol.*, **49**, 179–84.
58. Saunders, A.M., Mabbett, A.N., McEwan, A.G. and Blackall, L.L. 2007, Proton motive force generation from stored polymers for the uptake of acetate under anaerobic conditions, *FEMS Microbiol. Lett.*, **274**, 245–51.
59. Burow, L.C., Mabbett, A.N., McEwan, A.G., Bond, P.L. and Blackall, L.L. 2008, Bioenergetic models for acetate and

phosphate transport in bacteria important in enhanced biological phosphorus removal, *Environ. Microbiol.*, **10**, 87–98.
60. Stubbe, J. and Tian, J. 2003, Polyhydroxyalkanoate (PHA) homeostasis: the role of PHA synthase, *Nat. Prod. Rep.*, **20**, 445–57.
61. Snell, K.D., Feng, F., Zhong, L., Martin, D. and Madison, L.L. 2002, YfcX enables medium-chain-length poly(3-hydroxyalkanoate) formation from fatty acids in recombinant *Escherichia coli* fadB strains, *J. Bacteriol.*, **184**, 5696–705.
62. Kim, T.K., Vo, M.T., Shin, H.D. and Lee, Y.H. 2005, Molecular structure of the PHA synthesis gene cluster from new mcl-PHA producer *Pseudomonas putida* KCTC1639, *J. Microbiol. Biotechnol.*, **15**, 1120–4.
63. Vo, M.T., Lee, K.W., Jung, Y.M. and Lee, Y.H. 2008, Comparative effect of overexpressed *phaJ* and *fabG* genes supplementing (R)-3-hydroxyalkanoate monomer units on biosynthesis of mcl-polyhydroxyalkanoate in *Pseudomonas putida* KCTC1639, *J. Biosci. Bioeng.*, **106**, 95–8.
64. Zhang, H., Sekiguchi, Y., Hanada, S., et al. 2003, *Gemmatimonas aurantiaca* gen. nov., sp. nov., a gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultured representative of the new bacterial phylum *Gemmatimonadetes* phyl. nov., *Int. J. Syst. Evol. Microbiol.*, **53**, 1155–63.
65. Cui, Y.S., Im, W.T., Yin, C.R., Yang, D.C. and Lee, S.T. 2007, *Microlunatus ginsengisoli* sp. nov., isolated from soil of a ginseng field, *Int. J. Syst. Evol. Microbiol.*, **57**, 713–6.
66. An, D.S., Im, W.T. and Yoon, M.H. 2008, *Microlunatus panaciterrae* sp. nov., a β-glucosidase-producing bacterium isolated from soil in a ginseng field, *Int. J. Syst. Evol. Microbiol.*, **58**, 2734–8.