Polyphosphate metabolism

in

Acinetobacter johnsonii 210A

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1. De formule van van Wazer voor het berekenen van polyfosfaat dissociatie-constantes houdt onvoldoende rekening met het feit dat metaalionen niet aan alle P-groepen van de polyfosfaatketen even goed binden.

   Van Wazer JG, Campanella DA (1950) Structure and properties of the condensed phosphates. IV. Complex ion formation in polyphosphate solutions. J Am Chem Soc 72: 655-663

2. Het veranderen van de verenigingsnaam in een sponsornaam vergroot weliswaar de bekendheid van de sponsor maar gaat ten koste van de bekendheid van de club.

3. Elementanalyses van polyfosfaatgranules m.b.v. EDAX dienen aan ongefixeerde en niet-ingebedde cellen te worden uitgevoerd.

   Baxter M, Jensen Th (1980) A study of methods for in situ X-ray Energy Dispersive Analysis of polyphosphate bodies in *Plectonema boryanum*. Arch Microbiol 126: 213-215

4. Het 'last in first out' principe (het ontslaan van de laatst aangestelde docent) strookt niet met het streven naar vernieuwing van het onderwijs.

5. Bij het aanstellen van AIO's moet men beseffen dat deze onderzoekers behalve een salaris ook een budget nodig hebben voor het uitvoeren van het onderzoek.

6. Het feit dat de universiteit de enige onderwijsinstelling is waar de docenten geen lesbevoegdheid hoeven te bezitten, geeft aan dat onderwijs op dit niveau niet die prioriteit krijgt die het verdient.

7. Het aantonen van 0.001 % (w/w) PHB in *Acinetobacter* sp., zegt meer over de detectiegrens van de meetmethode dan over het vermogen van dit micro-organisme om deze reservestof op te hopen.

   Vierkant MA, Martin DW, Stewart JR (1990) Poly-ß-hydroxybutyrate production in eight strains of the genus *Acinetobacter*. Can J Microbiol 36: 657-663

8. De neiging om per fiets bergen te beklimmen is omgekeerd evenredig aan de hoogteverschillen in de eigen woonomgeving.

9. De verandering van 'the pet-organism' in een pet organisme heeft op menig lab veel vruchteloze experimenten tot gevolg gehad.

10. Ketenlengtebepalingen van polyfosfaten gebaseerd op Sigma polyfosfaatstandaarden zijn gezien de heterogeniteit van deze standaarden niet betrouwbaar.
    Clark JE, Wood HG (1987) Preparation of standards and determination of sizes of long-chain polyphosphates by gel electrophoresis. Anal Biochem 161: 280-290

11. De opdruk 'polyethyleen is milieu-veilig' op plastic tassen geeft de consument ten onrechte de indruk dat deze tassen zonder bezwaar weggegooid kunnen worden.

12. Gezien de papierwinkel die nodig is om zich in een ander EG-land te vestigen, is het ideaal van een Europa zonder binnengrenzen nog zeer veraf.

13. De minst belangrijke pagina's in een proefschrift worden het meest gelezen.

14. Als het salaris van een AIO de waardering weergeeft van de overheid voor zijn wetenschappelijk onderzoek, dan is het daarmee droevig gesteld.

Stellingen behorende bij het proefschrift 'Polyphosphate metabolism in *Acinetobacter johnsonii* 210A' van Kees Bonting.

Wageningen, 13 april 1993

CONTENTS

CHAPTER 1

Introduction

Introduction

Preview

In this chapter, the three main themes of the thesis will be introduced. These themes comprise: the microorganism *Acinetobacter*, the reserve polymer polyphosphate, and the enzymes involved in polyphosphate metabolism. In the first part, a short description of the genus *Acinetobacter* will be given, followed by a summary of some physiological characteristics of these microorganisms and the possible role of *Acinetobacter* sp. in biological phosphate removal. In the second part, several aspects of the reserve polymer polyphosphate are described. At the end of the introduction, a description of the enzymes which are able to synthesize or degrade polyphosphate is presented, followed by a short summary of data available on the regulation of polyphosphate metabolism. The chapter concludes by an outline of the thesis.

1. *Acinetobacter*

1.1 The Genus *Acinetobacter*

Acinetobacter strains have a wide distribution in nature. They can be obtained easily from soil, water and sewage (Baumann et al. 1968). It has been estimated that acinetobacters may constitute 0.001 % of the total heterotrophic aerobic population of soil and water (Baumann et al. 1968). In addition, acinetobacters are frequently found to be associated with humans or animals (Towner et al. 1991).

Members of the genus *Acinetobacter* are aerobic, Gram negative, non motile, catalase positive, and oxidase negative and their DNA composition varies from approximately 38-47 mol % G + C (Juni 1978). Most strains can grow at a temperature between 20°C - 30°C in a simple mineral medium and are able to use a wide variety of compounds as the carbon and energy source.

Only recently, a satisfactory classification of the genus *Acinetobacter* was achieved by using a combination of phenotypic properties (including carbon source utilization tests) and identification of genotypic species by means of modern taxonomic methods (genetic transformation, DNA hybridisation, and RNA sequence comparison) (Bouvet and Grimont 1986; Bouvet and Grimont 1987). Seventeen genomic species and nineteen biotypes were identified (Table 1). Independent work carried out by Tjernberg and Ursing (1989) and Nishimura et al. (1988) showed a good correlation with the groupings outlined by Bouvet and Grimont (1986). The results of these studies have resolved the earlier confusion concerning the taxonomic position of the oxidase-negative, non motile, Gram negative, diplobacilli. Members of the genus *Acinetobacter* are now well defined and recognized as a group clearly distinct from *Moraxella* and *Neisseria*.

1.2 Biochemical and Physiological Properties

Acinetobacters are able to grow only under aerobic conditions. No evidence has been

Table 1. Identification scheme for *Acinetobacter* genomic species

	1	2	3	4	5	6	7	8/9	10	11	12	13	14	15	16	17
Growth at : 44°C	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
: 41°C	-	+	+	-	90	-	-	-	-	-	-	-	-	-	-	-
: 37°C	+	+	+	+	+	+	-	+	+	+	+	89	+	+	+	75
Acid from D-Glucose	+	95	+	60	-	50	-	6	+	-	40	+	+	-	-	-
Gelatin hydrolysis	-	-	-	96	-	+	-	-	-	-	-	+	+	+	+	+
Utilization of:																
DL-Lactate	+	+	+	-	+	-	+	99	+	+	+	+	+	+	+	+
DL-4-Aminobutyrate	+	+	+	+	90	-	35	40	+	+	+	11	+	-	25	+
trans-Aconitate	+	99	+	52	-	-	-	-	-	-	-	11	67	-	-	50
Citrate	+	+	+	90	82	+	98	-	+	+	-	+	+	+	+	+
Glutarate	+	+	+	-	-	-	-	-	+	+	+	-	+	-	-	+
Aspartate	+	+	+	64	40	66	61	-	+	+	+	-	-	-	-	-
Azelate	+	90	+	-	-	-	-	+	50	25	+	-	+	-	-	-
ß-Alanine	+	95	95	-	-	-	-	-	+	+	-	-	+	-	75	+
L-Histidine	+	98	94	96	+	+	-	-	+	+	-	+	+	+	+	+
D-Malate	-	98	+	96	+	66	20	60	+	+	-	+	+	+	+	+
Malonate	+	98	85	-	-	-	15	-	-	-	+	11	+	-	50	50
Histamine	-	-	-	-	-	-	-	-	75	+	-	-	-	-	-	-
L-Phenylalanine	+	87	66	-	-	-	-	-	-	-	+	+	+	+	+	+
Phenylacetate	+	87	66	-	-	-	-	94	25	50	+	+	+	+	+	+

Named species are: 1 *A. calcoaceticus*; 2 *A. baumanii*; 4 *A. haemolyticus*; 5 *A. junii*; 7 *A. johnsonii*; 8/9 *A. lwoffii*; 12.*A. radioresistens*.

+ = all strains positive; - = all strains negative. Numbers are percentages of strains positive for a particular character. (Grimont and Bouvet, 1991)

obtained for the capability of these strains to use nitrate as an alternate electron acceptor in the process of anaerobic respiration (Juni 1978). The nutritional versality of *Acinetobacter* sp. is enormous. Utilizable carbon sources include fatty acids, aliphatic alcohols, dicarboxylic acids, certain amino acids, unbranched hydrocarbons, and many aromatic and alicyclic compounds (Baumann et al. 1968). The ability of *Acinetobacter* sp. to use long chain n-alkanes, alicyclic- and a variety of aromatic compounds has been used to elucidate the metabolic pathways of these substrates. (Asperger and Kleber 1991; Fewson 1991; Trudgill 1991). Another striking characteristic of almost the entire group is the inability to grow with disaccharides, sugar alcohols and hexoses (Baumann et al. 1968; Lautrop 1974). Although only a few acinetobacters are able to use glucose as the sole C- and energy source (Juni 1978), in all genospecies except genospecies 5 (*A. junii*), 7 (*A. johnsonii*) and 11, strains were found which were able to oxidize glucose to gluconic acid (Bouvet and Grimont 1986). The ability of these strains to form acid from D-glucose depends on the presence of a membrane-bound aldose dehydrogenase (Van Schie 1987). It was shown that this enzyme contains a co-factor which was given the semisystematic name pyrollo-quinoline quinone (PQQ) (Duine et al. 1979; Duine et al. 1980). The ability of acinetobacters to synthesize PQQ determines whether or not glucose can be oxidized (Van Schie et al. 1984). Addition of PQQ to 74 non-glucose-oxidizing acinetobacters resulted in glucose dehydrogenase activity in almost all strains tested, indicating an inherent deficiency in the PQQ synthesis in these organisms (Gerner-Smidt and Tjernberg 1990).

Many strains of *Acinetobacter* accumulate wax esters, polyphosphate or poly-ß-hydroxybutyric acid (PHB) as reserve polymer. The presence of wax esters is widespread but not universal within the genus *Acinetobacter*. Fixter et al. (1986) tested 19 strains of *A. calcoaceticus*. All strains contained some wax esters and 16 of these strains had increased wax ester contents when harvested from stationary phase N-limited batch cultures. Wax esters are probably used as energy reserves since they are degraded to carbon dioxide and water-soluble products during carbon and energy source starvation, yielding enough energy to contribute significantly to maintenance needs (Fixter and Sherwani 1991).

In 1975, Fuhs and Chen isolated several *Acinetobacter* strains from a wastewater treatment plant with biological phosphate removal, and showed the presence of polyphosphate in these organisms (Fuhs and Chen 1975). Since then, polyphosphate accumulating acinetobacters have been isolated in a number of laboratories. Polyphosphates are localized in the cytoplasm, mostly complexed in either large or small granules (Streichan and Schön 1991a). In *A. lwoffii* two forms of polyphosphate were found. The bulk of the polyphosphate was cytoplasmic, with a small fraction localized in the periplasmic space (Halvorson et al. 1987). The amount of polyphosphate accumulated by *Acinetobacter* sp. varies strongly. Fuhs and Chen (1975) found the phosphorus content to vary between 36 and 50 mg P/g dry weight. Deinema et al. (1985) reported values up to 120 mg P/g dry weight. Röske et al. (1988) investigated a strain able to accumulate phosphorus up to 130 mg per gram dry weight, whereas Hoffmeister et al. (1990) observed values for *A. calcoaceticus* between 34 and 67 mg P/g dry weight and for *A. lwoffii* of 35 mg P/g dry weight. The functions, synthesis and degradation of polyphosphate will be summarized in the second part of this chapter.

PHB is often found in polyphosphate storing acinetobacters isolated from sewage. Both polymers were detected in several *Acinetobacter* strains obtained from activated sludge showing enhanced biological phosphate removal (Fuhs and Chen 1975; Lötter et al. 1986). However,

these results must be viewed with caution since the staining procedures used to identify polyphosphate and PHB are not very specific (Rees et al. 1992). Only recently, the presence of PHB in pure cultures of *Acinetobacter* sp. able to accumulate polyphosphate was firmly established. Batch grown cells of *Acinetobacter* strain RA 3757 accumulated 71 μg P/mg protein when grown in high phosphate medium whereas no polyphosphate and 15 % (w/w) PHB were observed during growth in phosphate-limited medium (Rees et al. 1992).

1.3 Role of *Acinetobacter* in Phosphate Removal

Activated sludge subjected to alternate anaerobic and aerobic conditions in sewage treatment plants acquires the ability to store more phosphorus than required for normal growth (Barnard 1976). The excess phosphate is taken up by microorganisms and stored intracellularly as polyphosphate. Several reviews on the process of enhanced phosphate removal and its practical application have appeared recently (Toerien et al. 1990; Meganck and Faup 1988).

In 1975, Fuhs and Chen were the first to isolate bacteria of the genus *Acinetobacter* from activated sludge showing enhanced biological phosphate removal (Fuhs and Chen 1975). Since then, significant numbers of bacteria of the genus *Acinetobacter* have been isolated from anaerobic-aerobic activated sludge systems (Deinema et al. 1980; Buchan 1983; Murphy and Lötter 1986; Beacham et al. 1990; Beacham et al. 1992). Although some researchers state that the enhanced biological phosphate removal by activated sludge depends solely on the enrichment of *Acinetobacter* sp. in the system (Buchan 1983), the role of these microorganisms in biological phosphate removal is still not clearified. Lötter et al. (1986) found no significant differences in the amount of *Acinetobacter* strains present in anaerobic/denitrifying/aerobic systems that exhibit excess phosphate removal and completely aerobic systems that do not exhibit excess phosphate removal. Hiraishi et al. (1989) concluded that the introduction of anaerobic conditions into a batch aerobic system does not affect the bacterial population structure. Research on alternating anaerobic-aerobic activated sludge systems, which differed in organic loading and in the presence of nitrification and denitrification steps, showed a varying contribution of acinetobacters to the population of the sewage treatment plants. Members of the genus *Acinetobacter* represented only a minority of the total bacterial population in sludge from treatment plants with efficient phosphate removal and high organic loading (Auling et al. 1991). A similar result was obtained by Streichan et al. (1990) who found that the spectrum and population of polyphosphate accumulating bacteria depend both on the process design and on the sewage composition of pilot plants showing enhanced biological phosphate removal. Recently, the bacterial community structure of an anaerobic-aerobic activated sludge system was analyzed by using isoprenoid quinones as biomarkers. *Acinetobacter* sp. appeared to be only a minor constituent of the bacterial population. The most abundant isolates were identified as *Comamonas* and/or *Pseudomonas*, *Paracoccus* and *Flavobacterium-Cytophaga* (Hiraishi et al. 1989; Hiraishi and Morishima 1990).

The quantification of *Acinetobacter* sp. in activated sludge systems is still a major problem. For example, any enumeration method based on cultivation techniques alone is highly selective and can apply only to a small fraction of the total microflora present. Further, acinetobacters grow in large cell clusters which requires cell detachment e.g. physical methods to achieve a reliable enumeration. Finally, polyphosphate accumulating bacteria are mostly identified by cytochemical methods which are not completely

reliable (Streichan et al. 1990).
Summarizing, although acinetobacters are generally considered to be involved in biological phosphate removal, the exact role of these microorganisms in anaerobic/aerobic activated sludge systems is not yet completely clarified.

2. Polyphosphate

2.1 Physical and Chemical Properties

Polyphosphates have the general formula $M_{(n+2)}P_nO_{(3n+1)}$. They are composed of chains in which each phosphorus atom is linked to its neighbours through two oxygen atoms forming a linear unbranched structure (Kulaev 1979). The chain length may vary from 2 to around 10^4 phosphate residues (Harold 1966). In the absence of divalent cations, linear polyphosphates are stable to alkali. They are completely hydrolysed by 1 N acid to orthophosphate within 15 min at 100°C (Dawes and Senior 1973). All alkali metal salts of polyphosphates are soluble in water. Exceptions to this rule are the water-insoluble Kurrol's salts (a macromolecular crystalline potassium polyphosphate), and the compounds known as Maddrell's salts (crystalline sodium polyphosphates of very high molecular weight). Polyphosphates of divalent metals such as Ba^{2+}, Pb^{2+} and Mg^{2+} are either completely insoluble or dissolve to only a very limited extent in aqueous solutions (Kulaev 1979).
Polyphosphates are capable of forming complexes with other polymers especially proteins, basic polypeptides and nucleic acids. This property is due to a simple interaction between cations and anions. A similar polycation-polyanion interaction is found in the metachromatic reaction, in which high molecular weight polyphosphates cause a shift in the absorption maxima of cationic dyes, such as toluidine blue, towards shorter wavelengths (Kulaev 1979). Polyphosphates are known to be very good complexing agents for many metal ions and are able to act as dissolved ion-exchange agents (Van Wazer and Campanella 1950).

2.2 Detection Methods

Over the years, several methods have been developed to detect the presence of polyphosphate in living organisms. The oldest and most extensively used are based on the staining of cells by basic dyes, such as toluidine blue, neutral red, and methylene blue (Kulaev 1979). These staining reactions are rather unspecific and are therefore not a sufficient criterion for the identification of polyphosphate. Neisser stain, for example, may also stain other polymeric compounds such as PHB (Streichan et al. 1990). More recently, a fluorescence method was developed using fluorochromes of the type 4',6',-diamino-2-phenylindole hydrochloride (DAPI) (Allan and Miller 1980). A combination of this technique together with other methods was used to examine the presence of polyphosphate granules in bacteria isolated from activated sludge (Streichan et al. 1990). The use of DAPI has, however, also its drawbacks since Kjeldstad et al. (1991) concluded that this compound has no access to the intracellular polyphosphates of *Propionibacterium acnes* as long as cells are not damaged by UV-light. A less frequently used method involves the precipitation of polyphosphates from cell extracts in the form of insoluble Ba^{2+}-salts followed by acid hydrolysis. The amount of orthophosphate liberated is a measure of the amount of polyphosphate present (Kulaev 1979).
In the last decade, physical methods have been developed for detecting polyphosphate in the cell. The most important ones are ^{31}P NMR and energy dispersive X-ray analysis (EDAX). ^{31}P NMR is a non invasive technique able to monitor phosphorylated compounds like orthophosphate, pyrophosphate, ATP, ADP, AMP, NADP(H)

and the middle, ultimate and penultimate phosphate residues of a polyphosphate chain. ^{31}P NMR has been used in a number of studies to localize polyphosphate in bacteria (Ostrovskii et al. 1980; Streichan and Schön 1991a), yeasts (Beauvoit et al. 1989; Tijssen and Van Steveninck 1984) and algae (Sianoudis et al. 1986). Furthermore, sizes of polyphosphates can be estimated by ^{31}P NMR (Pilatus et al. 1989; MacDonald and Mazurek 1987). It is believed that the upper limit of the chain length that can be determined, is between 200 and 400 P-residues (Jacobson et al. 1982; Rao et al. 1985). Finally, information about the environment of the P-polymer can be obtained since the chemical shift, line intensity, line width, and line shape of the polyphosphate signal obtained by ^{31}P NMR are dependent on several factors such as: the chemical environment of the phosphorus atom and the phosphorus-containing compounds, its degree of mobility and complexation with ions, the presence and velocity of exchange processes (Sianoudis et al. 1986). For example, in extracts Glonek et al. (1971) showed that addition of Mg^{2+} to polyphosphate broadens the phosphorus resonance and shift it 0.5 to 0.6 ppm upfield. However, all this information can only be obtained from mobile, soluble pools of polyphosphate. Large polymers complexed in granular structures or tightly bound to nucleic acids or proteins will not be detected in a ^{31}P NMR spectrum (Roberts 1987).

Electron microscopy is another valuable tool in determining the presence of cellular polyphosphates. Polyphosphate complexed in granules are easily recognized since they are very electron dense. The presence of P and other elements in these granules can be proven by the use of energy dispersive X-ray analysis (EDAX). Sicko-Goad et al. (1975) were among the first in studying the elemental composition of polyphosphate bodies by the use of EDAX in conjunction with scanning transmission electron microscopy (STEM). Since then, the STEM-EDAX system has been used in various studies to establish the presence of P and other elements in polyphosphate granules (Baxter and Jensen 1980a; Baxter and Jensen 1980b; Buchan 1981; Buchan 1983; Heymann et al. 1989; Ogawa and Amano 1987; Webster et al. 1984). Although the STEM-EDAX system is a relatively simple and reliable technique, it can only be used in cells that contain large polyphosphate bodies. Fixation and embedding methods have a profound effect on the cation composition of polyphosphate granules (Baxter and Jensen, 1980a).

2.3 Extraction and Separation Procedures

The first step in many studies concerning the presence of polyphosphate in living cells is its extraction. Almost all methods which have been developed over the years are derived from the extraction schemes of Schmidt and Thannhauser (1945) and Schneider (1946) and yield polyphosphate in two fractions: 'soluble' polyphosphate which is extractable with cold 5 % trichloroacetic acid (TCA) or perchloric acid (PCA), and 'insoluble' polyphosphate which is recovered in the nucleic acid fraction. The nucleic acids are subsequently removed by adsorption to charcoal. The most widely used methods based on this principle are the procedures of Langen and Liss (1958) and Harold (1960). The method of Langen and Liss consists in the successive extraction of the cells in the cold with (i) 1 % TCA, (ii) a saturated solution of a salt such as $NaClO_4$, (iii) a dilute NaOH solution (pH 9-10), (iv) a more concentrated solution of alkali (1 N NaOH, 0°C), and (v) again a dilute NaOH solution (0.1 N, 20°C). With this procedure, four polyphosphate fractions which differed from each other in extractability and physiological behaviour were isolated from yeast (Liss and Langen 1962). The method of Harold comprises the following steps: extraction with (i) 0.5 N

PCA (20°C), (ii) ethanol (30 min, 20°C), (iii) ethanol-ether (3:1, 1 min boiled), and (iv) PCA (15 min, 70°C). Nucleic acids are removed by adsorbing them on activated charcoal. Recently, a new procedure for the isolation of intact polyphosphate was developed (Clark et al. 1986). This procedure yields three fractions, short chain polyphosphate which is soluble in TCA, long-chain polyphosphate which is soluble at neutral pH, and long-chain polyphosphate which is present in volutin granules. The authors claim that this method does not cause hydrolysis of the polyphosphate chains in contrast with the procedures of Langen and Liss, and Harold.

By using one of the extraction procedures, the chain length of the isolated polyphosphate can be roughly estimated since the acid-soluble fractions contain polyphosphate up to 20 residues whereas the degree of polymerization of the acid insoluble polyphosphate is much higher (Kulaev 1979). For a more precise determination of the sizes of polyphosphate, other methods have to be applied. Small polyphosphates ($n < 20$) can be sized by paper chromatography (Kulaev 1979; Rao et al. 1985), thin layer chromatography (Rao et al. 1985) or by the use of anion exchange columns (Kulaev 1979; Yamaguchi et al. 1979). Gel filtration has been used for fractioning polyphosphates with chain lengths up to 100-200 residues (Dürr et al. 1979; Kulaev 1979; Rao et al. 1985; Tijssen et al. 1983; Ueno et al. 1970). These columns are usually calibrated with standards of Sigma Co. These standards are, however, very heterogeneous (Clark and Wood 1987) which makes the determination of polyphosphate chain lengths with this technique less reliable. Another method for the separation of high-molecular-weight polyphosphate is the fractional precipitation of polyphosphate from aqueous solutions with acetone (Van Wazer 1950). The chain length of the fractionated polyphosphates can subsequently be determined by using a basic titration (Van Wazer et al. 1954).

Polyphosphate sizes can also be estimated by ^{31}P NMR. NMR is the only method available for determining the average chain length of soluble polyphosphate as it exists within the cell. It has a upper limit of about 200-400 residues (Jacobson et al. 1982; Rao et al. 1985).

Recently, two new methods have been developed for sizing microgram quantities of polyphosphates from 3 to nearly 1000 P-residues. The first method separates polyphosphates by using gel electrophoresis (Clark and Wood 1987). Chains shorter than about 100 resolve as individual bands on high percentage acrylamide gels. For longer polymers, low percentage acrylamide and agarose gels have to be used. The second method employs the enzyme polyphosphate glucokinase (Pepin et al. 1986). This enzyme shortens long chain polyphosphate having a wide range of sizes to a very narrow range of shorter chain lengths which can be determined on polyacrylamide gel. The number of moles of chains before and after reaction remains the same, therefore, the length of the polyphosphate of the original sample can be calculated. The longest chain length that can be sized in this way is about 600 residues.

2.4 Presence of Polyphosphate in living Organisms

Polyphosphates are widely distributed among the bacteria, blue green algae, fungi, protozoa and algae. They also have been found in some animals, mosses and in the higher flowering plants (Kulaev 1979). The presence of most of these polyphosphates has been detected by cytochemical methods which are inaccurate and unspecific. The absence of polyphosphate in organisms does not necessarily imply the inability of these organisms to synthesize polyphosphate since the polyphosphate content depends strongly on growth conditions.

In phosphate-starved cells, usually polyphosphate cannot be detected whereas in yeast the polyphos-

phate content can account for 20 % of the biomass dry weight when cells are transferred from a phosphate-depleted to a phosphate-rich medium (Liss and Langen 1962). Microorganisms such as *Aerobacter aerogenes* hardly contain any polyphosphate during rapid growth and accumulate this polymer only under conditions of nutritional imbalance (Harold 1966). Others, like *Propionibacterium shermanii*, synthesize polyphosphate during exponential growth (Clark et al. 1986). In *Mycobacterium phlei* and in *Escherichia coli*, polyphosphates were demonstrated during the lag phase and at the start of the logaritmic phase (Drews 1960; Nesmeyanova et al. 1973). *Acinetobacter* strain 210A accumulates phosphorus up to 10 % of its dry weight (Van Groenestijn 1988) irrespective of the growth phase (Van Groenestijn et al. 1989b).

2.5 Functions of Polyphosphate

There is a considerable variation in the amount, size, and localization of polyphosphates as well as in the interaction of polyphosphates with cellular processes in organisms able to accumulate these polymers. Therefore, it is likely that polyphosphates fulfil various roles in the metabolism of these organisms. In *Acinetobacter* sp. these P-polymers can perform at least two different functions. They can act as a phosphorus reserve since it was found that these microorganisms increase their biomass many fold during growth in phosphorus-free medium (Van Groenestijn and Deinema 1985). Further, it was shown that polyphosphate accumulating *Acinetobacter* sp. can maintain a relatively high level of ATP during anaerobiosis which suggests that the polymer can be used as an energy source (Van Groenestijn 1988; Streichan and Schön 1991b).

It has been hypothesized that polyphosphates are also involved in the regulation of intracellular concentrations of important metabolites including ATP, ADP, other nucleoside polyphosphates, pyro- and orthophosphate (Kulaev and Vagabov 1983). Furthermore, there is evidence for the participation of polyphosphate in glucose transport (Van Steveninck and Booij 1964) and nucleic acid metabolism (Kulaev 1979). Recently, complexes composed of PHB, polyphosphate and Ca^{2+} were extracted from the plasma membrane of competent *Escherichia coli* cells (Reusch and Sadoff 1988). It was suggested that these complexes form a channel which may play a role in the transport of Ca^{2+}, phosphate and DNA.

3. Enzymes of Polyphosphate Metabolism

3.1 Polyphosphate Synthesis

Over the years, several enzymes have been detected that are involved in either the polyphosphate synthesis or polyphosphate degradation (Wood and Clark 1988). Among these enzymes, polyphosphate kinase is the best studied. This enzyme catalyses the transfer of the high-energy phosphate residue from ATP to polyphosphate and back from polyphosphate to ADP to form ATP.

$$ATP + PP_n \rightleftarrows ADP + PP_{n+1}$$

Polyphosphate kinase was first detected in yeast by Yoshida and Yamataka (1953) and subsequently isolated and purified by Kornberg et al. (1956) from *Escherichia coli*. The enzyme has been detected in a variety of organisms including *Saccharomyces cerevisiae* (Felter and Stahl 1973), *Escherichia coli* (Kornberg et al. 1956), *Salmonella minnesota* (Mühlradt 1971), *Corynebacterium xerosis* (Muhammed 1961), *Arthrobacter atrocyaneus* (Levinson et al. 1975) and *Propionibacterium shermanii* (Robinson et al. 1984) (Table 2) and has been claimed to be the major enzyme responsible for polyphosphate

Table 2. Comparison of polyphosphate kinases from different organisms

Organism	$PP_n + ADP \rightarrow PP_{n-1} + ATP$				$PP_n + ATP \rightarrow PP_{n+1} + ADP$		
	Spec. activity	$K_m(PP_n)$	$K_m(ADP)$	pH opt.	Spec. activity	$K_m(ATP)$	pH opt.
	μmol/min·mg	mM	mM		μmol/min·mg	mM	
S. cerevisiae	0.45	0.13	0.2	6.7	0.02	-	-
P. shermanii	0.73	-	-	8.0	3.43	-	6.0
E. coli	2.8	0.03	0.05	7.0	2.9	1.4	7.2
A. atrocyaneus	-	-	-	-	0.82	0.53	6.0 - 7.0
C. xerosis	0.00	-	-	-	0.15	-	7.4

(Wood and Clark 1988)

formation in these and other microorganisms (Kulaev 1979). Polyphosphate kinase isolated from *P. shermanii* and from *E. coli* has been studied most extensively.

Polyphosphate kinase from *P. shermanii* has been purified to 70 % homogeneity and appeared to be a monomeric enzyme with a molecular weight of 83 kDa (Robinson et al. 1987). It was demonstrated that short chain polyphosphates can serve as a primer for synthesis of long chain polyphosphates with ATP. With polyphosphate as a primer, the majority of the synthesized polyphosphates possessed about 750 residues. With phosphate as a primer, chain lengths of about 200 residues were formed (Robinson et al. 1987). The mechanism of synthesis was shown to be processive, the elongation reaction occurred without dissociation of intermediate sizes of the polymer from the enzyme (Robinson and Wood 1986). The enzyme was activated by the presence of basic proteins like histone (Robinson et al. 1984).

Polyphosphate kinase of *E. coli* was already partially purified in 1956 (Kornberg et al. 1956). The enzyme required Mg^{2+}, the product of the enzymatic reaction was identified as highly polymerized polyphosphate. The inhibition of polyphosphate kinase by ADP appeared to be due to reversal of the reaction, the phosphorylation of ADP by polyphosphate (Kornberg 1957). Recently, the enzyme was purified to homogeneity and appeared to be a tetramer of 69 kDa-subunits (Ahn and Kornberg 1990). Addition of a primer for polyphosphate synthesis was not required. Polyphosphate formation at low ATP levels proceeded with an initial lag that could be removed by tetraphosphate. The stimulating effect of tetraphosphate on polyphosphate synthesis suggests that this compound may act as a primer. The apparent affinity of the enzyme for ATP was 2 mM. The enzyme was competitively inhibited by ADP. Ammonium sulfate stimulated polyphosphate synthesis whereas 50 mM KCl inhibited the activity by 50 % (Ahn and Kornberg 1990).

Polyphosphate is also synthesized by 1,3 diphosphoglycerate: polyphosphate phosphotransferase:

1,3 diphosphoglycerate + $PP_n \rightleftarrows$ 3 phosphoglycerate + PP_{n+1}

This reaction was detected in *Neurospora crassa* and subsequently in *P. shermanii* (Kulaev 1979). The status of this enzyme is uncertain. It has never been purified and the activity in crude extracts is extremely low. Moreover, the presence

of this enzyme in *P. shermanii* could not be confirmed (Wood and Goss 1985). Therefore, it was concluded that the evidence for its occurrence and its physiological significance is equivocal (Wood and Clark 1988).

Recently, the presence of another polyphosphate synthesizing enzyme was hypothesized. In the membrane fraction obtained from yeast protoplast lysate, an enzyme capable of synthesizing polyphosphate by employing the ß-phosphate groups of dolychylpyrophosphate was found. The enzyme, dolychylpyrophosphate: polyphosphate phosphotransferase, was solubilized from the membrane fraction. It appeared to be metal dependent, maximal activity was found at pH 8 in the presence of 0.1 % Triton X-100 (Kulaev 1990).

3.2 Polyphosphate Degradation

A considerable number of enzymes able to degrade polyphosphate have been found. The phosphorylation of glucose by polyphosphate was first discovered in 1956 in *Mycobacterium phlei*:

$$\text{Glucose} + PP_n \rightarrow \text{Glucose-6-P} + PP_{n-1}$$

The enzyme, polyphosphate glucokinase, was partially purified by Szymona and Ostrowski (1964) and required Mg^{2+} and a high concentration of neutral salts (e.g. 0.3 M KCl) for optimal activity. No utilization of either tri- or tetraphosphate could be detected whereas all enzymatic preparations exhibited some activity towards ATP. Recently, polyphosphate glucokinase of *M. phlei* was purified to apparent homogeneity. The enzyme consisted of eight identical subunits with a molecular weight of 34 kDa. Hexokinase activity was 8 to 10 times lower than the polyphosphate glucokinase activity in the purified preparation. The apparent affinities for the substrates glucose and polyphosphate were 0.48 mM and 7.2 µM, respectively (Girbal et al. 1989).

Polyphosphate glucokinase has also been found in other mycobacteria, corynebacteria, propionibacteria, *Nocardia minima, Micrococcus lysodeikticus,* and *Sarcina lutea* (Table 3) (Wood and Clark 1988). The enzyme was purified 960-fold from *P. shermanii* (Pepin and Wood 1986). During the purification, the ratio of polyphosphate glucokinase activity to ATP glucokinase activity remained approximately constant at 4 to 1. The apparent affinity of polyphosphate glucokinase for a variety of sizes of polyphosphate was tested. A remarkable decrease in K_m was observed with increase in chain length (Pepin and Wood 1986). The utilization of polyphosphate by polyphosphate glucokinase occurred by a non-processive (release of polyphosphate from the enzyme after each phosphorylation) or quasi-processive (release after several phosphorylations by successive reassociations with the enzyme and phosphorylations) mechanism (Pepin and Wood 1987).

Polyphosphate: AMP phosphotransferase degrades polyphosphate at the expense of AMP:

$$PP_n + AMP \rightarrow PP_{n-1} + ADP$$

This enzyme was first observed in crude extracts of *Mycobacterium smegmatis* (Winder and Denenny 1957) and later purified 60-fold from *Corynebacterium xerosis* (Dirheimer and Ebel 1965). The enzyme of *C. xerosis* did not exhibit activity towards ortho-, pyro- and trimetaphosphate, was specific for AMP and needed Mg^{2+} for activity. Van Groenestijn et al. (1987) reported the formation of ATP in cell extracts of *Acinetobacter* strain 210A by the combined action of polyphosphate: AMP phosphotransferase and adenylate kinase (Fig. 1). The activity of polyphosphate:AMP phosphotransferase was

Table 3. Comparison of polyphosphate glucokinases from different microorganisms

Organism	Purification (fold)	Spec. activity	$K_m(PP_n)$	K_m(glucose)
		μmol/min·mg	μM	μM
M. tuberculosis	600	156	3	-
C. xerosis	200	36	0.4	312
P. shermanii	960	15	4.3	-

(Wood and Clark 1988)

found to be maximal at pH 8.5 and 40 °C. A positive correlation between the activity of this enzyme and the accumulation of phosphate in six *Acinetobacter* strains was observed (Van Groenestijn et al. 1989a).

Polyphosphatases which hydrolyse polyphosphate to orthophosphate have been found in many microorganisms (Table 4):

$$PP_n + H_2O \rightarrow PP_{n-1} + P_i$$

Polyphosphatase was first investigated in detail in *Corynebacterium xerosis*. Muhammed et al. (1959) purified it about 100-fold and studied some of its properties. The enzyme was active on long-chain polyphosphates but inactive towards pyrophosphate, tri- and tetrametaphosphate, and hexametaphosphate. No short chain intermediates were detected during the polyphosphate degradation. The enzyme was inhibited by divalent cations including Mg^{2+}, and activated by EDTA.

Two polyphosphatase activities were found in cell extracts of *Neurospora crassa*. One of the polyphosphatases hydrolyzed high-polymer polyphosphate independently of its chain length to P_i. The other specifically catalyzed the splitting of tripolyphosphate to orthophosphate (Kulaev and Konoshenko 1971). The first enzyme was purified to a homogeneous state. Its molecular weight was 50 ± 3 kDa and an apparent affinity for polyphosphate of 6.8×10^{-4} M was found. The enzyme required the presence of divalent cations and exhibited no activity towards pyro- and triphosphate (Umnov et al. 1975).

Recently, the polyphosphatase localized in the cell wall of the yeast *Saccharomyces carlsbergensis* was purified 630-fold. The molecular mass of this enzyme was 40 kDa. The enzyme hydrolyzed polyphosphates with a chain length ranging from 3 to 200 residues. The affinity for polyphosphate increased with the chain length by several orders of magnitude (Kulaev 1990). Divalent cations were compulsory for activity whereas SH-reagents such as iodoacetamide were effective inhibitors of the enzyme (Andreeva and Okorokov 1990).

Polyphosphates were also hydrolyzed in cell extracts of *Acinetobacter* strain 210A (Fig. 1). The enzyme activity was stimulated by the presence of NH_4Cl or KCl. Mg-polyphosphate was the only polymer degraded. Hardly any orthophosphate was produced in the presence of sodium- or potassium polyphosphate (Van Groenestijn et al. 1989a).

Other polyphosphate degrading enzymes detected in microorganisms are polyphosphate depolymerase, polyphosphate dependent NAD kinase,

Table 4. Comparison of polyphosphatases from different microorganisms

Organism	Purification (fold)	Spec. activity	$K_m(PP_n)$	pH optimum
		μmol/min·mg	mM	
E. coli		190	0.13	3
C. xerosis	97	0.14	0.77	7
S. cerevisiae	23	3	0.35	7.5

(Wood and Clark 1988)

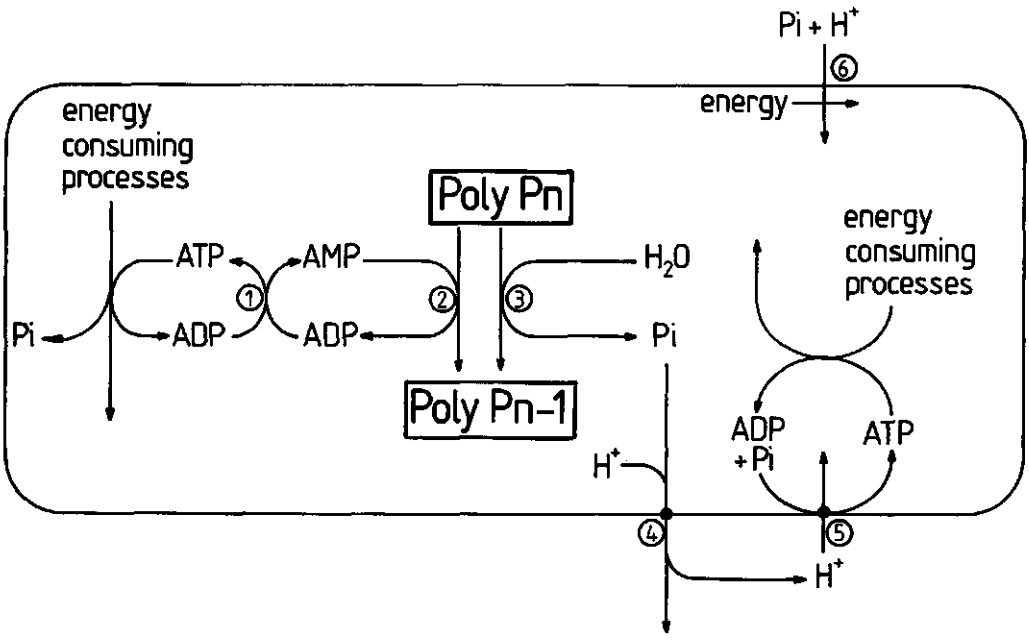

Figure 1. Energetic aspects of polyphosphate metabolism in *Acinetobacter johnsonii* 210A

1) Adenylate kinase
2) Polyphosphate:AMP phosphotransferase
3) Polyphosphatase
4) Phosphate efflux
5) ATPase
6) Phosphate influx

polyphosphate fructokinase, polyphosphate mannokinase, and polyphosphate gluconatekinase (Kulaev 1979; Kulaev and Vagabov 1983).

4. Regulation of Polyphosphate Metabolism

The regulation of enzymes involved in polyphosphate metabolism is largely unknown. Only in two microorganisms, *Escherichia coli* and *Aerobacter aerogenes*, the control of polyphosphate metabolism has been studied in detail.

In *Escherichia coli*, it was shown that phosphate starvation induced an increase in the activity of alkaline phosphatase, tripolyphosphatase, and polyphosphatase. Addition of orthophosphate to a previously starved culture resulted in a cessation of the increase in the activity of these enzymes (Nesmeyanova et al. 1974). When cells of *E. coli* were subjected to phosphate starvation in the presence of chloramphenicol, a specific inhibitor of protein synthesis, no increase in the activity of alkaline phosphatase, tripolyphosphatase, and polyphosphatase was observed. This indicates that the biosynthesis of these enzymes is controlled by orthophosphate, and the increase in their activity during phosphate starvation results from derepression of synthesis rather than activation of pre-existing enzymes. Mutants which were impaired in one of the regulatory genes for alkaline phosphatase were used to study the regulation of these enzymes on the genetic level. It was found that tripolyphosphatase, polyphosphatase and alkaline phosphatase of *E. coli* are regulated by a orthophosphate, possess a common regulator gene, and constitute a single regulon (Kulaev 1979).

The effect of growth conditions on polyphosphate metabolism in *Aerobacter aerogenes* is mediated by two entirely different mechanisms. The first of these comes into operation when nucleic acid synthesis is blocked as a result of the absence of an essential metabolite other than phosphate. A dual competitive relation between the accumulation of polyphosphate and of nucleic acid was observed. Resumption of nucleic acid synthesis reduced the rate of concurrent polyphosphate synthesis and at the same time stimulated its degradation (Harold 1963; Harold and Harold 1965). A fundamentally different regulatory mechanism was found in cells previously subjected to phosphate starvation. Addition of phosphate resulted in a rapid accumulation of polyphosphate. This appeared to be caused by the derepression of polyphosphate kinase synthesis during phosphate starvation (Harold 1964). The rate of polyphosphate formation was directly proportional to the amount of polyphosphate kinase indicating polyphosphate kinase as the only enzyme involved in polyphosphate synthesis. Analysis of two mutants blocked in polyphosphate accumulation (Harold and Harold 1963) supported this conclusion (Harold 1964). Mutants in which the polyphosphate metabolism was modified, were also used to examine the regulation of polyphosphate formation and degradation on the genetic level. It was found that the synthesis of alkaline phosphatase, polyphosphate kinase, and polyphosphatase was permanently repressed in mutant Pn-1 and constitutively elevated in mutant Pn-3. This indicates that these enzymes are controlled by a common regulator gene (Harold and Harold 1965). However, the enzymes do not constitute a single operon. Synthesis of the enzymes was not coordinate and partial revertants which had regained the capacity for derepression of alkaline phosphatase but not of polyphosphate kinase could be obtained (Harold and Harold 1965).

5. Outline of this Thesis

Although the exact role of *Acinetobacter* sp. in alternating anaerobic/aerobic activated sludge systems is still not completely clarified, these organisms are generally considered to be involved in the process of biological phosphate removal.

Acinetobacter sp. have the ability to accumulate large amounts of phosphorus as polyphosphate in the cell. The fundamental principles that underlie the polyphosphate metabolism in these microorganisms are, however, still not fully understood. A thorough understanding of polyphosphate synthesis and degradation at the molecular level is needed to optimize the process of biological phosphate removal. Therefore, the aim of this thesis was to study the enzymatic processes responsible for the accumulation and degradation of polyphosphate in *Acinetobacter johnsonii* 210A.

A. johnsonii 210A has been used in a large number of studies on polyphosphate metabolism (Deinema et al. 1985; Van Groenestijn 1988), the strain was, however, not identified. Chapter 2 describes the classification of this organism as *A. johnsonii* by the use of a combination of biochemical and genetic methods. Additional characteristics of this organism such as the storage of PHB, oxidation of glucose, and the occurence of plasmids are reported and compared to other *Acinetobacter* species. Polyphosphates are localized in the cytoplasm and mostly complexed in one or two large and several small granules. Polyphosphate granules are composed of negatively charged P-polymers and positively charged counterions such as K, Mg and Ca. Chapter 3 shows the effect of the cation composition of the growth medium on the elemental composition of the polyphosphate granules and discusses the mechanism that possibly determines the presence of different cations in the large polyphosphate granules. Polyphosphates usually cover a wide range of molecular sizes and several polyphosphate fractions each containing polymers with different chain lengths can be isolated. Chapter 4 presents the size of the phosphate polymers during polyphosphate synthesis and degradation in *A. johnsonii* 210A as determined by gel electrophoresis. Furthermore, the correlation between intracellular ATP levels and polyphosphate formation is shown, in order to demonstrate a possible involvement of polyphosphate kinase in polyphosphate synthesis. Polyphosphate degradation was studied in detail by isolating the enzymes involved in this process. The partial purification and some properties of polyphosphate:AMP phosphotransferase are described (Chapter 5). This enzyme phosphorylates AMP to ADP with polyphosphate and makes it possible for *A. johnsonii* 210A to conserve the energy liberated from the cleavage of polyphosphate. Chapter 6 presents some properties of polyphosphatase, an enzyme which catalyzes the hydrolytic cleavage of high polymeric polyphosphates in *Acinetobacter johnsonii*. The isolation and characteristics of the inorganic pyrophosphatase are shown in chapter 7. The possible involvement of pyrophosphate in polyphosphate metabolism is discussed. Although *A. johnsonii* 210A is known for its ability to accumulate large amounts of phosphate in C- and N-limited continuous cultures, the response of this organism to different phosphate concentrations during growth was not examined so far. Chapter 8 reports on the effect of phosphate on biomass, cellular composition, phosphate uptake rates, and activities of enzymes involved in (poly)phosphate metabolism of *A. johnsonii* 210A during growth in a chemostat at different phosphate levels.

6. References

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CHAPTER 2

Additional characteristics of the polyphosphate-accumulating *Acinetobacter* strain 210A and its identification as *Acinetobacter johnsonii*

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Additional characteristics of the polyphosphate-accumulating *Acinetobacter* strain 210A and its identification as *Acinetobacter johnsonii*

Abstract

Acinetobacter strain 210A, a non-motile, strict aerobic, rod shaped bacterium was isolated from activated sludge in 1980. The strain was unambiguously identified as *Acinetobacter johnsonii*, using a combination of biochemical and genetic properties. The organism was able to synthesize polyphosphate as well as poly-ß-hydroxybutyric acid during growth in batch cultures. Storage of these reserve polymers depended on the amount of phosphate in the growth medium. At excess phosphate, polyphosphate was formed. The amount of phosphate accumulated was about 4 mg P/100 mg dry weight and was unaffected by the 'energy content' of the substrates tested. Poly-ß-hydroxybutyric acid was formed when phosphate was the limiting nutrient. Cells grown in media with an initial phosphate concentration of 30 μM contained 13 % (w/w) poly-ß-hydroxybutyric acid. Intact cells were able to oxidize a variety of monosaccharides in the presence of PQQ. Disaccharides were not oxidized, neither in intact cells nor in cell free extracts. The strain contained several plasmids.

Introduction

The taxonomical position of acinetobacters has been subject to various investigations and it became clear that this group of bacteria was phenotypically and genetically heterogeneous (Baumann et al. 1968). In 1969 a highly efficient transformation system was demonstrated in a strain of *A. calcoaceticus (Bacterium anitratum)* (Juni and Janik 1969). This finding was used to establish genetic relatedness amongst acinetobacters. It was demonstrated that DNA from all 265 strains of *Acinetobacter* studied, was able to transform a stable auxotroph of a competent strain (strain BD413 *trp* E27) (Juni 1972). *Acinetobacter* strains were therefore recognised as a natural group and defined as follows: strictly aerobic, non-motile, oxidase-negative coccobacilli; Gram-negative, but sometimes difficult to destain; grows well on complex media between 20°C and 30°C without growth factor requirements; nitrate is rarely reduced; extracted DNA is able to transform strain BD413 *trp* E27; the G+C content of the DNA is between 39 and 47 mol% (Juni 1984).

Recently Grimont and Bouvet (1991) identified 17 genospecies by using a combination of phenotypic and genotypic properties. This work together with studies of Tjernberg and Ursing (1989) and Nishimura et al. (1988) have resulted in a satisfactory classification of *Acinetobacter* strains. The genus *Acinetobacter* harbours bacteria which are clearly distinct from bacteria belonging to the genera *Moraxella* and *Neisseria*. Acinetobacters are widely distributed in nature

and are easily isolated from soil, water and sewage (Baumann et al. 1968). They are considered to be the main bacteria responsible for the excess phosphate uptake in activated sludge (Buchan 1983; Fuhs and Chen 1975). Deinema et al. isolated several *Acinetobacter* strains from samples of activated sludge (Deinema et al. 1980; Deinema et al. 1985). The strains were able to accumulate large amounts of phosphate as polyphosphate. One of the isolates, *Acinetobacter* strain 210A, was used in a large number of studies on polyphosphate metabolism (Bonting et al. 1991; Bonting et al. 1992; Van Groenestijn 1988). Up to now this strain was not identified.

The aim of this study was to identify strain 210A with a combination of biochemical and genetic methods and to characterise it with respect to formation of polyphosphate and poly-ß-hydroxybutyric acid, glucose oxidation and the occurrence of plasmids. The possible involvement of *Acinetobacter johnsonii* in biological P-removal is discussed.

Material and methods

Organisms and cultivation

Acinetobacter strains 210A and 132 were isolated from activated sludge with the method described by Deinema et al. (1980). Both strains were grown in 300 ml Erlenmeyer flasks at 20°C. The flasks contained 100 ml butyrate medium as described by Van Groenestijn et al. (1987) and were placed on a rotating incubator (140 rpm). To study the polyphosphate accumulation in *Acinetobacter* strain 210A during growth on different C-sources, butyrate was replaced by a variety of substrates. The total C content in the media was always 1 g/L. In the media with butanol and ethanol, the TRIS-buffer was replaced by a K-phosphate buffer (pH 7.0). Growth at limiting amounts of phosphate was examined in media containing phosphate concentrations ranging from 0.03 to 1 mM. In these media 0.215 g/L KCl was added as K-source.

DNA relatedness

DNA from *Acinetobacter* strain 210A was hybridized against reference strains of all DNA groups and unclassified strains of *Acinetobacter* as described by Tjernberg and Ursing (1989) using the filter method of Tjernberg et al. (1989).

DNA transformation

Transformation of DNA between *Acinetobacter* strain 210A and the competent strain BD413 *trp* E27 was performed as described by Juni (1972).

Determination of DNA composition

The base composition of DNA from *Acinetobacter* strain 210A was estimated by using the thermal denaturation method (Sandstedt et al. 1983).

Isolation of plasmid DNA

Plasmid DNA was isolated according to Kado and Liu (1981).

Biochemical identification

Biochemical characteristics of *Acinetobacter* strain 210A were studied by using the identification scheme as described by Bouvet and Grimont (1987).

Extract preparation and enzyme assays

Cells were collected by centrifuging at 8,000 x *g* for 10 min at 4°C, washed in 50 mM K-PIPES (pH 6.7) with 2 mM $CaCl_2$ and directly

used for the enzyme assays.
Cell extracts were prepared according to the method described by Matsushita et al. (1988). The last step of this method was modified. Crude membrane and soluble fractions were separated by centrifugation at 100,000 x *g* for 60 min. Pyrollo-quinoline quinone (PQQ) dependent aldose oxidation was assayed by following the rate of oxygen consumption with a Clark type oxygen electrode at 30°C. The assay mixture contained air-saturated 50 mM K-PIPES buffer (pH 6.7), 2 mM $CaCl_2$ and 5 μM PQQ when whole cells were used. The aldose oxidation in cell free extracts was measured in air-saturated 50 mM TRIS buffer (pH 8) with 2 mM $CaCl_2$, 5 μM PQQ and 2.8 mM phenazine methosulfate. The assay mixture was pre-incubated for 5 min. The reaction was started by the addition of sugar to a final concentration of 20 mM.

Analytical methods

Protein was estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Orthophosphate was measured spectrometrically according to the Standard Methods (1976). The amount of poly-ß-hydroxybutyric acid was estimated according to the method of Braunegg et al. (1978). The presence of polyphosphate in bacteria isolated from sludge was estimated microscopically after staining according to Neisser (Gurr; 1965). The oxidation of glucose in isolates was determined by growing these organisms on agar plates with acetate (15 mM), glucose (10 mM), PQQ (0.8 nM) and bromocresol purple (0.003 %). Bacterial dry weight was quantified by centrifuging culture samples at 8,000 x *g* for 10 min, washing the pellets twice with demineralized water and drying them to constant weight at 105°C. The phosphorus content of the biomass was calculated from the increase in the biomass dry weight and the decrease in the orthophosphate concentration of the medium.

Chemicals

All chemicals were of analytical grade and commercially available.

Results

Identification of Acinetobacter *strain 210A*

DNA extracted from *Acinetobacter* strain 210A, a strictly aerobic, non-motile and gram-negative organism, was able to transform the competent strain BD413 *trp* E27. The G+C content of the DNA was 42.9 ± 0.2 mol%. DNA from *Acinetobacter* strain 210A was hybridized against reference strains of all DNA groups and unclassified strains examined by Tjernberg and Ursing (1989). The delta-Tm value of strain 210A versus the type strain of *Acinetobacter johnsonii* was 1.5. The delta-Tm value versus other *Acinetobacter* groups varied between 5.9 and 11.2. The organism was phenotypically characterized by the use of the identification scheme as described by Bouvet and Grimont (1987). Both the DNA hybridization and the phenotypic data (Table 1) supported identification of strain 210A as a strain of *Acinetobacter johnsonii*. *Acinetobacter* strain 210A will therefore be referred to as *Acinetobacter johnsonii* 210A.

Reserve polymers

Until now polyphosphate and wax-esters have been identified in a large number of acinetobacters (Fixter and Sherwani 1991). Recently, poly-ß-hydroxybutyric acid was also detected in several *Acinetobacter* strains (Vierkant et al. 1990). The ability of *Acinetobacter johnsonii* 210A to synthesize polyphosphate was already described by Deinema et al. (1985). Polyphos-

Table 1. Phenotypic characteristics of *Acinetobacter* strain 210A and several other *Acinetobacter* genospecies

Characteristic	*Acinetobacter* strain 210A	*A. johnsonii*	*A. calcoaceticus*	*A. baumanii*	*A. lwoffii*
Growth at : 44°C	-	-	-	+	-
: 41°C	-	-	-	+	-
: 37°C	-	-	+	+	+
Acid from D-glucose	-	-	+	+	d
Gelatin hydrolosis	-	-	-	-	-
Utilization of:					
DL-Lactate	+	+	+	+	+
DL-4-Aminobutyrate	+	d	+	+	d
trans-Aconitate	-	-	+	+	-
Citrate	+	+	+	+	-
Glutarate	-	-	+	+	-
L-Aspartate	+	d	+	+	-
Azelate	-	-	+	+	+
ß-Alanine	-	-	+	+	-
L-Histidine	-	-	+	+	-
D-Malate	+	d	-	+	d
Malonate	-	d	+	+	-
Histamine	-	-	-	-	-
L-Phenylalanine	-	-	+	d	-
Phenylacetate	-	-	+	d	d

Symbols: + = 90 to 100 % positive; - = 0 to 10 % positive; d = 11 to 89 % positive

phate is formed during growth in the presence of excess energy and phosphate. The influence of the 'energy content' of the substrates on the accumulation of polyphosphate was studied by growing the organism on highly reduced or oxidized compounds. The amount of polyphosphate formed was not severely affected by the 'energy content' of the substrate and ranged from 3.7 to 5.7 mg P/ 100 mg dry weight. Grown on acetate, the organism accumulated 4.7 ± 0.3 mg P/ 100 mg dry weight whereas during growth on a more reduced substrate such as butanol, 4.5 ± 0.5 mg P/ 100 mg dry weight was formed. Addition of glucose/PQQ which can be used as an auxiliary energy source did not increase the amount of polyphosphate accumulated either (not shown).

When cells were grown in media that contained less than 1 mM phosphate, poly-ß-hydroxybutyric acid was formed. The amount of poly-ß-hydroxybutyric acid accumulated, was inversely proportional to the phosphate concentration in the medium. The organism contained 13 % (w/w) of poly-ß-hydroxybutyric acid when grown in media with an initial phosphate concentration of 30 μM.

Glucose oxidation

Acinetobacters which were able to produce acid from D-glucose were found in all genospecies except genospecies 5 (*A. junii*), 7 (*A. johnsonii*) and 11 (Bouvet and Grimont 1987). The ability of *Acinetobacter* strains to form acid aerobically from glucose depends on the presence of a quinoprotein aldose dehydrogenase (E.C. 1.1.99.17) that oxidizes aldose to aldono lactone (Van Schie 1987).

Intact cells of *A. johnsonii* 210A were not able to produce acid from D-glucose (Table 1). However, in the presence of PQQ, glucose (Van Veen et al. 1993) and a wide variety of aldose sugars were oxidized. Oxidation of disaccharides was not observed, neither in whole cells nor in cell free extracts (Table 2). The apparent affinity of intact cells for D-glucose was 2.6 mM. A similar value was reported for *A. calcoaceticus* and *A. haemolyticus* (Bouvet and Bouvet 1989). In cell free extracts a K_m-value for D-glucose of 3.2 mM was found.

Table 2. Substrate specificity of whole cells and in cell free extracts of *A. johnsonii* 210A for oxidation of mono- and disaccharides

Substrate	Activity (%)	
	Whole cells	Cell free extract
D-Glucose	100 (± 0)[1)]	100 (± 0)[2)]
D-Galactose	48 (± 14)	50 (± 1)
D-Mannose	10 (± 5)	9 (± 1)
D-Xylose	89 (± 5)	95 (± 5)
D-Ribose	42 (± 6)	59 (± 4)
L-Arabinose	61 (± 4)	69 (± 1)
Lactose	0	0
Cellobiose	0	0
Saccharose	0	0

[1)]100 % is 0.18 μmol O_2 consumed/min · mg dry weight
[2)]100 % is 1.5 μmol O_2 consumed/min · mg protein

Plasmids

Indigenous plasmids have been found in the majority of *Acinetobacter* isolates that have been examined. Although many of these are cryptic plasmids, others have been associated with antibiotic and heavy metal resistance, aromatic hydrocarbon degradation, conjugal fertility and restriction/modification systems (Towner 1991). Recently, it was shown that *Acinetobacter* strains which were isolated from sludge and able to accumulate polyphosphate, carried several plasmids (Bayly et al. 1991).

We investigated the presence of plasmids in two

Acinetobacter strains, *A. johnsonii* 210A and *Acinetobacter* strain 132. *Acinetobacter* strain 132 does not accumulate large amounts of phosphate in contrast with *A. johnsonii* 210A (Van Groenestijn 1988). Plasmids were observed in extracts of both strains. The plasmid profiles showed, however, clear differences. Both strains contained a large plasmid while in *A. johnsonii* 210A at least one additional large and a small plasmid was found (Fig. 1).

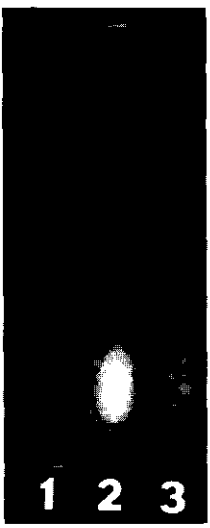

Figure 1. Plasmid profiles of *Acinetobacter johnsonii* 210A and *Acinetobacter* strain 132.

lane 1. Hind III digest of λDNA
lane 2. Plasmid profile of *A. johnsonii* 210A
lane 3. Plasmid profile of *Acinetobacter* strain 132

Presence of Acinetobacter johnsonii *in activated sludge*

The bacterial population of two sludge types both showing enhanced biological phosphate removal was investigated. Renpho sludge was obtained from a pilot plant of the Department of Water Pollution Control of the Wageningen Agricultural University, The Netherlands (Rensink et al. 1989). Oxidation of glucose in the presence of PQQ was found in 412 of the 1579 colonies isolated. Polyphosphate was found in 128 of the 412 glucose oxidizing isolates. Since the P-accumulating strains were all able to grow on acetate and showed the same morphology as *Acinetobacter* sp. previously isolated, it was suggested that the new isolates also belonged to the genus *Acinetobacter*. Five isolates were identified to the genospecies level. Three isolates appeared to be *A. johnsonii*, the other two belonged to the genospecies of *A. lwoffii*.
Sludge obtained from a fill and draw system (Appeldoorn et al. 1992) showed a different picture. Only 23 of the 1020 colonies isolated were able to oxidize glucose in the presence of PQQ. In 609 isolates, the presence of polyphosphate could be demonstrated whereas only 14 isolates exhibited the capacity of both glucose oxidation and polyphosphate synthesis.

Discussion

Description of Acinetobacter johnsonii 210A.
The morphological and biochemical characteristics of *Acinetobacter* strain 210A fit in with the description of the genus (Juni 1984, Bouvet and Grimont 1986) and the description of the species (Bouvet and Grimont 1986). The organism was isolated from activated sludge and is deposited in the Netherlands Culture Collection of Microorganisms under the access number LMAU A130.
Acinetobacter johnsonii 210A is able to synthesize both polyphosphate and poly-ß-hydroxybutyric acid. Polyphosphate can act as an energy reserve and is accumulated during growth at excess phosphate (Van Groenestijn 1988). The amount of polyphosphate formed is not greatly altered by the 'energy content' of the substrate during growth in batch cultures. Even an addition of an auxiliary energy source

does not significantly increase the amount of polyphosphate. This observation may be explained by assuming conditions in the batch experiments of energy spillage rather than energy limitation regardless the 'energy content' of the substrate used. The amount of poly-ß-hydroxybutyric acid accumulated depended on the initial phosphate concentration in the medium. At phosphate concentrations below 1 mM, poly-ß-hydroxybutyric acid was synthesized. Similar results were found when this organism was grown in P-limited chemostat cultures (Bonting et al. 1992). *Acinetobacter johnsonii* 210A is not unique in its ability to synthesize both polyphosphate and poly-ß-hydroxybutyric acid since these polymers were also observed in *Acinetobacter* sp. isolated from activated sludge (Lötter et al. 1986).

The ability of *Acinetobacter johnsonii* 210A to oxidize glucose only in the presence of PQQ is in accordance with the study of Gerner-Smidt who investigated 74 asaccharolytic strains of *Acinetobacter* (Gerner-Smidt and Tjernberg 1990). Like *Acinetobacter calcoaceticus* LMD 79.41, intact cells of *A. johnsonii* 210A oxidize monosaccarides but not disaccharides (Dokter et al. 1987). However, in cell free extracts of *A. johnsonii* 210A only monosaccharides are oxidized whereas the glucose dehydrogenase enzyme present in cell free extracts of *A. calcoaceticus* LMD 79.41 can also use disaccharides as a substrate (Matsushita et al. 1988). Matsushita et al. (1988) concluded that *A. calcoaceticus* LMD 74.41 contains two glucose dehydrogenase enzymes. Besides a membrane bound enzyme which shows a high activity towards monosaccharides, the organism contains a soluble enzyme that oxidizes preferentially glucose and disaccharides. Since we were not able to detect any difference in substrate specificity between whole cells and cell free extracts, we conclude that *A. johnsonii* 210A most probably contains only one glucose dehydrogenase.

Acinetobacter johnsonii 210A contains several plasmids whereas strain 132 which is not able to accumulate large amounts of phosphate harbours only one large plasmid. This observation is in line with a report of Bayly et al. (1991) who found that at least two plasmids, present in a polyphosphate accumulating strain, were absent in its derivative which was unable to form polyphosphate. They suggested that the polyphosphate synthesizing system is encoded on a 20 kb plasmid. More research will be needed to investigate the role of plasmids in the polyphosphate accumulating *A. johnsonii* 210A.

Acinetobacters have been isolated from various enhanced phosphorus removing activated sludges. Duncan et al. (1988) studied the presence of acinetobacters in a pilot-scale sewage-treatment plant. They reported that 78% of the acinetobacters isolated, belonged to genospecies 7 (*Acinetobacter johnsonii*). Beacham et al. (1990) investigated a modified University of Cape Town configured pilot plant for the presence of different strains of *Acinetobacter*. They found a very diverse *Acinetobacter* population with *A. junii, A. johnsonii* and *A. lwoffii* as the most common isolated species. Polyphosphate accumulating bacteria other than *Acinetobacter* sp. have also been isolated from activated sludge. Hiraishi and Morishima (1990) found *Comamonas* and/or *Pseudomonas* as the most abundant isolates of anaerobic-aerobic activated sludge. Significant numbers of *Paracoccus* and *Flavobacterium-Cytophaga* were also detected whereas *Acinetobacter* sp. was only a minor constituent of the bacterial population. Recently, Streichan et al. (1990) showed that the number of *Acinetobacter* sp. differs considerable between pilot plants with different sewages as well as in sludges with the same sewage input but with different modes of processes. In this study it is shown that *Acinetobacter* sp. form an important part of the bacterial population of Renpho sludge

obtained from a pilot plant showing enhanced biological phosphate removal. Part of the strains isolated from this sludge could be identified as *A. johnsonii*. This suggests that *A. johnsonii* probably plays an important role in biological phosphate removal in this sewage treatment system. The investigation of the bacterial population of fill and draw sludge showed that about 60 % of the organisms isolated were able to store polyphosphate whereas only 2 % of the isolates exhibited the capacity of glucose oxidation. Since nearly all acinetobacters are able to oxidize glucose in the presence of PQQ (Grimont and Bouvet 1991), the allmost complete absence of glucose oxidizing isolates suggests that *Acinetobacter* sp. represent only a minority of the population in the fill and draw system. The difference in bacterial population of Renpho sludge and fill and draw sludge clearly shows that the presence of *Acinetobacter* sp. in activated sludge systems showing enhanced biological phosphate removal strongly depends on process design and influent composition of the treatment system.

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CHAPTER 3

The elemental composition dynamics of large polyphosphate granules in *Acinetobacter* strain 210A

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The elemental composition dynamics of large polyphosphate granules in *Acinetobacter* strain 210A

Abstract

Electron microscopy and energy dispersive X-ray micro-analysis were used to examine the elemental composition of large polyphosphate granules in unfixed and unstained intact cells of *Acinetobacter* strain 210A. When grown in medium with butyrate, *Acinetobacter* strain 210A possessed 1 or 2 large granules with a diameter of 0.4 μm besides a relatively large number of small granules. The large granules were composed of phosphorus, magnesium and potassium. A decrease in the Mg/Ca-ratio of the medium from 5.95 to 0.0073 resulted in a decline in the intracellular Mg/Ca-ratio from 15 to 0.56. At a high intracellular Mg/Ca-ratio, magnesium was the dominant counterion in the polyphosphate granule. Calcium became the major cation in the polyphosphate bodies at a low intracellular Mg/Ca-ratio. Omission of Ca^{2+} or modification of the K/Mg ratio in the medium did not significantly affect the cation composition of the polyphosphate granules. The dissociation constants for Mg- and Ca-polyphosphate were 9.3 x 10^{-2} mol/l and 1.5 x 10^{-1} mol/l, respectively.

Introduction

Volutin is found inside the cell of numerous microorganisms and is usually identified by staining reactions. These cellular inclusions display the special property of metachromasy, *i.e.* they shift the absorbance spectrum of a wide variety of basic dyes to shorter wavelengths. The metachromatic nature of these granules has been ascribed to the presence of polyphosphate (Kulaev 1979) but in earlier studies it was demonstrated that granules which consist largely of poly-ß-hydroxybutyric acid also stain metachromatically (Martinez 1963; Williamson and Wilkinson 1958).

The pioneering use of energy dispersive X-ray analysis (EDAX) by Jensen and co-workers (Baxter and Jensen 1980a, b; Sicko-Goad et al. 1975) to determine the elemental composition of cellular inclusions demonstrated that the electron-dense bodies observed in a wide variety of microorganisms consist of polyphosphate (Harold 1966; Widra 1959). This was also reported by Webster et al. (1984) who studied the metachromatic inclusions in *Spirillum itersonii*, *Corynebacterium diphtheriae* and *Micrococcus luteus*. More recently, electron-dense bodies were detected in bacteria present in certain activated sludges. These bacteria take up phosphorus in excess of normal metabolic requirements (Buchan 1981, 1983; Heymann et al. 1989; Röske et al. 1989). *Acinetobacter calcoaceticus* and possibly other *Acinetobacter* strains are considered to be

the main bacteria responsible for the excess phosphate uptake in activated sludge (Buchan 1983; Fuhs and Chen 1975). The electron-dense polyphosphate bodies have also been encountered in pure cultures of *Acinetobacter* sp. which were grown aerobically in simple media with acetate or butyrate as the sole source of carbon and energy (Buchan 1981, 1983; Van Groenestijn 1988).

Van Groenestijn and Deinema (1985) reported that with EDAX, the elements phosphorus, magnesium, calcium and potassium were found in polyphosphate granules of *Acinetobacter* strain 210A. From the uptake and release rates of phosphate and several cations in *Acinetobacter* strain 210A and in *Acinetobacter* strain 132 which does not accumulate polyphosphate, it was deduced that magnesium was the most important counterion of polyphosphate in *Acinetobacter* strain 210A (Van Groenestijn et al. 1988). Entirely different results were obtained by other research groups. By using EDAX, calcium was identified as dominant cation in polyphosphate granules in activated sludge and in pure cultures of *Acinetobacter* sp. (Buchan 1981, 1983). Very recently this finding was corroborated by Röske et al. (1989) who studied *A. calcoaceticus*.

In this paper the elemental composition of polyphosphate bodies detectable by Scanning Transmission Electron Microscope (STEM) in *Acinetobacter* strain 210A grown under a variety of conditions is quantified and shows that the cation composition of polyphosphate depends on the relative and absolute abundance of magnesium and calcium in the medium.

Table 1. Cation composition of the growth media

Medium	Mg^{2+} (mM)	Ca^{2+} (mM)	K^+ (mM)	Mg/Ca ratio
Standard[1)]	2.44	0.41	3.23	5.95
Medium I	2.44	0	3.23	∞
Medium II	2.44	4.1	3.23	0.595
Medium III	0.03	0.41	3.23	0.073
Medium IV	0.03	4.1	3.23	0.0073
Medium V	0.03	0	3.23	∞
Medium VI	1.22	0.41	36.7	2.98
Medium VII	4.88	0.41	0.083	11.9

[1)] The standard medium contained, per liter: 2.29 g of Na-butyrate, 0.1 g of $Na_2S_2O_3 \cdot 5\ H_2O$, 1.0 g of NH_4Cl, 0.6 g of $MgSO_4 \cdot 7\ H_2O$, 0.44 g of KH_2PO_4, 0.06 g of $CaCl_2$, 6 g of TRIS and 2 ml of a trace mineral solution (Van Groenestijn et al. 1987)

Material and methods

Organism and cultivation

Acinetobacter strain 210A was isolated and described by Deinema et al. (1985). The organism was grown in Erlenmeyer flasks of 300 ml at 18 to 20°C. The flasks contained 100 ml medium and were placed on a rotating incubator (140 rpm). The standard medium was prepared according to Van Groenestijn et al. (1987). The amounts of K^+, Ca^{2+} and Mg^{2+} of the standard medium and the other media used in this study are shown in Table 1.
0.6 g of Na_2SO_4/l was added to medium III, IV and V as a sulphur source instead of $MgSO_4$. In medium VI, potassium was added as KH_2PO_4 (0.44 g/l) and as KCl (2.5 g/l). To medium VII, 0.6 g of $Na_2HPO_4 \cdot H_2O$/l was added.
The organisms were always precultivated in the same medium as used for growth, 1 ml of a full grown culture (OD_{623} = 2) was used as inoculum.

Growth measurements

Growth of cultures was monitored by measuring the optical density at 623 nm using a Vitatron colorimeter (Vitatron, Dieren, The Netherlands)

Determination of intracellular concentrations of Mg^{2+}, Ca^{2+} and K^+

Cells were centrifuged for 10 min at 8,000 x *g* at 4°C, concentrated in 5 % of the remaining supernatant and separated from the medium by centrifugation through silicon oil (d = 1.023) (Bakker and Harold 1980). After removal of the supernatant and the silicon oil, the pellet was resuspended in a small volume of water, boiled for 10 min and centrifuged for 5 min at 13,000 rpm in a microcentrifuge (Heraeus biofuge 13, Amsterdam, The Netherlands). The supernatant was divided into two parts. One part was directly used for cation determinations. To the second part, streptomycin sulfate was added to a final concentration of 1 % (w/v). After centrifugation for 5 min at 13,000 rpm in a microcentrifuge, the supernatant was also used for cation determinations. Cation concentrations were measured in an atomic absorption spectrophotometer (Perkin Elmer 560). Streptomycin sulfate was added to estimate the amount of cations present in polyphosphategranules. Streptomycin sulfate precipitates polyphosphate. Since polyphosphates are complexed with cations, it was assumed that precipitation of polyphosphate by streptomycin sulfate also results in a precipitation of the counterions. A specific internal cell volume of 2.9 μl/mg of protein was used for the calculation of intracellular cation concentrations (Bakker and Mangerich 1981).

Determination of the dissociation constants of K- Mg- and Ca polyphosphate

The dissociation constants of K- Mg- and Ca-polyphosphate were determined according to Van Wazer and Campanella (1950). Graham's salt was prepared by heating $NaH_2PO_4 \cdot 2H_2O$ during 3 hours at 750°C (Kulaev 1979). After rapid cooling, the polymerized product was dissolved in water. Dowex 50 WX8 was added, the suspension was mixed for 1 hour and filtered (Whatman no. 5 filterpaper). Two ml of the polyphosphoric acid (39 g P/l) was titrated with 0.1 N tetramethylammonium hydroxide in a volume of 10 ml. The effect of salts on the titration was studied by the addition of 5 x 10^{-4} mol KCl, $CaCl_2$ or $MgCl_2$ to the polyphosphoric acid solution.

Protein measurements

Total cell protein was determined according to Lowry et al. (1951) with bovine serum albumin

as the standard.

Electron microscopy

Cells were harvested by centrifugation at 8,000 x *g* for 10 min at 4°C, washed twice in demineralized water and concentrated 10-fold in a small amount of demineralized water.
The scanning transmission mode of the scanning electron microscope was used to image the polyphosphate bodies in whole unfixed cells of *Acinetobacter* strain 210A. The elemental composition of the polyphosphate granules was examined by *in situ* energy-dispersive X-ray micro-analysis. At low temperature the procedure was as follows: a small droplet of the bacterial suspension was placed on a thin Formvar film which in turn was supported by a copper grid. The film was blotted dry after 30 seconds with filter paper and immediately mounted on a special cryo-holder (Philips model) for the Philips EM 400T scanning transmission electron microscope. The specimen holder was evacuated in the prevacuum chamber of the microscope and as a result the bacterial film froze on the grid. Specimens were kept frozen during the observation and analysis in the electron microscope. The accelerating voltage was 80 kV and the specimens were tilted over 18^0 towards the X-ray detector for accurate X-ray collecting geometry. Backscattered electron imaging was used to visualize the bacteria. Granules were analysed with the EDAX 9100 system using an electron beam with a diameter of 100 nm.
For analysis at room temperature the procedure was slightly modified: A small droplet of the suspension was placed on a thin Formvar film which in turn was supported on a copper grid. The film was blotted dry after 30 seconds with filter paper and mounted on a specimen holder for the Philips EM 400T. Backscattered electron imaging and the 80 kV accelerating voltage were used to image the specimen. Granules were analysed as above.
For each sample studied, spectra were collected from 3 to 6 individual granules. Spectra of the cytoplasm served as controls.

Chemicals

All chemicals were of analytical grade and commercially available.

Results

Standard medium

Log phase cells of *Acinetobacter* strain 210A grown in batch cultures of standard medium contained granules which stained blue-violet with Neisser staining (Gurr 1965). The cells possessed one or two large granules with a diameter ranging from 0.2 to 0.4 μm (Fig. 1).

Figure 1. STEM image of *Acinetobacter* strain 210A grown in standard medium. Bar represents 1 μm.

In addition to these large granules, numerous smaller electron dense bodies were present varying in diameter from 0.01 to 0.1 μm (not shown). Elements consistently present in the large polyphosphate bodies were phosphorus, magnesium and potassium (Fig. 2A). Calcium

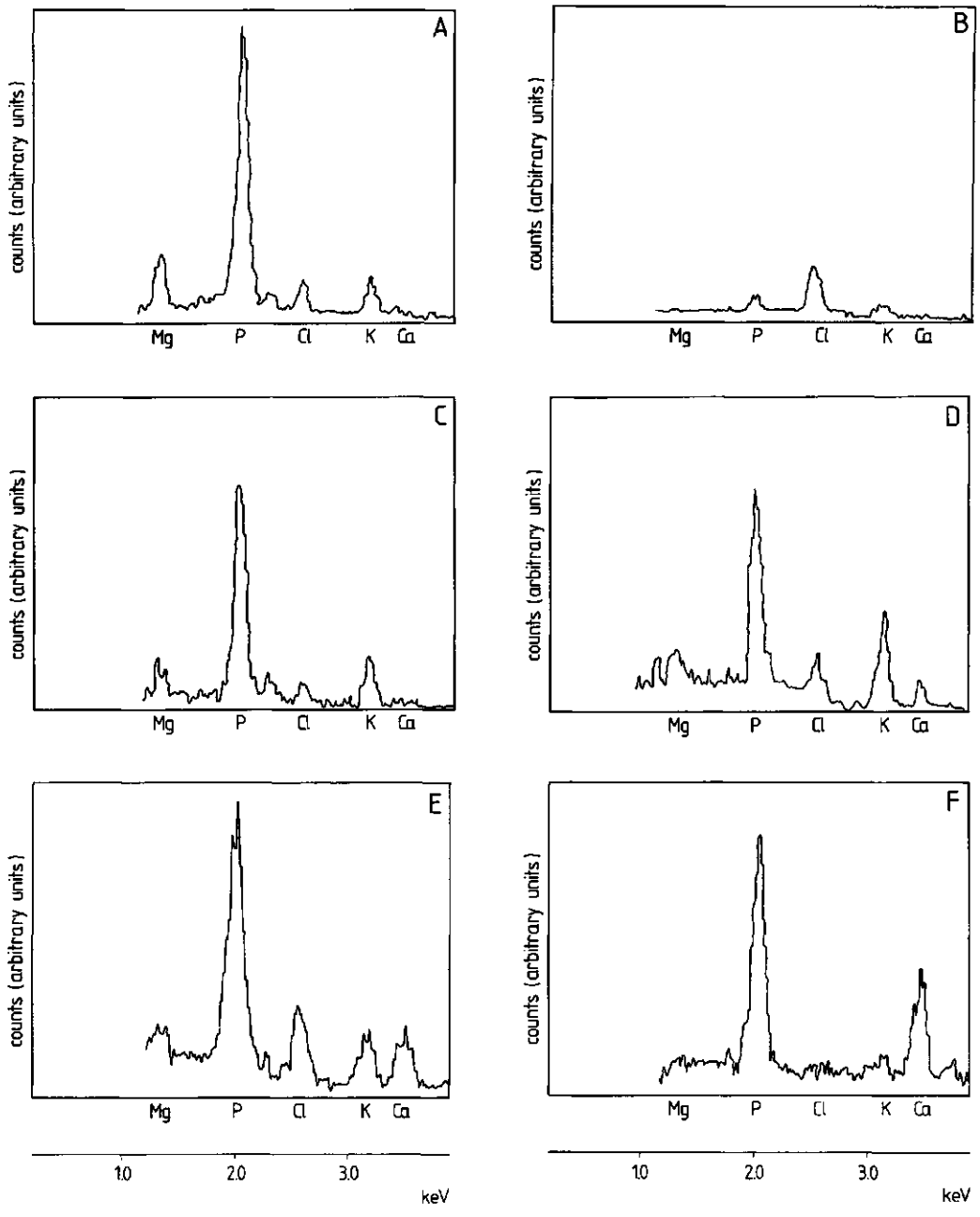

Figure 2. EDAX spectrum of (A) a large polyphosphate body of *Acinetobacter* strain 210A grown in standard medium; (B) cytoplasm of *Acinetobacter* strain 210A grown in standard medium; (C) large polyphosphate bodies of *Acinetobacter* strain 210A grown in medium I; (D) grown in medium II; (E) grown in medium III; (F) grown in medium IV.

was always completely absent, and chlorine is probably not specific for the granules because similar concentrations were present in the cytoplasm (Fig. 2B).

Ratio between magnesium and calcium

EDAX images. To study whether magnesium could be replaced by calcium as counterion of the large polyphosphate granules, the Mg/Ca-ratio in the medium was varied. Omission of Ca^{2+} from the medium (medium I) did not affect the formation of the large polyphosphate granules. The elemental composition of the large polyphosphate granules was similar to cells grown in standard medium. Only magnesium and potassium could be detected as counterions (Fig. 2C). Growth of the organism in medium without Ca^{2+} was slower. The doubling time increased from 4-5 hours to about 20 hours. Addition of extra Ca^{2+} in the standard medium to a concentration of 4.1 mM (medium II) resulted in polyphosphate granules that contained magnesium, potassium and calcium (Fig. 2D). By lowering the amount of Mg^{2+} 80-fold, the Mg/Ca-ratio in the medium was further decreased (medium III). The EDAX spectrum of cells grown under these conditions showed magnesium, potassium, and calcium peaks which were equally high (Fig. 2E). The lowest Mg/Ca-ratio used in this study was obtained by a 80-fold reduction of the amount of Mg^{2+} and a simultaneous 10-fold increase in the Ca^{2+} concentration (medium IV). Cells grown in this medium possessed large polyphosphate granules. Spectra of these granules showed that only calcium was present as counterion (Fig. 2F). About 20 % of the granules examined, showed small potassium peaks in the EDAX spectrum. All granules lacked magnesium as counterion. Chlorine was not detected.

Growth in a medium, that contained only 0.03 mM Mg^{2+} and no Ca^{2+}, was slow (doubling time more than 30 hours). Large polyphosphate granules were not formed (not shown).

Intracellular cation concentrations. Intracellular concentrations of Mg^{2+} and Ca^{2+} were studied in cells grown in standard medium, medium I and medium IV. From Table 2 it is obvious that the Mg/Ca-ratio in the cell is determined by the Mg/Ca-ratio in the medium. In cells which were grown in media that contained 2.44 mM Mg^{2+} (standard medium and medium I), the intracellular Mg^{2+} concentration was relatively high. The concentration of Mg^{2+} was 2-fold lowered when polyphosphates were precipitated by the addition of streptomycin sulfate, indicating that some magnesium in the cell is part of the polyphosphate granule. This finding is in line with the results obtained by EDAX (Fig. 2A, C). By lowering the amount of Mg^{2+} in the medium 80-fold (medium IV), the Mg^{2+} concentration in the cell was decreased three times. Addition of streptomycin sulfate did not precipitate Mg^{2+}. This is in accordance with the EDAX image (Fig. 2F).

By raising the Ca^{2+} concentration in the medium from 0 *via* 0.41 to 4.1 mM (medium I, standard medium and medium IV), the intracellular concentration of Ca^{2+} markedly increased (Table 2). Precipitation of polyphosphate by streptomycin sulfate resulted only in a decrease of the Ca^{2+} concentration in extracts obtained from cells grown at 0.41 and 4.1 mM Ca^{2+}. This suggests that under these conditions calcium is present as counterion in the polyphosphate granule. The EDAX image of cells grown at 4.1 mM Ca^{2+} shows a large calcium peak (Fig. 2F). This peak is, however, absent in cells grown at 0.41 mM Ca^{2+} (Fig. 2A). The amount of calcium present in the polyphosphate granule of cells grown at 0.41 mM Ca^{2+} is probably too low to be detected by EDAX.

The intracellular concentration of K^+ showed a relatively constant value of 180 mM which

Table 2. Intracellular concentrations of Mg^{2+} and Ca^{2+} in *Acinetobacter* strain 210A before and after addition of streptomycin sulfate.

Media	Mg^{2+}-concentration (mM)		Ca^{2+}-concentration (mM)	
	Before	After	Before	After
Standard	90 (± 14)	38 (± 20)	6.3 (± 1.2)	3.6 (± 1.1)
Medium I	87 (± 18)	46 (± 8)	1.8 (± 0.9)	1.3 (± 0.5)
Medium IV	33 (± 12)	35 (± 17)	59 (± 6)	45 (± 18)

was not lowered by the addition of streptomycin sulfate.

Dissociation constants of K- Mg- and Ca-polyphosphate

To examine whether the substitution of magnesium by calcium is caused by the relative abundance of each cation and their solubility constants with polyphosphate, the dissociation constants of Ca- and Mg-polyphosphate were determined. Since addition of metal ions has a pronounced effect on the pH of a polyphosphoric acid solution, we used pH measurements to estimate the dissociation constants (Van Wazer and Campanella 1950). Titration curves of polyphosphoric acid in the absence and in the presence of Ca^{2+} and Mg^{2+} are shown in Figure 3. The shape of the pH titration curve of the polyphosphoric acid solution is altered by addition of Ca^{2+} or Mg^{2+}. This is due to complex formation between the added metal ion and the phosphate polymer which gives rise to exchange of the weakly acidic protons of the polyphosphate chain by the metal ions. This results in a decrease of the pH. From the titration curves, dissociation constants for Mg- and Ca-polyphosphate can be calculated on the basis of elementary mass action law and mass balances (Van Wazer and Campanella 1950). Values of 9.3×10^{-2} mol/l and 1.5×10^{-1} mol/l were found for Mg- and Ca-polyphosphate, respectively. Addition of KCl to the polyphosphoric acid solution did not affect the titration curve which suggests that K-polyphosphate has a high dissociation constant and is therefore very soluble (not shown).

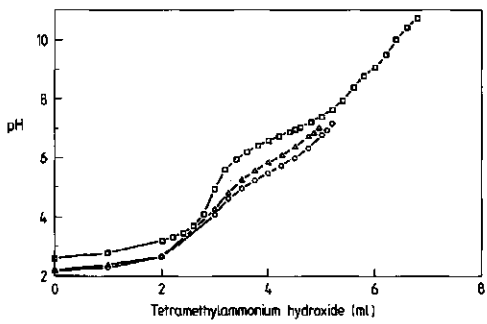

Figure 3. Titration of polyphosphoric acid with 0.1 N tetramethylammonium hydroxide. □ , polyphosphoric acid; ▵ , polyphosphoric acid + $CaCl_2$; ○ , polyphosphoric acid + $MgCl_2$

Ratio between magnesium and potassium

When it became clear that calcium was able to function as sole counterion in the polyphosphate granule, it was interesting to investigate whether magnesium and potassium could act in the same way. *Acinetobacter* strain 210A was grown in the standard medium with different magnesium and potassium concentrations. Cells grown in potassium-enriched medium (medium VI) as well as in potassium-deficient medium (medium VII), contained polyphosphate granules with

magnesium and potassium as counterions. Growth of cells in potassium-deficient medium was strongly reduced, and large granules were much less numerous. A number of smaller electron dense bodies were distributed throughout the cytoplasm (not shown).

Discussion

The successful use of air-dried, unfixed and unstained cells for *in situ* energy dispersive X-ray micro-analysis of polyphosphate granules has been described for *Spirillum itersonii*, *Corynebacterium diphtheriae* and *Micrococcus luteus* (Webster et al. 1984) and *Plesiomonas shigelloides* (Ogawa and Amano 1987). By using these methods, *Acinetobacter* strain 210A grown in the standard medium was found to have large granules containing phosphorus, magnesium and potassium. The composition of the small granules in strain 210A could not be measured since they were below the limit of detection of the STEM-EDAX system. A similar situation has been encountered in *Plectonema boryanum* (Baxter and Jensen 1980a). Assumedly the smaller black bodies in strain 210A are also polyphosphate bodies. In *Plesiomonas shigelloides* only small polyphosphate bodies were observed at the onset and early stage of growth, ranging from 50 to 150 nm in diameter. During the log phase these bodies increased in size to 550 nm in diameter (Ogawa and Amano 1987).

The large polyphosphate bodies in *Acinetobacter* strain 210A have a dynamic elemental composition. By decreasing the Mg/Ca-ratio in the growth medium, magnesium was replaced by calcium as counterion of the large polyphosphate granules. The results obtained by EDAX correlate well with the intracellular cation concentrations. At a relatively high intracellular Mg^{2+} concentration, magnesium is the predominant counterion in the polyphosphate granule whereas calcium becomes the major cation in the polyphosphate body if the intracellular Ca^{2+} concentration increases. The presence of magnesium or calcium in the polyphosphate granules was not very surprising since both Mg- and Ca-polyphosphate have a relative low solubility. The cation composition of the large polyphosphate granules correlated well with the Ca/Mg-ratio in the cell. These results suggest a chemical precipitation as the mechanism that determines the cation composition in the polyphosphate body. The presence of potassium in the polyphosphate granules was not expected since K-polyphosphate is a very soluble compound. However, polyphosphate of *Acinetobacter* strain 210A appeared to be far higher polymerized than the synthetic polyphosphate used in this study (data not shown). Kurrol's salt, a highly polymeric K-polyphosphate is insoluble in water (Kulaev 1979). The presence of potassium in the large polyphosphate granules may therefore also be explained by chemical precipitation. The possible involvement of the enzymatic synthesis in the cation composition of the large polyphosphate granules is not known since the biosynthesis of polyphosphate is yet unclear. *Acinetobacter* strain 210A lacks polyphosphate kinase activity (Van Groenestijn 1988). Potassium is known to be required for the bacterial uptake of phosphate (Rosenberg 1987). Potassium has been shown to stimulate the uptake of phosphate by *Acinetobacter* strain 210A and by activated sludge (Van Groenestijn et al. 1988). It is therefore not surprising that only a few smaller granules were seen in potassium deficient cells of *Acinetobacter* strain 210A.

Plectonema boryanum fixed in glutaraldehyde and then embedded in either Epon or Durcupon possessed large granules which contained almost only phosphorus and calcium when thick sections were examined *in situ* with X-ray energy dispersive analysis (Sicko-Goad et al. 1975). Air-dried cells, however, possessed polyphosphate

bodies with high concentrations of phosphorus and potassium and low concentrations of magnesium and calcium. The discrepancy between these results could be ascribed to fixation and embedding procedures. Fixation of cells in glutaraldehyde resulted in the loss of the potassium peak and in the enhancement of the calcium peak while magnesium was lost during embedding in Epon (Baxter and Jensen 1980a). In previous studies dealing with the elemental composition of large polyphosphate granules in activated sludge or *A. calcoaceticus* var. *lwoffii*, samples for EDAX analysis were prepared by fixation in glutaraldehyde (Buchan 1981, 1983; Heymann et al. 1989). The composition of the polyphosphate bodies reported by these authors must therefore be regarded with caution in view of the profound effects of glutaraldehyde on the elemental composition of polyphosphate granules. Suspensions of *Acinetobacter* strain 210A containing significant amounts of polyphosphate released phosphate together with magnesium and potassium when incubated in the absence of oxygen (Van Groenestijn et al. 1988). The results of the experiments reported here, indicate that magnesium and potassium are the counterions in the large polyphosphate granules during growth in the standard medium. By modifying the amount of Ca^{2+} and Mg^{2+} in the medium, the intracellular concentration of Ca^{2+} and Mg^{2+} as well as the elemental composition of the polyphosphate granules of *Acinetobacter* strain 210A can be changed. Therefore, the mechanism that determines the presence of cations in the large polyphosphate granules is probably based on chemical precipitation.

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CHAPTER 4

Properties, synthesis and degradation of polyphosphate of *Acinetobacter johnsonii* 210A

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Properties, synthesis and degradation of polyphosphate of *Acinetobacter johnsonii* 210A

Abstract

Polyphosphates accumulated by *Acinetobacter johnsonii* 210A were isolated and characterized. The polymers consisted of about 700 P-groups and were localized in the cytoplasm mostly complexed in one or two large and several small granules. Polyphosphate formation was followed by ^{31}P NMR spectroscopy. Cells grown in low P_i-medium were energized with glucose/PQQ in the presence of orthophosphate. The amount of polyphosphate formed, correlated positively with the intracellular ATP concentration suggesting an involvement of polyphosphate kinase in polyphosphate synthesis. Dense non aerated suspensions of cells aerobically grown in high P_i-medium were used to follow polyphosphate degradation. Initially, polyphosphate was degraded relatively rapidly. The polyphosphate degradation rate decreased during the experiment. Only the amount but not the size of the P-polymers did alter. The polymers had about 700 P-residues.

Introduction

Inorganic polyphosphates are linear polymers in which orthophosphate groups are linked by energy-rich phosphate anhydride bonds. The number of phosphate residues in these compounds may vary noticeably: from two in pyrophosphate to several hunderds and thousands in high molecular-weight polymer phosphates (Kulaev and Vagabov 1983). Microorganisms are able to accumulate considerable amounts of polyphosphate. In yeasts, the polyphosphate content can account for 20 % of the biomass dry weight (Liss and Langen 1962). Strains of *Acinetobacter* are able to accumulate phosphorus up to 10 % of their dry weight (Deinema et al., 1985).

The presence of polyphosphate can be demonstrated by high-resolution ^{31}P NMR spectroscopy which is a non-invasive technique that has been used in a large number of studies to localize polyphosphate in bacteria (Ostrovskii et al. 1980; Streichan and Schön 1991), yeasts (Beauvoit et al. 1989; Tijssen and Van Steveninck 1984), and algae (Sianoudis et al. 1986). ^{31}P NMR can also be applied to study polyphosphate sizes (Niere et al. 1990; Rao et al. 1985). It is the only method available for determining the average chain length of soluble polyphosphate as it exists within the cell. Polyphosphate complexed in granular structures or bound to nucleic acids or proteins will, due to its immobile nature, not be detected in a high-resolution ^{31}P NMR spectrum (Roberts 1987).

Several enzymes have been found which mediate polyphosphate synthesis and degradation (Wood and Clark 1988). Polyphosphate kinase has been claimed to be the major enzyme responsible for

polyphosphate formation in most microorganisms (Kulaev 1979). The mechanism of polyphosphate formation in *Acinetobacter* sp. is, however, still unclear. T'Seyen et al. (1985) studied polyphosphate kinase activity in cell extracts of several pure cultures of *Acinetobacter* sp. They found very low activities ranging from 0.7 to 4.7 nmol/min · mg protein. Similar values were reported by Van Groenestijn et al. in *Acinetobacter* strain B8 and P. Other *Acinetobacter* strains tested did not show any activity of this enzyme (Van Groenestijn et al. 1989). The degradation of polyphosphate in *Acinetobacter johnsonii* 210A is mediated by two enzymes (Van Groenestijn et al. 1989). Polyphosphatase catalyzes the hydrolysis of polyphosphate to P_i: PP_n (polyphosphate) + $H_2O \rightarrow PP_{n-1} + P_i$. Polyphosphate:AMP phosphotransferase phosphorylates AMP to ADP with polyphosphate: AMP + $PP_n \rightarrow$ ADP + PP_{n-1} (Van Groenestijn et al. 1987).

The aim of this work was to isolate and characterize polyphosphate of *A. johnsonii* 210A and to investigate its degradation and synthesis by the use of polyphosphate extractions and *in vivo* ^{31}P NMR spectroscopy.

Material and methods

Organism and cultivation

Acinetobacter johnsonii 210A was grown at 20°C in a buffered (pH 7) medium. The medium contained per liter: 2.29 g of sodium butyrate, 0.1 g of $Na_2S_2O_3 \cdot 5\ H_2O$, 1.0 g of NH_4Cl, 0.6 g of $MgSO_4 \cdot H_2O$, 0.06 g of $CaCl_2$, 6 g of Tris, 0.1 g of EDTA and trace elements as described by Van Groenestijn et al. (1987). The high P_i-medium contained in addition to these compounds per liter: 0.44 g of KH_2PO_4. The low P_i-medium contained per liter an additional 0.044 g of KH_2PO_4 and 0.215 g of KCl.

Polyphosphate synthesis

0.5 L of cells grown in low P_i-medium were washed in 100 mM K-PIPES buffer (pH 6.7) and resuspended in 100 mM K-PIPES buffer (pH 6.7) with 1 mM EDTA. After 10 min of incubation at 30°C, $MgSO_4$ was added to a final concentration of 10 mM and the suspension was centrifuged for 20 min at 8,000 x *g*. The pellet was resuspended in 10 ml 100 mM K-PIPES buffer (pH 6.7). 1 ml of D_2O, 0.5 ml of K-phosphate buffer (0.5 M, pH 7), 0.5 ml of glucose (1.0 M) and 0.2 ml of Pyrrolo-quinoline quinone (PQQ) (1 mM) were added after which the suspension with an A_{660nm} of about 25 was incubated anaerobically for 3 hours at room temperature. Polyphosphate synthesis was started by gassing the suspension with 100% O_2. To investigate the effect of N,N'-dicyclohexylcarbodiimide (DCCD) on polyphosphate synthesis an additional 0.1 ml of DCCD (5 mg/ml) was added to the cells and samples were taken at different time intervals during the anaerobic preincubation. ^{31}P NMR was used to follow the polyphosphate formation and samples for ATP measurements and electron microscopy were taken directly out of the NMR tube.

Polyphosphate degradation

1 L of cells grown in high P_i-medium were washed and resuspended in 22.5 ml 50 mM Tris-HCl buffer (pH 7.6). 2.5 ml D_2O was added after which the cell suspension with an A_{660nm} of about 20 was transferred into an NMR tube. ^{31}P NMR scans were recorded and samples for phosphate measurements and polyphosphate isolation were taken simultanuously at different time intervals.

Analytical methods

Protein was estimated by the method of Lowry

et al. (1951) with bovine serum albumin (BSA) as the standard. Orthophosphate and total phosphorus (persulfate digestion method) were determined spectrophotometrically according to Standard Methods (1976). Bacterial dry weight was quantified by centrifuging culture samples at 8,000 x *g* for 10 min, washing the pellets twice with demineralized water and drying them to constant weight at 105°C. ATP concentrations were determined with the firefly luciferase assay as described by Otto et al. (1984). A specific internal cell volume of 2.9 µl/mg protein was used for the calculation of intracellular ATP concentrations (Bakker and Mangerich 1981). Polyphosphates were isolated according to Clark et al. (1986) with modifications as described by Bonting et al. (1992a). Chain lengths of the isolated polyphosphates were determined according to Clark and Wood (1987) by using 1.5 % agarose gels.

^{31}P NMR

In vivo ^{31}P NMR spectra were recorded at 121.5 MHz using a 300 MHz Bruker AMX spectrometer equipped with a 20 mm triple probehead tuned to ^{31}P. Spectra were obtained at 25°C using 20 mm outer diameter NMR tubes containing 12 ml (polyphosphate synthesis experiments) or 25 ml (polyphosphate degradation experiments) of cells. Acquisition parameters were the following: 60° pulse angle, 0.4 acquisition time, 0.1 s of delay, 1800 free induction decay's. In order to determine cytoplasmic pH values, a calibration curve relating the chemical shift of cytoplasmic P_i and the internal pH, was obtained using cells suspended in buffer as described by Nicolay et al. (1981) with valinomycin (0.2 mM) and nigericin (0.2 mM). External pH values were measured with a pH electrode and were close to those determined by use of the calibration curve.

Chemicals

The NRB/LUMIT-PM kit was obtained from Perstorp Analytical (Oud-Beijerland, The Netherlands). O_2 and N_2 gas were from Hoek Loos (Schiedam, The Netherlands). All other chemicals were of analytical grade and commercially available.

Results

Properties of polyphosphate

Localization. Acinetobacter johnsonii 210A is able to accumulate polyphosphate when grown in the presence of excess phosphate and energy (Van Groenestijn 1988). Cells grown in high P_i-medium possessed one or two large and several smaller polyphosphate granules. The large granules were mostly localized at the polar ends of the cell. The smaller granules were distributed throughout the cytoplasm (not shown). To examine the possible presence of polyphosphate in the periplasm, cells grown in high P_i-medium were centrifuged and concentrated in 5 % of the remaining supernatant. Subsequently, EDTA was added to a final concentration of 10 mM. EDTA can freely diffuse into the periplasm and will bind divalent cations originating from the polyphosphate when present in this compartment. Since the lineshape of mobile polyphosphate is sensitive for removal of these bound cations (Ostrovskii et al. 1980), a change in the polyphosphate resonance is expected when polyphosphate is present in the periplasmic space. Cells treated with EDTA did, however, not show such a change in the lineshape. This indicates that *A. johnsonii* 210A does not contain periplasmic polyphosphate.

Isolation. Polyphosphates were extracted from cells grown in high P_i-medium as described by

Table 1. Amount of phosphorus in the polyphosphate fractions extracted from *Acinetobacter johnsonii* 210A before (ortho-P) and after (total-P) boiling with 1 N HCl

Fraction	Ortho-P (mg P · g dry weight^{-1})	Total-P (mg P · g dry weight^{-1})
LPP	1.1	1.2
Non granular HPP	0.1	8.0
Granular HPP	0.1	9.5

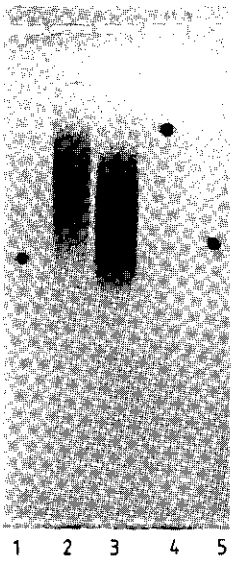

Figure 1. Electrophoresis of polyphosphates extracted from *A. johnsonii* 210A on a 1.5 % agarose gel. Lanes: 1, bromphenol blue; 2, granular HPP; 3, non granular HPP; 4, xylene cyanol; 5, bromphenol blue

Bonting et al. (1992a). The amount of phosphorus in the low polymeric PP_n fraction (LPP, $n<20$) was always low and consisted mainly of orthophosphate. High amounts of phosphorus were present in the high polymeric PP_n fractions (HPP, $n>20$). In these fractions the orthophosphate content was negligible (Table 1).

The recovery of the method was rather low, less than 60 % of the cellular phosphorus was present in the isolated fractions.

Chain length. From Table 1 it can already be concluded that polyphosphates synthesized by *A. johnsonii* 210A are at least 20 P-groups long. To determine the exact chain length of the non granular and granular polyphosphates, the polymers were electrophoresed on a 1.5 % agarose gel. Bromphenol blue and xylene cyanol were used as markers. These dyes comigrate with polyphosphate chains of 470 and 1200 P-groups, respectively (Clark and Wood 1987). Figure 1 shows a 1.5 % agarose gel with two small smears derived from a non granular and granular HPP fraction. From the positions of the smears and the marker dyes, a polyphosphate chain length of about 700 ($\pm$ 300) P-groups was calculated.

Polyphosphate synthesis

Since it was not possible to detect polyphosphate synthesizing activity *in vitro*, a method was developed to study polyphosphate synthesis *in vivo* in *A. johnsonii* 210A. Polyphosphates are high energy phosphorus compounds and therefore sufficient amounts of energy and phosphate must be present in the cell to allow the organism to synthesize these polymers. *A. johnsonii* 210A cells were energized by using their ability to oxidize glucose to gluconic acid in the presence of PQQ (Bonting et al. 1992b). Oxidation of glucose is coupled to the respiratory chain and recently it was shown that addition of glucose and PQQ resulted in a stimulation of the proton

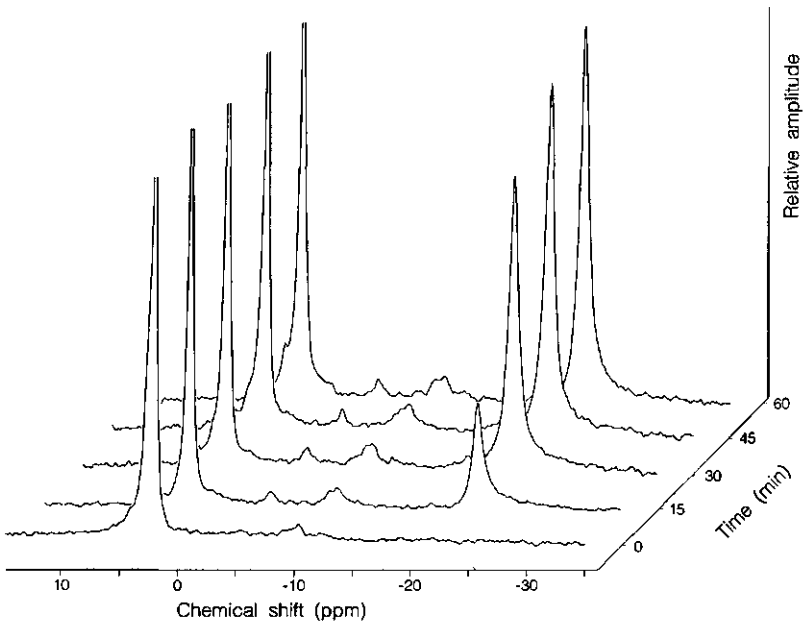

Figure 2A. Time course of the synthesis of polyphosphate as measured in NMR spectra during incubation of *A. johnsonii* 210A in the presence of P_i, glucose and PQQ. Chemical shifts: 2.8 ppm P_i (intracellular, t = 60 min), 1.3 ppm P_i (extracellular, t = 60 min), -5.0 ppm γ-ATP, -5.4 ppm ß-ADP, -9.6 ppm α-ADP, -10.0 ppm α-ATP, -18.7 ppm ß-ATP, -22.7 ppm polyphosphate

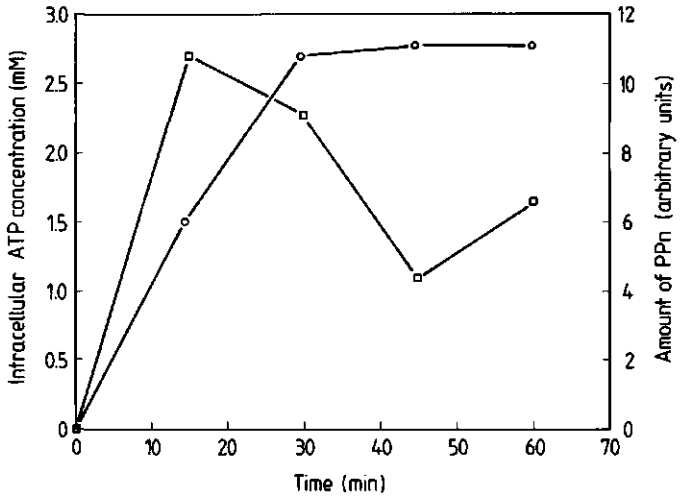

Figure 2B. Time course of the area of the polyphosphate signal (○) and the intracellular ATP concentration (□) of cells energized with glucose and PQQ.

motive force (Van Veen et al. 1993).

Cells used in this study were grown in low P_i-medium in order to prevent polyphosphate synthesis during growth. Cells grown at low P_i-concentrations show a high P_i-uptake rate (Bonting et al. 1992a). ^{31}P NMR was applied to follow polyphosphate formation *in vivo*.

Figure 2A shows NMR spectra of cells without polyphosphate energized with glucose/PQQ in the presence of 20 mM extracellular phosphate. Within 15 min, orthophosphate was taken up and polyphosphate was formed. Next to polyphosphate formation there was a rapid increase in the intracellular ATP level (Fig. 2B) and a stimulation of the transmembrane pH gradient (Δ pH). After 60 min of incubation a Δ pH value of 1.3 could be calculated from the chemical shifts of the internal P_i (2.84 ppm) and the external P_i (1.34 ppm) signals in the ^{31}P NMR spectrum and the titration curve.

To investigate whether ATP is involved in polyphosphate synthesis, cells were pre-incubated with the H^+-ATPase inhibitor DCCD and subsequently energized with glucose/PQQ. After a pre-incubation of 45 min with DCCD (7.5 μg/mg protein), a relatively high intracellular ATP level and a substantial formation of polyphosphate were observed. When cells were pre-incubated for 135 min, both the intracellular ATP concentration and the amount of polyphosphate decreased significantly. After a pre-incubation of 225 min, the cells were not able to synthesize polyphosphate anymore. The ATP levels in these cells were very low (Fig 3). The positive correlation between the intracellular ATP levels and the amount of polyphosphate, suggests a possible involvement of ATP in polyphosphate synthesis. In none of the polyphosphate synthesis experiments, the resonances of short chain polyphosphates like pyro-, tri-and tetraphosphate or resonances of the terminal, and penultimate phosphate groups of longer polyphosphate chains were observed. The P-polymers synthesized by

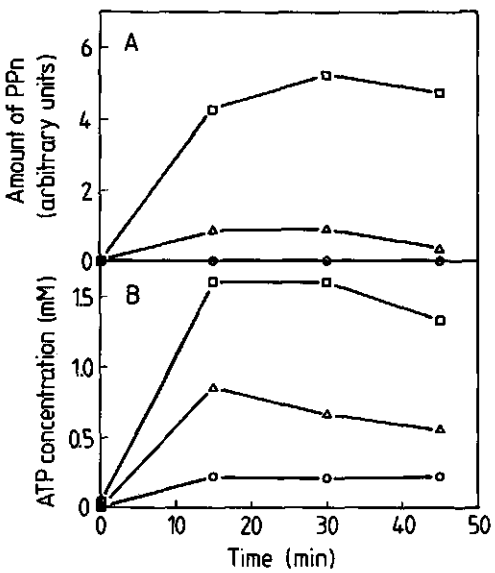

Figure 3. Time course of (A) polyphosphate resonance intensities and (B) intracellular ATP levels of cells pre-incubated with DCCD and energized with glucose and PQQ. Pre-incubation with DCCD was for 45 min (□), 135 min (△), and 225 min (○).

the use of glucose/PQQ showed on a 1.5 % agarose gel similar migration patterns as polyphosphate extracted from batch grown cells (not shown). This points to a direct synthesis of highly polymerized polyphosphates via a processive mechanism.

Polyphosphate degradation

Polyphosphate degradation was monitored by ^{31}P NMR. A dense cell suspension was incubated in a NMR tube and samples for polyphosphate isolation and for phosphate measurements were taken at different time intervals. Initially, the polyphosphate degradation rate was relatively high (Fig. 4). When the suspension was incubated for more than 4 hours, the rate of polyphosphate degradation decreased. The polyphosphate signal in the ^{31}P NMR spectrum gradually declined during the experiment (not shown). Mostly, the signal had completely disappeared after 24 hours

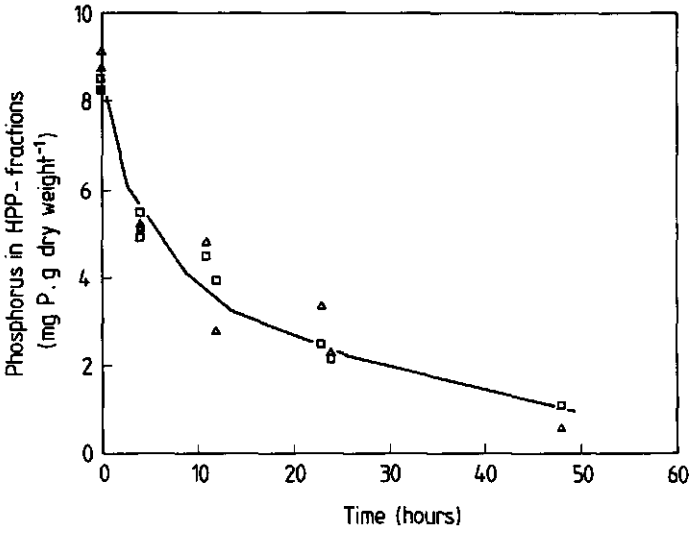

Figure 4. Amount of phosphorus present in the high polymeric polyphosphate fractions during polyphosphate degradation in non aerated dense cell suspensions of *Acinetobacter johnsonii* 210A; (□) non granular HPP, (▵) granular HPP.

of incubation although still some polyphosphate was present in the cells. The slowly degradable polyphosphate is probably complexed in granules which can not be detected by high-resolution ^{31}P NMR.

During the incubation, the amount of phosphate in the supernatant increased due to polyphosphate degradation. The phosphate release showed a relatively constant rate of 1 mg P/g dry weight · hour. The discrepancy between the rate of decrease in the amount of phosphorus present in the polyphosphate fractions and the rate of increase in the level of phosphorus in the supernatant is possibly due to the low recovery of the polyphosphate isolation procedure and lysis of the cells.

Polyphosphate degradation was also followed by gelelectrophoresis. Polyphosphates isolated during the experiment were electrophoresed on a 1.5 % agarose gel. The HPP fractions contained only high polymerized polyphosphates with a mean chain length of about 700 P-groups (not shown). No small polyphosphates were detected. The amount of phosphorus in the LPP fraction was always low (< 1 mg P/g dry weight).

Discussion

Under all conditions tested, polyphosphates of *Acinetobacter johnsonii* 210A exhibit a chain length of about 700 P-groups. This is remarkable since polyphosphates obtained from microorganisms usually cover a wide range of molecular sizes and several polyphosphate fractions each containing polymers with different chain lengths can be isolated (Liss and Langen 1962, Kulaev 1979). Time courses of polyphosphate accumulation and degradation normally show a change in the content of the polyphosphate fractions resulting in a change in the average polyphosphate chain length (Rao et al. 1985, Langen and Liss 1958). In *A. johnsonii* 210A, however, neither during polyphosphate synthesis nor during polyphosphate degradation intermediate sized P-polymers were observed.

Polyphosphates present in cells of *A. johnsonii* 210A are localized exclusively in the cytoplasm and are mostly complexed in either large or small granules. The localization of polyphosphate in several *Acinetobacter* sp. and *Moraxella* sp. isolated from activated sludge, was studied by Streichan and Schön (1991). They observed only

cytoplasmic polyphosphates in the *Acinetobacter* sp., whereas the *Moraxella* strains stored polyphosphate intracytoplasmically as well as in the periplasm. The presence of polyphosphate in both the cytoplasmic and periplasmic space was also reported for *Acinetobacter lwoffii*, *Propionibacterium acnes*, and *Mycobacterium smegmatis* (Halvorsson et al. 1987; Kjeldstad et al. 1991; Ostrovskii et al. 1980).

In order to quantify the amount of polyphosphate in the cell, the isolation method developed by Clark et al. (1986) and ^{31}P NMR were used. Neither the extraction procedure nor ^{31}P NMR appeared to be completely reliable for this purpose. The isolation procedure had a low recovery and the discrimination between non granular and granular polyphosphate is doubtful. High-resolution ^{31}P NMR, although widely used in studying polyphosphate metabolism, only selects mobile, soluble pools of polyphosphate. Large polymers immobilized by complexation into rigid, high molecular weight structures in granules, or polyphosphates tightly bound to nucleic acids or proteins will not be detected in a high-resolution ^{31}P NMR spectrum (Roberts 1987). This probably explains the lack of a polyphosphate signal in NMR in cells which were incubated for more than 24 hours anaerobically and still contained polyphosphate.

Polyphosphate synthesis in *A. johnsonii* 210A was also followed by ^{31}P NMR spectroscopy. When cells without polyphosphate were energized by glucose/PQQ in the presence of phosphate, a polyphosphate signal emerged within a few minutes. The relatively rapid polyphosphate formation was only observed when ATP generation was possible. As soon as the formation of ATP was blocked, polyphosphate synthesis was impaired. This suggests that polyphosphate is formed *via* ATP by polyphosphate kinase. This hypothesis is supported by the absence of intermediate sized polyphosphates in *A. johnsonii* 210A. Polyphosphate kinase synthesizes polyphosphates without dissociation of intermediate sizes of the polymer from the enzyme. As a consequence, only high molecular weight polyphosphates are formed (Robinson and Wood 1986). However, the activity of polyphosphate kinase in cell extracts of *Acinetobacter* sp. is very low or even absent (Van Groenestijn et al. 1989, 'T Seyen et al. 1985). The discrepancy between the polyphosphate synthesis rate *in vivo* and the polyphosphate kinase activity *in vitro* can be interpreted in two ways. Either the polyphosphate kinase enzyme looses its activity during extract preparation or other polyphosphate synthesizing enzymes are involved in the formation of polyphosphate in *A. johnsonii* 210A. Two enzyme systems different from the polyphosphate kinase can be hypothesized: (1) a system in which a phosphorylated compound other than ATP is the P-donor for polyphosphate formation or (2) a membrane bound proton translocating polyphosphate synthesizing enzyme system. Both possibilities are, however, not supported by our results. Addition of several phosphorylated compounds such as acetylphosphate, phosphoenolpyruvate, ADP and glucose-6-phosphate to cell extracts of *A. johnsonii* 210A did not result in the formation of a polyphosphate signal in the ^{31}P NMR spectrum. The second hypothesis, which was already put forward by Comeau et al. (1986) is less likely since neither the 100,000 x *g* membrane fraction nor right side out vesicles contained any polyphosphatase activity. Therefore, we assume that despite its low activity in cell extracts, polyphosphate kinase is the most likely candidate for polyphosphate synthesis in *A. johnsonii* 210A.

Incubation of *A. johnsonii* 210A cells under anaerobic conditions resulted in a degradation of polyphosphate and a release of orthophosphate. The change in degradation rate of polyphosphate (Fig. 4) suggests the existence of two different polyphosphate fractions in the cell. A mobile

fraction which is readily degradable and an immobile fraction possibly complexed in granules which is degraded very slowly. The changes in the polyphosphate component in *Propionibacterium acnes* as observed by ^{31}P NMR were also assigned to the presence of two different polyphosphate pools. A long chained ^{31}P NMR 'invisible' pool complexed in granules and a short chain 'free' polyphosphate fraction in the cytoplasm (Kjeldstad et al. 1989).

In this study it is shown that polyphosphates synthesized by *A. johnsonii* 210A always consist of about 700 P-groups, are localized in the cytoplasm and are slowly degraded. Recently, it was shown that the combined action of polyphosphate:AMP phosphotransferase and adenylate kinase enables *A. johnsonii* 210A to use its polyphosphate as a source of ATP when energy generation is not otherwise possible (Van Groenestijn et al., 1987; Van Groenestijn, 1988). Data presented in this study support the possible involvement of ATP in polyphosphate synthesis in *A. johnsonii* 210A. As a consequence a close relationship between polyphosphate and energy metabolism is proposed.

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CHAPTER 5

Properties of Polyphosphate:AMP Phosphotransferase of *Acinetobacter* strain 210A

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Properties of polyphosphate:AMP phosphotransferase of *Acinetobacter* strain 210A

Abstract

Polyphosphate:AMP phosphotransferase, an enzyme which catalyzes the phosphorylation of AMP to ADP at the expense of polyphosphate, was purified more than 1,500-fold from *Acinetobacter* strain 210A by streptomycin sulfate precipitation and by Mono-Q, Phenyl Superose, and Superose column chromatography. Streptomycin sulfate precipitation appeared to be an effective step in the purification procedure. During the following chromatographic steps, there was a 29-fold increase in specific activity but the yield was low (0.3 %).

Kinetic studies showed apparent Km-values of 0.26 mM for AMP and 0.8 μM for polyphosphate with an average chain length of 35 phosphate groups. The highest activities were found with polyphosphate molecules of 18 to 44 phosphate residues. The polyphosphate chain was degraded completely to ADP. The mechanism of degradation is processive. No activity was obtained with ortho-, pyro-, tri- and tetraphosphate. The enzyme was inhibited by pyro-, tri- and tetraphosphate. The inhibition by tri- and tetraphosphate was mixed with polyphosphate as a substrate. The inhibition constants for the dissociation of the enzyme-inhibitor complex and for the enzyme-inhibitor-substrate complex were 0.9 and 6.5 mM, respectively, for triphosphate and 0.7 and 1.5 mM, respectively, for tetraphosphate.

Introduction

During growth, *Acinetobacter* strain 210A is able to accumulate up to 300 mg phosphate per g dry weight. The exact amount of phosphate depends on growth rate, substrate, limiting nutrients, and temperature (Van Groenestijn et al. 1989b). When energy generation is no longer possible (e.g., in absence of oxygen or an electron donor), polyphosphate is degraded and phosphate is released into the medium (Van Groenestijn 1988). Several polyphosphate-utilizing enzymes have been described: polyphosphate kinase in *Escherichia coli* (Kornberg 1957) and in *Propionibacterium shermanii* (Robinson et al. 1984), polyphosphate glucokinase in *Mycobacterium phlei* (Szymona and Ostrowski 1964), polyphosphate dependent NAD kinase in *Acetobacter, Achromobacter, Brevibacterium, Corynebacterium*, and *Micrococcus* spp. (Murata et al. 1980), 1,3-diphosphoglycerate:polyphosphate phosphotransferase in *Neurospora crassa* (Kulaev et al. 1968), polyphosphate:AMP phosphotransferase in *Corynebacterium xerosis* (Dirheimer and Ebel 1965) and polyphosphatase in *C. xerosis* (Muhammed et al. 1959). Only two of these enzymes, polyphosphatase and poly-

phosphate:AMP phosphotransferase, have been found in *Acinetobacter* strain 210A (Van Groenestijn et al. 1989a). Polyphosphatase catalyzes the hydrolysis of polyphosphate to orthophosphate: PP_n (polyphosphate) + $H_2O \rightarrow PP_{n-1} + P_i$. Polyphosphate:AMP phosphotransferase phosphorylates AMP to ADP with polyphosphate :$PP_n + AMP \rightarrow PP_{n-1} + ADP$. This second enzyme makes it possible for *Acinetobacter* strain 210A to conserve the energy liberated from the cleavage of polyphosphate. With adenylate kinase, ATP can be formed from two molecules of ADP. The combined action of polyphosphate:AMP phosphotransferase and adenylate kinase allows *Acinetobacter* strain 210A to use its polyphosphate as a source of ATP when energy generation is not otherwise possible (e.g., absence of oxygen) (Van Groenestijn et al. 1987). Little is known about polyphosphate:AMP phosphotransferase. Only one previous attempt has been made to purify the enzyme (Dirheimer and Ebel 1965). In the present study a partial purification and some properties of this enzyme of *Acinetobacter* strain 210A are reported.

Material and methods

Organism and cultivation

Acinetobacter strain 210A, isolated and described by Deinema et al. (1985), was mass cultured in a 300-liter fermenter filled with 200 liters of medium. The medium contained, per liter, 2.29 g of sodium butyrate, 0.1 g of $Na_2S_2O_3 \cdot 5\,H_2O$, 1.0 g of NH_4Cl, 0.6 g of $MgSO_4 \cdot 7\,H_2O$, 0.06 g of $CaCl_2 \cdot 2\,H_2O$, 0.1 g of EDTA, 3 g of K_2HPO_4, 2 g of KH_2PO_4, and trace elements as described by Van Groenestijn et al. (1987). Cells were harvested at the mid log phase by continuous centrifugation and stored until use at -20°C.

Enzyme purification

Unless stated otherwise, all purification steps were performed at 4°C. Cells (50 g) were thawed, diluted with 4 mM EDTA in 50 mM Tris-HCl (pH 7.6) in a 1:5 ratio, and disrupted at 0°C by sonication (ten times, 30 s each; interval pauses of 30 s; 40 W; Branson Sonic Sonifier). The broken cells were centrifuged for 12 min at 3,500 x *g*. The supernatant was collected and centrifuged again for 12 min at 13,000 x *g*. This supernatant contained about 10 mg of protein per ml and is referred to as crude extract.

To 20 ml of the crude extract, 1 ml of 20% (wt/vol) streptomycin sulfate was added. The precipitated material was collected by centrifugation at 30,000 x *g* for 15 min, and the supernatant was discarded. The precipitate was resuspended several times in 50 mM Tris-HCl (pH 7.6) and centrifuged at 30,000 x *g* for 15 min. In this way, the enzyme was extracted from the pellet. The supernatants from the pellet were collected and pooled. To this fraction, called streptomycin fraction, 0.5 mg DNase, and 0.13 mmol of $MgCl_2$ were added. This mixture was incubated for 2 h at 30°C to hydrolyze the DNA contaminating this fraction.

The following steps were performed with a high-resolution fast protein liquid chromatography system (Pharmacia/LKB, Woerden, The Netherlands) at room temperature. All columns were equilibrated with the starting buffers of the gradients. Linear gradients were used throughout. To prevent overloading, four aliquots of the streptomycin fraction were loaded separately onto a Mono-Q HR 5/5 anion-exchange column. A 35-ml gradient from 0 to 1.0 M NaCl in Tris-HCl (pH 7.6) was applied at a flow rate of 1.0 ml/min. Fractions with the highest activities were pooled and dialyzed overnight in a dialysis tube with a cutoff range of 1,000 Da against 50 mM Tris-HCl (pH 7.6) at 4°C. The dialyzed fraction

was applied again to a Mono-Q HR 5/5 anion-exchange column. Proteins were eluted with a 30 ml gradient from 0 to 0.5 M of NaCl in Tris-HCl (pH 7.6) at a flow rate of 1.0 ml/min. The most active fractions were pooled and mixed in a 1:1 ratio with 2 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl (pH 7.6). This sample was adsorbed to a Phenyl Superose HR 5/5 column. A 10 ml gradient from 1 to 0 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl (pH 7.6) at a flow rate of 0.5 ml/min was used to elute the protein. The fraction with the highest specific activity was injected on a Superose 6 HR 10/30 gel filtration column, equilibrated with 150 mM NaCl in 50 mM Tris-HCl (pH 7.6) and eluted at a flow rate of 0.2 ml/min. Fractions with the highest specific activities were collected and stored at -20°C until use.

Enzyme Assays

Polyphosphate:AMP phosphotransferase was measured photometrically at 340 nm by monitoring the reduction of $NADP^+$ as described by Van Groenestijn et al. (1987) (method A) or by monitoring the oxidation of NADH (method B). One liter of reaction mixture for method A contained the following: Tris-HCl (pH 8.5), 100 mmol; $MgCl_2$, 8 mmol; glucose, 5 mmol; $NADP^+$, 0.4 mmol; AMP, 1 mmol; polyphosphate (n = 35), 0.2 g; adenylate kinase, 1 U; hexokinase, 2 U; and glucose-6-phosphate dehydrogenase, 1 U. The reaction was started by the addition of AMP.

One liter of reaction mixture for method B contained the following: Tris-HCl (pH 8.5), 100 mmol; $MgCl_2$, 8 mmol; NADH, 0.15 mmol; phosphoenolpyruvate, 1 mmol; AMP, 1 mmol; polyphosphate (n = 35), 0.2 g; lactate dehydrogenase, 3 U; and pyruvate kinase, 0.8 U. The reaction was started by the addition of polyphosphate.

Method A was used to measure activity during the purification procedure. Method B could not be applied because of endogenous oxidation of NADH in the crude extract. However, method B had the advantage of being more rapid than method A and was therefore used in the determination of the kinetic properties of the enzyme.

Adenylate kinase (EC 2.7.4.3) was measured by monitoring the oxidation of NADH. One liter of reaction mixture contained the following: Tris-HCl (pH 8.5), 100 mmol; $MgCl_2$, 8 mmol; NADH, 0.15 mmol; PEP, 1 mmol; AMP, 1 mmol; ATP, 1 mmol; lactate dehydrogenase, 3 U; and pyruvate kinase, 0.8 U. The reaction was started by the addition of ATP.

Polyphosphatase activity was determined by monitoring the formation of P_i from polyphosphate. One liter of the reaction mixture contained the following: Tris-HCl (pH 7.6), 50 mmol; NH_4Cl, 300 mmol; $MgCl_2$, 10 mmol; and polyphosphate (n = 35), 0.4 g. The reaction was started by the addition of polyphosphate.

All three enzymes were assayed at 30°C. The assay mixtures of 1.0 ml contained 10 µl of crude extract or partially purified enzyme. Under the assay conditions used, the activities were proportional to the amount of enzyme applied.

Separation of small polyphosphates

Phosphate and pyro-, tri-, and tetraphosphate (P_2, P_3, and P_4) were separated by elution on a Mono-Q HR 5/5 anion-exchange column with a 20-ml gradient from 0 to 0.36 M KCl in 0.02 M acetate buffer (pH 4.5) at a flow rate of 1.0 ml/min. Portions of each fraction were hydrolyzed by boiling with 0.6 N H_2SO_4 and $K_2S_2O_8$ and then analyzed for phosphate.

Analytical methods

Protein was determined by the method of Lowry et al. (1951) or Bradford (1976) with bovine serum albumin as the standard. The purity of

Table 1. Purification of polyphosphate:AMP phosphotransferase of *Acinetobacter* strain 210A

Step	Protein (mg)	Activity (U)[a]	Spec. act. (U/mg)	Purification (fold)	Yield (%)
Crude extract	2,313	156	0.067	1	100
Streptomycin fraction	21.1	75	3.54	53	48
Mono-Q 1	5.2	37	7.13	106	24
Mono-Q 2	1.9	26	13.7	205	17
Phenyl Superose	0.092	3.9	42.8	639	2.5
Superose	0.0045	0.46	101.2	1,511	0.3

[a] One unit equals 1 μmol of product formed per min

the enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Superose 6 HR and Sephadex G-75 were used to estimate the molecular size of the enzyme. P_i and total phosphorus (persulphate digestion method) were determined spectrophotometrically according to the Standard Methods (1976).

Chemicals

Toluidine blue O and polyphosphate glasses with differently labelled chain lenghts (n=2, 3, 4, 5, 18, 28, 35, 44, 64, or 91) were purchased from Sigma Chemical Co. (Amsterdam, The Netherlands). These commercial samples contain a relatively large number of polyphosphates with widely different chain lengths (Clark and Wood 1987). Acrylamide and *N,N'*-methylenebis-acrylamide were from Bio-Rad Laboratories (Utrecht, The Netherlands). All other biochemicals were obtained from Boehringer Mannheim (Almere, The Netherlands). Polyphosphate (n=155 ±7) was a gift from H.G. Wood (Case Western University, Cleveland, Ohio, U.S.A.)

Results

Enzyme purification

The polyphosphate:AMP phosphotransferase enzyme was purified 1,500-fold with a recovery of 0.3% (Table 1). The enzyme could be precipitated by 1% (wt/vol) streptomycin sulfate. Similar to the situation with polyphosphate kinase in *E. coli* B (Murata et al. 1988), this treatment appeared to be an effective first step in the purification procedure. During the column chromatography steps, the increase in specific activity was good but the recovery of the enzyme was poor.

In the absence of polyphosphate in the crude extract, less polyphosphate:AMP phosphotransferase activity was precipitated (results not shown). This finding suggests an association between the enzyme and the bacterial polyphosphate.

Characterization

In the most purified fractions, no adenylate kinase and polyphosphatase activity could be detected. The fractions showed one major band on SDS-PAGE corresponding to a molecular mass of 55

kDa. In a second purification procedure, the successive fractions of the Phenyl Superose HR 5/5 column were screened for activity and subjected to SDS-PAGE. The gel revealed two bands, one of 55 kDa and one of 41 kDa. Only the fractions which contained the 55-kDa band possessed a significant polyphosphate:AMP phosphotransferase activity (Fig. 1). From these results, it is deduced that the activity is associated with the 55-kDa band.

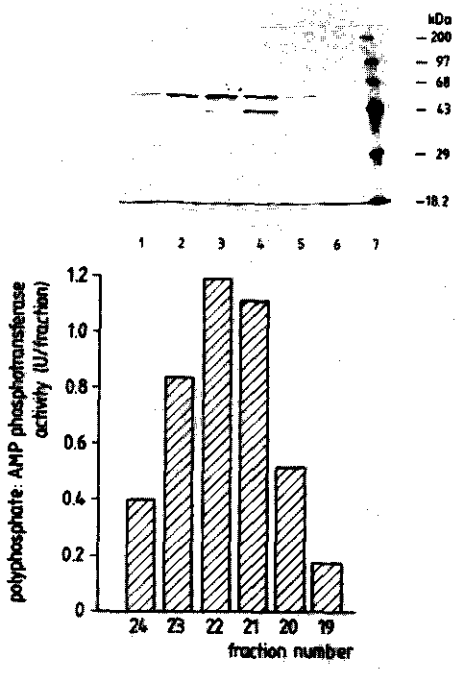

Figure 1. Gelelectrophoresis and activity profile of the most purified fractions on Phenyl Superose HR 5/5. Lanes: 1, fraction 24; 2, fraction 23; 3, fraction 22; 4, fraction 21; 5, fraction 20; 6, fraction 19; 7, molecular mass markers (200, 97, 68, 43, 29, and 18.4 kDa).

The enzyme was stable at moderate temperatures. There was no activity loss during incubation period for 2 h at 30°C. Incubation at 45°C for 15 min resulted in a 50% reduction and at 50°C resulted in a complete loss of activity. Storage at -20°C for 1 month gave a decrease in activity of about 50% which was not due to freezing and thawing. The enzyme showed a broad pH optimum from 6.5 to 8.5. No activity was observed at pH values below 5.5 or above 10.

Substrate specificity

No activity was obtained with P_i, P_2, P_3, and P_4. The highest activities were found with polyphosphate of a degree of polymerization between 18 and 44 phosphate groups (Table 2).

Degradation of polyphosphate

Mechanism. To determine the mechanism of polyphosphate degradation, an assay mixture containing polyphosphate:AMP phosphotransferase, polyphosphate ($n=155 \pm 7$), AMP (1 mM), $MgCl_2$ (8 mM), and Tris-HCL (100 mM, pH 8.5) was incubated at 30°C. At different time intervals, aliquots were taken and diluted 1:1 with EDTA (100 mM). After addition of sample dye, the polyphosphates were electrophoresed on a 10% polyacryamide gel and stained with toluidine blue. As shown in Figure 2, no additional bands were formed during the polyphosphate degradation. From this result, we concluded that the mechanism of utilization is processive.

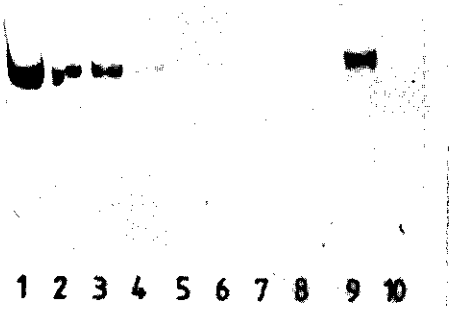

Figure 2. Degradation of polyphosphate. Lanes: 1, polyphosphate ($n=155 \pm 7$); 2 to 8, samples of assay mix after 0, 1, 5, 10, 20, 30, and 60 min of incubation; 9, assaymix without AMP after 60 min of incubation; 10, assay mix without polyphosphate after 60 min of incubation.

Table 2. Effect of chain length on polyphosphate utilization by polyphosphate:AMP phosphotransferase of *Acinetobacter* strain 210A

Polyphosphate chain length	Relative activity (%)
1	0
2	0
3	0
4	0
5	56 ± 3
18	85 ± 15
28	86 ± 9
35	100 ± 0
44	82 ± 11
64	70 ± 8
91	65 ± 6

Endproduct. To investigate whether small polyphosphates (P_2 to P_4) are the end products of polyphosphate degradation, we incubated the enzyme with AMP and polyphosphate for 3 h. Samples were taken and eluted on a Mono-Q HR 5/5 column. Each fraction was hydrolyzed and analyzed for phosphate. No peaks were observed which corresponded with either P_2, P_3 or P_4. From this result, we concluded that the polyphosphate chain was degraded completely to ADP.

Kinetic properties

The reaction rate at different AMP and polyphosphate concentrations followed Michaelis-Menten kinetics. Half maximal rates were obtained at 0.26 ± 0.04 mM AMP and 3.0 ± 1.0 mg of polyphosphate per liter (ca. 0.8 μM at an average chain length of 35 phosphate groups) (Figs. 3 A, B).

Inhibition studies

Polyphosphate:AMP phosphotransferase was inhibited by P_2, P_3, and P_4. For P_2, no inhibition constants could be determined because precipitates were formed in the assay mixture at higher P_2 concentrations (above 2 mM). The inhibition by P_3 and P_4 was mixed with polyphosphate as the substrate. The inhibition constants for dissociation of the enzyme-inhibitor complex (K_{IE}) and of the enzyme-inhibitor-substrate complex (K_{IES}) for P_3 were 0.9 ± 0.4 and 6.5 ± 0.9 mM, respectively, and those for P_4 were 0.7 ± 0.1 and 1.5 ± 0.2 mM, respectively. The values for K_{IE} and K_{IES} were determined by the method of Cornish-Bowden (1974). Figures 4A and B represent typical inhibition experiments with P_3 and P_4, respectively. ATP affected the enzyme activity only slightly, 2 mM ATP had no influence, and 6 mM ATP resulted in a reduction in activity of only 30%.

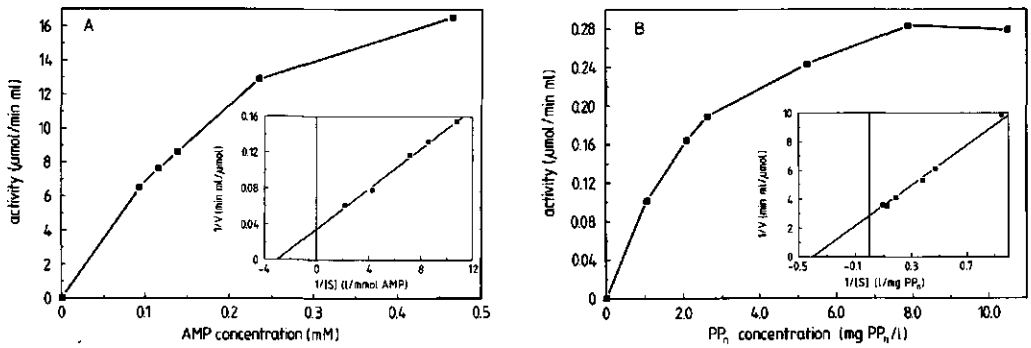

Figure 3 (A) Relationship between AMP concentration and activity of the polyphosphate:AMP phosphotransferase enzyme at a polyphosphate concentration of 0.2 g/l. Inset: Lineweaver-Burk plot of the same data. (B) Relationship between polyphosphate concentration and activity of the polyphosphate:AMP phosphotransferase enzyme at an AMP concentration of 1 mM. Inset: Lineweaver-Burk plot of the same data.

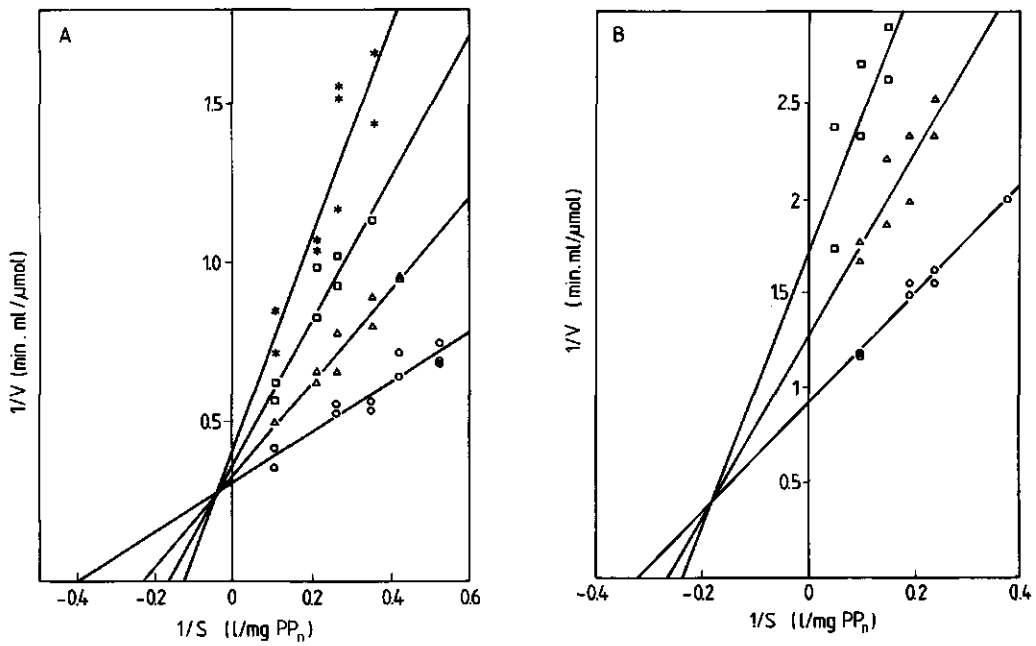

Figure 4 (A) Inhibition of polyphosphate:AMP phosphotransferase by P_3. Symbols for P_3 concentrations: ○, 0 mM; △, 0.97 mM; □, 1.94 mM; ∗, 2.91 mM. (B) Inhibition of polyphosphate:AMP phosphotransferase by P_4. Symbols for P_4 concentrations: ○, 0 mM; △, 0.61 mM; □, 1.22 mM.

Discussion

From the increase in specific activity upon purification, it can be calculated that only a minor part of the total soluble cell protein of *Acinetobacter* strain 210A consists of polyphosphate:AMP phosphotransferase. The specific activity of the enzyme is very high. So far, only a few polyphosphate-degrading enzymes have been purified to a similarly high specific activity (Wood and Clark 1988).

Determination of the molecular weight of the native protein was not entirely straightforward. From the elution pattern using Superose 6 HR, calibrated with the molecular mass standards BSA (67 kDa) and aldolase (158 kDa), a molecular mass of less than 67 kDa was estimated. On SDS-PAGE, a 55 kDa band corresponded with the polyphosphate:AMP phosphotransferase activity. These results suggest that the native enzyme exists as a monomer. However, in view of the delicate stability of the enzyme which resulted in a low recovery, it cannot entirely be excluded that the 55-kDa protein represents a stabilized, active subunit of the holoenzyme.

Polyphosphate:AMP phosphotransferase has a high affinity for its substrates. The K_m value for AMP is 0.26 mM. This value is in good agreement with results found by Van Groenestijn et al. (1989a) who reported a K_m for AMP of 0.6 mM in cell free extracts. The K_m of 0.8 μM for polyphosphate is extremely low. Pepin and Wood (1986) found a similar low K_m of 0.94 μM for polyphosphate ($n = 40 \pm 3$) for the enzyme polyphosphate glucokinase. Generally, K_m values range between 10 μM and 1 mM for polyphosphate of several polyphosphate-degrading enzymes (Felter and Stahl 1973, Kornberg 1957, Suzuki et al. 1972, Szymona and Ostrowski 1964).

P_2, P_3, and P_4 cannot serve as substrates for polyphosphate:AMP phosphotransferase of *Acinetobacter* strain 210A. The inability of this enzyme to use low-molecular-weight polyphosphates as donors in the phosphorylation of AMP has also been reported by Dirheimer and Ebel (1965) for *C. xerosis*. Inhibition of polyphosphate:AMP phosphotransferase by P_2, P_3, and P_4 was not reported previously. The inhibition of the enzyme of *Acinetobacter* strain 210A may well explain the low activity towards P_5. The commercial P_5 used in this study contained P_2, P_3, and P_4 as contaminants. About 50% of the total amount of P of the P_5 was in the form of P_2, P_3, and P_4.

Although P_2, P_3, and P_4 are not substrates for the enzyme, the polyphosphate chain is degraded completely. This has also been found by Muhammed et al. (1959) for the enzyme polymetaphosphatase which is most probably a polyphosphatase from *C. xerosis*. These authors were not able to detect the formation of short-chain polyphosphate polymers during polyphosphate degradation.

The absence of small polyphosphate polymers during polyphosphate degradation by polyphosphate:AMP phosphotransferase and the inhibition of the enzyme by these compounds are intriguing. Apparently, the binding of a long polyphosphate chain slightly alters the catalytic site of the enzyme in such a way that the chain is degraded completely. Small polyphosphates, however, are probably not able to bind directly at the catalytic site but possibly bind in the neighbourhood of it, resulting in a change of the catalytic properties of the enzyme. More research is needed to investigate this hypothesis and to elucidate the inhibition mechanism.

The mechanism of polyphosphate degradation by polyphosphate:AMP phosphotransferase is processive. Once bound, the polyphosphate chain does not dissociate from the enzyme until it has been utilized completely. This has also been observed by Robinson and Wood (1986) for the enzyme polyphosphate kinase.

The role of polyphosphate:AMP phosphotrans-

ferase was doubted because several authors (Dawes and Senior 1973) state that Dirheimer and Ebel (1965) found a K_m for AMP of 20 mM. However, no data on the K_m in this or other references are found (Wood and Clark 1988). Our data now clearly show a relatively high affinity of this enzyme for AMP with a K_m of 0.26 mM. From our results, it is obvious that polyphosphate can act directly as an energy reserve in *Acinetobacter* strain 210A by means of polyphosphate:AMP phosphotransferase and adenylate kinase activities.

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CHAPTER 6

Properties of polyphosphatase of *Acinetobacter johnsonii* 210A

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Submitted

Properties of polyphosphatase of *Acinetobacter johnsonii* 210A

Abstract

Polyphosphatase, an enzyme which hydrolyses high polymeric polyphosphates to P_i, was purified 77-fold from *Acinetobacter johnsonii* 210A by Q-Sepharose, hydroxylapatite and mono-Q column chromatography. The native molecular mass, estimated by gelfiltration and native gelelectrophoresis, was 55 kDa. Results obtained with SDS-polyacrylamide gelelectrophoresis indicated that polyphosphatase of *Acinetobacter johnsonii* 210A is a monomer. The enzyme was specific for high polymeric polyphosphates and showed no activity towards pyrophosphate and organic phosphate esters. The enzyme was inhibited by iodoacetamide and in the presence of 10 mM Mg^{2+} by pyro- and triphosphate. Analysis of kinetic properties revealed an apparent K_m-value for polyphosphate with an average chain length of 64 residues of 30 mg polyphosphate per liter and for tetraphosphate of 1.2 mM. Polyphosphate chains were degraded to short chain polymers by a processive mechanism. Polyphosphatase activity was maximal in the presence of Mg^{2+} and K^+.

Introduction

In the presence of excess phosphate and energy, *Acinetobacter johnsonii* 210A, a strict aerobic microorganism, is able to accumulate large amounts of phosphate intracellularly as polyphosphate (Van Groenestijn 1988). This polymer can, depending on growth conditions, account for 30 % of the biomass dry weight (Deinema et al. 1985). When energy generation is not possible (e.g., in the absence of oxygen), polyphosphate is degraded (Van Groenestijn 1988). Two polyphosphate degrading enzymes have been found in *Acinetobacter johnsonii* 210A (Van Groenestijn et al. 1989).

Polyphosphate:AMP phosphotransferase catalyzes the degradation of polyphosphate at the expense of AMP: PP_n (polyphosphate) + AMP $\rightarrow PP_{n-1}$ + ADP. By the combined action of polyphosphate:AMP phosphotransferase and adenylate kinase, *Acinetobacter johnsonii* 210A is able to use its polyphosphate as a source of ATP (Van Groenestijn et al. 1987). Polyphosphate:AMP phosphotransferase has been purified partially and characterized (Bonting et al. 1991).

Polyphosphatase hydrolyzes polyphosphate to P_i: $PP_n + H_2O \rightarrow PP_{n-1} + P_i$. This enzyme of *A. johnsonii* 210A has not been studied in detail. It was speculated that the enzyme has a role in the degradation of polyphosphate in cells of *Acinetobacter* strain 210A growing under P-limitation (Van Groenestijn and Deinema 1985). This paper reports on the partial purification and characterization of polyphosphatase of *Acinetobacter johnsonii* 210A. Some possible functions of this enzyme are discussed.

Material and methods

Organism and cultivation

Acinetobacter johnsonii 210A was grown on 20 mM sodium butyrate in 10 L carboys containing 10 L of medium as described by Van Veen et al. (1993a). Cells were harvested by continuous centrifugation at the mid-log phase (OD_{660} = 1).

Purification procedure

Unless stated otherwise, all purification steps were performed at 4°C. Cells were treated according to the method used for preparation of membrane vesicles as described by Van Veen et al. (1993b). Ammonium sulfate (500 g) was added to 1 L of suspension obtained after removal of whole cells and vesicles. The precipitated material was collected by centrifugation at 20,000 x *g* for 10 min, resuspended in 50 ml 30 mM Tris-HCl (pH 7.2) + 10 mM $(NH_4)_2SO_4$ (= buffer A) and dialyzed overnight against 5 L of buffer A. The dialysate was centrifuged for 20 min at 30,000 x *g*. The supernatant was collected and stored at -20°C until use. This supernatant is referred to as crude extract.

The following steps were performed with a high-resolution fast protein liquid chromatography system (Pharmacia/LKB, Woerden, The Netherlands) at room temperature. All columns were equilibrated with the starting buffers of the gradients. Linear gradients were used throughout. The crude extract (45 ml) was injected on a Q-Sepharose column (3.2 by 10 cm). A 360 ml gradient from 0 to 0.6 M KCl in buffer A was applied at a flow rate of 3 ml/min. The most active fractions were pooled and adsorbed to a hydroxylapatite column (2.2 by 20 cm). Elution was with a 200 ml gradient (10 - 500 mM sodium phosphate buffer (pH 6.8) with 10 mM $(NH_4)_2SO_4$) at a flow rate of 1 ml/min. Fractions with the highest activities were pooled and dialyzed overnight against buffer A. The dialysate was applied to a Mono-Q HR 5/5 anion exchange column. A 20 ml gradient from 0 to 0.6 M KCl in buffer A at a flow rate of 1 ml/min was used to elute the protein. Fractions with the highest specific activities were collected and used for enzyme assays.

Enzyme assays

Polyphosphatase (E.C. 3.6.1.11) was determined by following the formation of P_i from polyphosphate. The reaction mixture contained (per liter): Tris-HCl (pH 7.6), 50 mmol; NH_4Cl, 300 mmol; $MgCl_2$, 10 mmol; polyphosphate (n = 64), 0.2 g. The reaction was started by the addition of polyphosphate.

Alkaline phosphatase (E.C. 3.1.3.1) was assayed as described by Bonting et al. (1992).

All enzyme activities were measured at 30°C. The assay mixtures of 0.5 ml contained 100 μl crude extract or 20 μl purified enzyme. The reactions were initiated by the addition of enzyme or substrate.

Analytical methods

Protein was determined according to Bradford (1976) with bovine serum albumin as the standard. Superose 6 HR 10/30 was used to estimate the molecular size of the enzyme. Native polyacrylamide gel electrophoresis (PAGE) and activity staining of polyphosphatase were performed as described (Baykov and Volk 1985). The purity of the enzyme was determined by sodium dodecyl sulfate (SDS)-PAGE according to Laemmli (1970). A Mono-Q HR 5/5 column was used to separate short chain polyphosphates (Bonting et al. 1991). Electrophoresis of polyphosphates by SDS-PAGE was performed as described by Clark and Wood (1987). P_i and

Table 1. Purification of polyphosphatase of *Acinetobacter johnsonii* 210A

Step	Protein (mg)	Activity (U)[a]	Spec. act. (mU/mg)	Purification (Fold)	Yield (%)
Crude extract	423	11,414	27	1	100
Q-Sepharose	46.4	8,777	189	7.0	77
Hydroxylapatite	8	4,840	605	22.4	42
Mono-Q	1.4	2,926	2090	77.4	26

[a]One unit equals 1 μmol of product formed per min

total phosphorus (persulfate digestion method) were determined spectrometrically according to Standard Methods (1976).

Chemicals

Toluidine blue and polyphosphate glasses (n=2, 3, 4, 5, 18, 28, 35, 44, 64, 91) were obtained from Sigma Chemical Co. (Amsterdam, The Netherlands). Acrylamide, N,N'-methylenebisacrylamide, sodium dodecyl sulfate and hydroxylapatite were from Bio-Rad Laboratories (Utrecht, The Netherlands). Q-Sepharose, Mono-Q HR 5/5, Superose 6 HR 10/30 and molecular mass standards for gelfiltration and native-PAGE were purchased from Pharmacia Fine Chemicals (Woerden, The Netherlands). All other biochemicals were obtained from Boehringer Mannheim (Almere, The Netherlands).

Results

Enzyme purification

The results of a typical purification are summarized in Table 1. In three steps, a 77-fold purified protein was obtained with 26 % recovery. The final polyphosphatase activity was 2 μmol · mg^{-1} protein · min^{-1}. The enzyme rapidly lost its activity during dialysis and chromatographic steps. When dialyzed against 30 mM Tris-HCl (pH 7.6), the enzyme activity decreased about 3-fold. Similar to the situation in *Saccharomyces cerevisiae*, addition of ammonium sulfate (10 mM) to the buffer stabilized the enzyme (Felter et al. 1970). Addition of Mg^{2+} (10 mM), glycerol (10 % v/v), dithiotreitol (1 mM), EDTA (1 mM), or triton (0.05 % w/v) had no effect. The recovery of the enzyme activity during ion exchange chromatography improved significantly by using KCl instead of NaCl in buffer A.

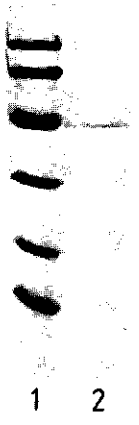

Figure 1. Analysis of polyphosphatase purity by SDS-PAGE. lanes: 1, molecular mass markers (94, 67, 43, 30, 20.1, 14.4 kDa); 2, fraction from native-PAGE

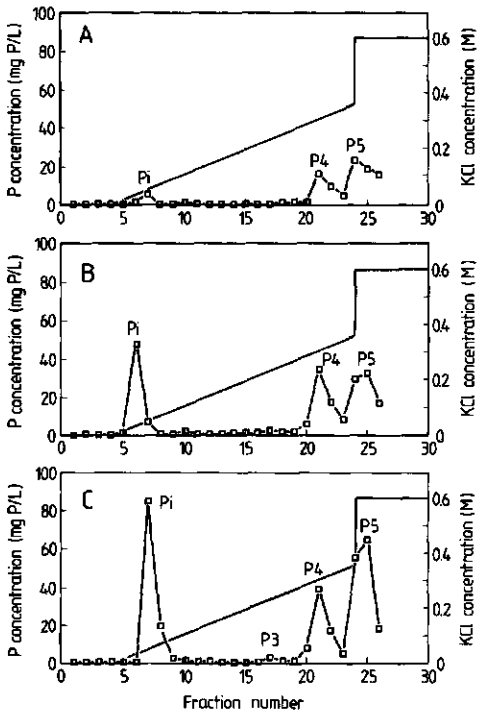

Figure 2. Degradation of high polymeric polyphosphate (n = 64) by polyphosphatase followed by anion exchange column chromatography. A) t=0; B) t=1 hour; C) t=3 hours. Orthophosphate (P_i), triphosphate (P_3), tetraphosphate (P_4), and pentaphosphate (P_5) are indicated.

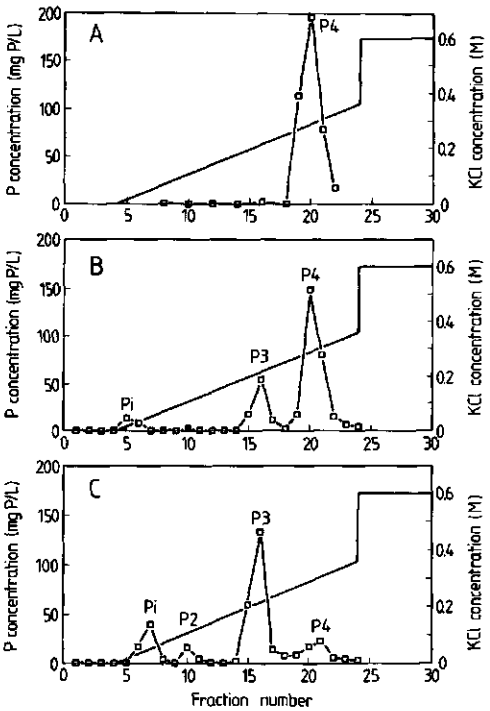

Figure 3. Degradation of tetraphosphate by polyphosphatase followed by anion exchange column chromatography. A) t=0; B) t=2 hours; C) t= 3.5 hours. Orthophosphate (P_i), pyrophosphate (P_2), triphosphate (P_3), and tetraphosphate (P_4) are indicated.

Characterization

Polyphosphatase was eluted from the Superose 6 HR 10/30 column at a volume indicating a molecular mass of 50 ± 20 kDa, as compared to standards of known molecular mass. By the use of native gels with different percentages acrylamide (5, 6, 7, 8, 9 and 10 %), the molecular mass of the native protein was also estimated (Hedrick and Smith 1968). The gels were after electrophoresis incubated at 37°C for 30 min in a mixture containing: Tris-HCl (pH 7.6), 50 mM; KCl, 0.1 M; $MgCl_2$, 2 mM; PP_n (n = 64), 0.2 g/L and stained with ammonium-heptamolybdate and methyl green in 1 M H_2SO_4.

The mobility of polyphosphatase as compared to standards of known molecular mass indicated a molecular mass of 55 ± 15 kDa. To investigate the subunit composition of the enzyme, a small part of the native gel which contained polyphosphatase activity was boiled with SDS and electrophoresed on a 10 % SDS-polyacrylamide gel. After staining, one major band was seen which corresponded to a molecular mass of 43 kDa (Fig. 1). This suggests that the native enzyme exists as a monomer.

Storage of the enzyme at -20°C hardly affected its activity. After 10 days less than 10 % of the activity was lost.

Substrate specificity

The enzyme was specific for high polymeric polyphosphates. No activity was obtained with pyrophosphate (P_2). The highest activities were found with polyphosphates with chain lengths of more than 28 P-groups (Table 2). Organic phosphates (ATP, ADP, PEP, glucose-6-P, paranitrophenylphosphate) tested at a concentration of 2 mM were not hydrolyzed by the enzyme. The activity towards triphosphate (P_3) and tetraphosphate (P_4) depended on the presence of certain ions. In an assay mixture containing 300 mM NH_4^+ and 10 mM Mg^{2+}, the activities for P_3 and P_4 were 11 and 80 %, respectively. In the presence of 0.1 M K^+ and 2 mM Mg^{2+}, the enzyme did not show any activity towards P_3, with P_4 an activity of only 30 % was obtained (Table 2).

Table 2. Effect of chain length on polyphosphate hydrolysis by polyphosphatase of *A. johnsonii* 210A

Polyphosphate chain length	Relative activity (%)
2	0
3	11 ± 1; 0[a]
4	80 ± 5; 30 ± 10[a]
5	35 ± 2
18	55 ± 4
28	92 ± 1
35	85 ± 5
44	99 ± 1
64	95 ± 5
91	97 ± 3

[a]Activity obtained in the presence of 0.1 M K^+ and 2 mM Mg^{2+}

Degradation of polyphosphate

Mechanism. The mechanism of polyphosphate degradation was determined as described for polyphosphate:AMP phosphotransferase (Bonting et al. 1991). A mixture containing polyphosphatase, polyphosphate (n = 71 ± 18), $MgCl_2$ (2 mM), KCl (0.1 M) and Tris-HCl (50 mM, pH 7.6) was incubated at 30°C. At different time intervals, samples (10 µl) were taken and after addition of 5 µl EDTA (0.1 M) and 5 µl sample dye, immediately frozen in liquid N_2. Just before electrophoresis, samples were rapidly thawed and electrophoresed on a 15 % PAA-gel. No additional bands were formed during polyphosphate degradation suggesting a processive mechanism of hydrolysis (not shown).

Endproduct. To investigate whether the polyphosphate chain is degraded completely to P_i, the enzyme was incubated for 3 h with polyphosphate (n = 64, 4 g/L), $MgCl_2$ (2 mM), KCl (0.1 M) and Tris-HCl (50 mM, pH 7.6). Samples were taken and eluted on a Mono-Q HR 5/5 column. After three hours of incubation, next to P_i, small amounts of P_3, P_4, and pentaphosphate (P_5) were detected, suggesting that polyphosphate chains are not completely hydrolyzed to P_i (Fig. 2). By using P_4 (8 mM) as a substrate, P_i, P_2, and P_3 were formed (Fig. 3).

Kinetic properties

The reaction rate was followed at different polyphosphate concentrations. By applying high polymeric polyphosphate, a K_m-value of 30 ± 8 mg of polyphosphate per liter (n = 64) could be calculated. The apparent affinity for P_4 was 1.2 mM.

Inhibition studies

Polyphosphatase was inhibited completely by

2 mM iodoacetamide. Addition of EDTA (5 mM) decreased the activity only in an assay mixture with 2 mM Mg^{2+}. In the presence of 10 mM Mg^{2+}, no effect of EDTA was found, suggesting that EDTA inhibits the enzyme by competition for Mg^{2+}. Pyrophosphate (2 mM) and triphosphate (2 mM) partially inhibited the enzyme (45 % and 70 %, respectively) only in the presence of a high concentration of Mg^{2+} (10 mM). The enzyme activity was not affected by KCN (2 mM), KF (2 mM) and NaN_3 (0.3 mM).

Cations

In the presence of Mg^{2+} (2.5 mM) and K^+ (0.1 M), polyphosphatase showed maximal activity (100 %). No activity was obtained in the absence of cations whereas the activity in the presence of Mg^{2+}, K^+ or NH_4^+ alone was low (< 15 %). Addition of NH_4^+ (0.3 M) or K^+ (0.1 M) strongly enhanced the activating effect of Mg^{2+} (3-fold and 7-fold, respectively). Other divalent cations were less effective in stimulating the polyphosphatase activity. In the presence of 0.1 M K^+, low activities (< 20 %) were detected with Ca^{2+}, Mn^{2+} or Fe^{2+} (2.5 mM). Hydrolysis of polyphosphate was not observed in an assay mixture containing 0.1 M K^+ and 2.5 mM of either Zn^{2+}, Co^{2+} or Ba^{2+}.

Discussion

Polyphosphatases have been found in numerous organisms (Kulaev and Vagabov 1983) and have been isolated from several eukaryotes. The enzyme was purified 23-fold from *Saccharomyces cerevisiae* (Felter et al. 1970), 27-fold from *Endomyces magnusii* (Afanas'eva et al. 1976), 630-fold from *Saccharomyces carlsbergensis* (Kulaev 1990) and to a homogeneous state from *Neurospora crassa* (Umnov et al. 1975). Only once the enzyme has been purified and characterized from a prokaryote. Muhammed et al. (1959) studied a polymetaphosphatase, which is most probably a polyphosphatase, from *Corynebacterium xerosis*. The enzyme was purified 97-fold and characterized with respect to substrate specificity, dependence of cations and formation of short-chain polyphosphates. All these purification procedures were hampered by the lability of the enzyme. Polyphosphatase of *Acinetobacter johnsonii* 210A also rapidly lost its activity during dialysis and chromatographic steps. Similar to the situation in *S. cerevisiae*, addition of ammonium sulfate stabilized the enzyme (Felter et al. 1970).

The molecular mass of native polyphosphatase of *A. johnsonii* 210A was estimated to be 55 kDa by the use of Superose 6 and native PAGE. Similar values were reported for polyphosphatases of *N. crassa* (50 kDa) and of *E. magnusii* (48 kDa). (Umnov et al. 1975; Afanas'eva et al. 1976).

As reported for many polyphosphatases, the enzyme of *A. johnsonii* 210A was specific for high polymeric polyphosphates (Muhammed et al. 1959; Felter et al. 1970; Umnov et al. 1975; Andreeva and Okorokov 1990). Pyrophosphate and organic phosphate esters were not hydrolyzed by the enzyme.

The mechanism of polyphosphate degradation is processive. The P-polymer remains bound to the enzyme during hydrolysis. However, the formation of short-chain polyphosphates after prolonged incubation indicates that the polyphosphate chain dissociates from the enzyme before it has been hydrolyzed completely. Formation of short-chain polyphosphates during degradation of high polymeric polyphosphates has not been reported earlier and is in contrast to the findings of Muhammed et al. (1959) and Harold and Harold (1965) who showed that P_i was the only product formed during utilization of polyphosphate by polyphosphatase of *C. xerosis* and *A. aerogenes*, respectively.

The affinity of polyphosphatase of *A. johnsonii* 210A for high polymeric polyphosphates is in the same range as reported for polyphosphatases of other microorganisms (Muhammed et al. 1959; Felter et al. 1970; Umnov et al. 1975). The low affinity of the enzyme for short chain polyphosphates is in accordance with the results of Kulaev who reported that the affinity of polyphosphatase of *S. carlsbergensis* increased with polyphosphate chain length by several orders of magnitude (Kulaev 1990).

Iodoacetamide appeared to be an effective polyphosphatase inhibitor whereas fluoride had no effect on the enzyme activity. Inhibition by iodoacetamide was also found for polyphosphatase of *S. carlsbergensis* (Andreeva and Okorokov 1990). High concentrations of fluoride (0.2 M) partially inhibited the polyphosphatase activity of *E. magnusii* (65 %) and *N. crassa* (60-80 %) (Afanasieva and Kulaev 1973; Kulaev and Konoshenko 1971). Inhibition of polyphosphatase by pyro-and triphosphate was not reported previously. Only in the presence of high Mg^{2+}-concentrations the enzyme was inhibited by these short chain polyphosphates. This suggests that Mg^{2+} is required for the interaction of the short chain P-polymers with polyphosphatase. More investigations on the effect of pyro- and triphosphate on polyphosphatase are needed to clarify the inhibition mechanism.

Polyphosphatases differ substantially from each other in their requirement for metal ions. Most polyphosphatases exhibit their activity only in the presence of these ions (Felter et al. 1970; Afanasieva and Kulaev 1973; Umnov et al. 1975; Harold and Harold 1965; Andreeva and Okorokov 1990) but the enzyme of *C. xerosis* was inhibited by them (Muhammed et al. 1959). Polyphosphatase of *A. johnsonii* 210A is activated by Mg^{2+}. The enzyme activity is stimulated to a lesser extent by Ca^{2+}, Mn^{2+} or Fe^{2+}. A similar situation was observed for polyphosphatase of *N. crassa* (Umnov et al. 1975) whereas the enzyme of *E. magnusii* required Mn^{2+} or Co^{2+} for optimal activity (Afanasieva and Kulaev 1973). The stimulating effect of K^+ in the presence of Mg^{2+} on the polyphosphatase activity was observed earlier in *N. crassa* and *A. aerogenes* (Kulaev and Konoshenko 1971; Harold and Harold 1965). Polyphosphatase of *A. johnsonii* 210A exhibited maximal activity in the presence of 2.5 mM Mg^{2+} and 0.1 M K^+. Cells of *A. johnsonii* 210A contain high levels of K^+ (0.18 M) and Mg is present as counterion in the polyphosphate granules (Bonting et al. 1993).

Since polyphosphatases hydrolyze polyphosphate to P_i, this enzyme is thought to be involved in the regulation of intracellular P_i-concentrations (Kulaev 1979). Recently, it was suggested that *A. johnsonii* 210A is able to generate an electrochemical ion gradient by transporting P_i across the membrane in symport with ions (Van Veen et al. 1993a). This indicates a possible involvement of polyphosphatase in the energy metabolism of the cell. More research is needed to elucidate the exact role of this enzyme in polyphosphate metabolism of *A. johnsonii* 210A.

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CHAPTER 7

Purification and properties of pyrophosphatase of *Acinetobacter johnsonii* 210A

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Purification and properties of pyrophosphatase of *Acinetobacter johnsonii* 210A

Abstract

Inorganic pyrophosphatase (E.C. 3.6.1.1) from *Acinetobacter johnsonii* 210A was purified 200-fold to apparent homogeneity. The enzyme catalyzed the hydrolysis of inorganic pyro-and triphosphate. No activity was found with other polyphosphates and with a variety of organic phosphate esters. The molecular mass was estimated to be 141 kDa by gelfiltration. SDS-gel electrophoresis indicated a subunit composition of six identical polypeptides with a molecular mass of 23 kDa. The cation Mg^{2+} was required for activity; Mn^{2+}, Co^{2+} and Zn^{2+} supported 48%, 48%, and 182% of the activity observed with Mg^{2+}, respectively. The enzyme was heat-stable and inhibited by fluoride and iodoacetamide. Analysis of kinetic properties revealed an apparent K_m for pyrophosphate of 0.26 mM.

Introduction

Acinetobacter johnsonii 210A exhibits a special phosphate metabolism. During normal growth, excess phosphate is taken up, polymerized to polyphosphate and stored inside the cell in polyphosphate granules (Deinema et al. 1985; Van Groenestijn 1988). Recently, polyphosphate:AMP phosphotransferase and polyphosphatase, enzymes which are both involved in the degradation of polyphosphate, have been isolated from this organism and characterized (Bonting et al. 1991; Bonting et al. 1993). It was shown that both enzymes are unable to use short chain polyphosphates such as pyro-and triphosphate as a substrate. These compounds are known to be hydrolyzed by another enzyme: pyrophosphatase. This enzyme is in contrast to the polyphosphate degrading enzymes, widely distributed in nature and has been purified from several microorganisms (Hachimori et al. 1975; Jetten et al. 1992; Josse 1966; Kunitz and Robbins 1961; Lahti and Niemi 1981; Tominaga and Mori 1977; Ware and Postgate 1971).

Pyrophosphate is formed in a variety of nucleoside triphosphate dependent reactions such as deoxyribo-and ribonucleic acid polymerizations, coenzyme synthesis, and amino acid or fatty acid activation. In addition, pyrophosphate can be synthesized by photophosphorylation, oxidative phosphorylation, and glycolysis (Kukko-Kalske and Heinonen 1985; Lahti 1983). Since pyrophosphate inhibits various enzymes (Kukko-Kalske and Heinonen 1985), cells have to utilize it rapidly to prevent growth inhibition. Besides pyrophosphatase, pyrophosphate-dependent phosphotransferases are able to lower the intracellular pyrophosphate concentration. These enzymes phosphorylate compounds such as L-serine and acetate at the expense of pyrophosphate. They have been found in

Propionibacterium shermanii and *Entamoeba histolytica*, respectively (Wood et al. 1977). Although the role of pyrophosphatase in cellular metabolism, and reactions in which pyrophosphate is involved, have been studied extensively, little is known about the effect of pyrophosphatase and pyrophosphate on polyphosphate metabolism. This paper describes the purification and properties of pyrophosphatase of the polyphosphate accumulating organism *Acinetobacter johnsonii* 210A. The role of pyrophosphate in polyphosphate metabolism is discussed.

Material and methods

Organism and cultivation

Acinetobacter johnsonii 210A was grown in two 10 L carboys each containing 8 L of medium as described by Bonting et al. (1991). Cells were harvested at the log-phase (OD_{660} = 1.5) by continuous centrifugation and washed two times in 50 mM Tris-HCl (pH 7.6).

Purification procedure

Unless stated otherwise, all purification steps were performed at 4°C. Washed cells were resuspended in 150 ml 50 mM Tris-HCl (pH 7.6), disrupted at 0°C by sonication (10 times, 30 s each; interval pauses of 30 s; 40 W; Branson Sonic Sonifier, Danbury, Conn., USA) and centrifuged for 30 min at 30,000 x *g*. The supernatant contained about 10 mg of protein per ml and is referred to as crude extract.
After addition of $MgCl_2$ to a final concentration of 17 mM, the crude extract was placed for 10 min in a water bath with a temperature of 70 °C and subsequently rapidly cooled on ice. Denaturated proteins were removed by centrifugation at 100,000 x *g* for 60 min. Under vigorously stirring, a saturated $(NH_4)_2SO_4$-solution was added to the supernatant. The 50-80 % precipitate was collected by centrifugation (20,000 x *g*, 10 min) and dissolved in 50 mM Tris-HCl (pH 7.6).
The following steps were performed with a high-resolution fast protein liquid chromatography system (Pharmacia/LKB, Woerden, The Netherlands) at room temperature. All columns were equilibrated with the starting buffers of the gradients. Linear gradients were used throughout. The fraction (50 ml) obtained after ammonium sulfate precipitation was loaded on a hydroxylapatite column (2.2 by 20 cm). A 200 ml gradient from 0.2 to 1 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl (pH 7.6) was applied at a flow rate of 2 ml/min. Fractions with pyrophosphatase activity were pooled and ammonium sulfate (2 M) was added to a final concentration of 1 M. To prevent overloading, two aliquots of this fraction were injected on a Phenyl Superose HR 5/5 column. Elution was with a 10 ml gradient (1-0 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl (pH 7.6)) at a flow rate of 0.45 ml/min. Fractions with the highest activity were combined and passed in six separate runs through a Superose 6 HR 10/30 column equilibrated with 150 mM NaCl in 50 mM Tris-HCl (pH 7.6) at a flow rate of 0.2 ml/min. The most active fractions were pooled and mixed in a 1:1 ratio with 50 mM Tris-HCl (pH 7.6). The diluted fraction was applied to a Mono-Q HR 5/5 anion exchange column. Purified pyrophosphatase was eluted at 0.27 M NaCl in a 43 ml gradient from 0 to 0.4 M NaCl in 50 mM Tris-HCl (pH 7.6) at a flow rate of 1.1 ml/min.

Enzyme assays

Pyrophosphatase (E.C. 3.6.1.1) was assayed by following the liberation of phosphate from pyrophosphate. The reaction mixture contained (per liter): Tris-HCl (pH 7.6), 50 mmol; $MgCl_2$, 10 mmol; pyrophosphate, 2 mmol.

Table 1. Purification of pyrophosphatase of *A. johnsonii* 210A

Step	Protein (mg)	Activity (U)[a]	Spec. act. (U/mg)	Purification (fold)	Yield (%)
Crude extract	1,241	4219	3.4	1	100
Heat (70°C, 10 min)	694	4093	5.9	1.7	97
Ammonium sulfate	105	2604	24.8	7.3	62
Hydroxylapatite	13	1394	108	31.8	33
Phenyl Superose	3.0	662	218	64.1	16
Superose HR 10/30	1.0	486	472	139	12
Mono-Q	0.37	257	695	204	6

[a]One unit equals 1 μmol of phosphate formed per min

Alkaline phosphatase (E.C. 3.1.3.1) was measured according to Bonting et al. (1992) with 2 mM *p*-nitrophenylphosphate as the substrate. The assay mixtures (0.5 ml) contained 5 μl of a solution of the purified enzyme. The reactions were started by the addition of enzyme.

Analytical methods

Protein was estimated according to Bradford (1976) with bovine serum albumin (BSA) as the standard. The purity of the enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970). Native PAGE and activity staining of the pyrophosphatase were performed as described by Baykov and Volk (1985). Superose 6 HR 10/30 was used to estimate the molecular size of the enzyme. Orthophosphate was determined spectrometrically according to the Standard Methods (1976).

Chemicals

Tetrasodium pyrophosphate was from Janssen (Beerse, Belgium). Tri-and tetraphosphate were obtained from Sigma Chemical Co. (Amsterdam, The Netherlands). Acrylamide, N,N'-methylenebisacrylamide, sodium dodecyl sulfate and hydroxylapatite were from Bio-Rad Laboratories (Utrecht, The Netherlands). Phenyl Superose HR 5/5, Superose 6 HR 10/30, Mono-Q HR 5/5 and molecular mass standards for gelfiltration and SDS-PAGE were purchased from Pharmacia Fine Chemicals (Woerden, The Netherlands). All other biochemicals were from Boehringer Mannheim (Almere, The Netherlands).

Results

Enzyme purification

The process of purification, in which the specific pyrophosphatase activity increased 200-fold, is summarized in Table 1. Like many pyrophosphatases of Gram-negative bacteria, the enzyme of *Acinetobacter johnsonii* 210A appeared to be remarkably heat-resistant (Blumenthal et al. 1967). This property was used in the purification procedure by heating the crude extract for 10 min at 70 °C. Heat labile proteins coagulated whereas pyrophosphatase could be recovered in the supernatant.

Characterization

The molecular mass of the native pyrophosphatase was estimated to be approximately 141 kDa by gelfiltration (Fig. 1). The subunit molecular mass of the enzyme was determined by SDS-PAGE. The pyrophosphatase enzyme was found to be composed of only one species of polypeptide with a molecular mass of 23 kDa (Fig. 2). This suggests pyrophosphatase to be a hexameric protein with six identical subunits. When native gels were incubated for 1 min in pyrophosphatase assay mixture and stained with ammonium heptamolybdate and methylgreen in 1 M H_2SO_4, both the crude extract and the purified enzyme showed one band with the same mobility. This points to the presence of only one pyrophosphatase enzyme in *A. johnsonii* 210A. Purified pyrophosphatase that had been kept for several weeks at -20°C showed two bands on native gel after activity staining. By the use of gel filtration, molecular masses of approximately 140 and 70 kDa were found, respectively. This indicates a partial degradation of pyrophosphatase during storage at -20°C in two active parts each consisting of three subunits.

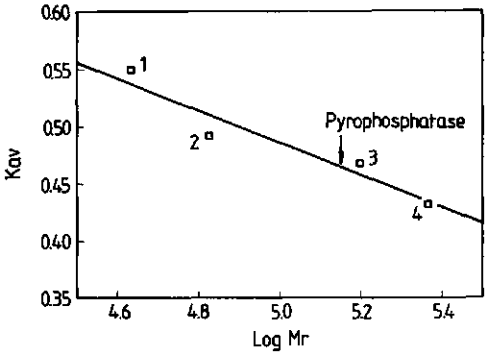

Figure 1. Molecular mass determination of the native pyrophosphatase on Superose 6 HR 10/30. Standards were ovalbumin (45 kDa, 1); bovine serum albumin (67 kDa, 2); aldolase (158 kDa, 3); catalase (232 kDa, 4). The position of pyrophosphatase of *A. johnsonii* 210A is indicated by the arrow.

Heat stability

Pyrophosphatase of *A. johnsonii* 210A appeared to be quite resistant to heat treatment. In the presence of 17 mM Mg^{2+}, 60% of the activity remained after 10 min at 80°C. The enzyme lost 90% of its activity when heated for 10 min at 90°C. In the absence of Mg^{2+}, the enzyme was less heat stable. Only about 25% of the activity was retained after 10 min at 70°C.

Substrate specificity

The purified enzyme was only able to use pyro- and triphosphate as a substrate. Polyphosphates (n=4, n=35) and organic phosphate esters were not hydrolyzed (Table 2). Pyro-and triphosphate were only utilized in the presence of divalent cations. Of the cations tested, Mg^{2+} (100%), Zn^{2+} (182%), Mn^{2+} (48%) and Co^{2+} (48%) were effective. Ba^{2+} and Ca^{2+} did not support pyrophosphatase activity.

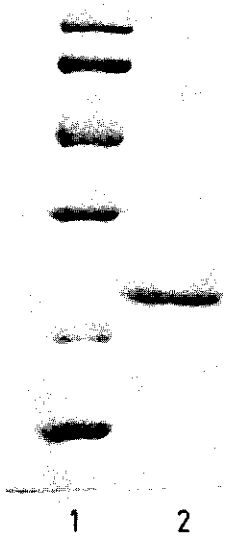

Figure 2. Analysis of purity by SDS-PAGE. Lanes: 1, molecular mass markers (94, 67, 43, 30, 20.1, 14.4 kDa); 2, purified pyrophosphatase of *A. johnsonii* 210A.

Table 2. Substrate specificity of pyrophosphatase

Substrate	Concentration	Relative rate of hydrolysis
Pyrophosphate	2 mM	100
Triphosphate	2 mM	27
Tetraphosphate	2 mM	0
Polyphosphate (n=35)	0.2 mg/ml	0
ATP	2 mM	0
ADP	2 mM	0
AMP	2 mM	0
Glucose-6-phosphate	2 mM	0
Phosphoenol pyruvate	4 mM	0
p-Nitrophenylphosphate	2 mM	0

Kinetic properties

The initial reaction rates at different pyrophosphate concentrations were followed to determine the affinity of the enzyme for this compound. Half maximal rates were obtained at 0.26 (± 0.02) mM pyrophosphate at a molar Mg to pyrophosphate ratio of 3. The enzyme activity depended on this ratio. At ratios below 1, the hydrolysis of pyrophosphate was reduced by approximately 50 %.

Inhibition studies

Similar to the situation in *Escherichia coli* (Josse and Wong 1971), *Ferrobacillus ferrooxidans* (Howard and Lundgren 1970), *Thiobacillus thiooxidans* (Tominaga and Mori 1977) and *Bacillus stearothermophilus* (Hachimori et al. 1975), pyrophosphatase of *A. johnsonii* 210A was strongly inhibited by fluoride (100%, 2 mM). Addition of NaN_3 (2 mM) or KCN (2 mM) did not affect the activity. Pyrophosphatase was completely inhibited in the presence of 2 mM iodoacetamide suggesting an involvement of sulfhydryl groups in the active site of the enzyme.

Discussion

From the increase in specific activity upon purification it can be calculated that about 0.5% of the soluble cell protein of *Acinetobacter johnsonii* 210A consists of pyrophosphatase. This is somewhat more than reported for *Methanotrix soehngenii* (0.2%) (Jetten et al. 1992) and *Escherichia coli* (0.2%) (Josse 1966) which is possibly due to a difference in P-metabolism between these organisms and *A. johnsonii* 210A. *A. johnsonii* 210A is able to accumulate large amounts of phosphate which are subsequently polymerized to polyphosphate and stored inside the cell in polyphosphate granules (Van Groenestijn 1988).

Pyrophosphatase of *A. johnsonii* 210A has been purified to a state of apparent homogeneity. The molecular mass of the purified enzyme was estimated to be approximately 141 kDa. This value is similar to the molecular mass of pyro-

phosphatase from *M. soehngenii* (139 kDa) (Jetten et al. 1992) but differs from molecular masses of pyrophosphatases from *E. coli* (120 kDa) (Wong et al. 1970), *Bacillus stearothermophilus* (120 kDa) (Hachimori et al. 1975), *Streptococcus faecium* (65-70 kDa) (Starr and Oginski 1971), *Thiobacillus thiooxidans* (88 kDa) (Tominaga and Mori 1977) and *Streptococcus faecalis* (128 kDa) (Lahti and Niemi 1981). The subunit structure of the enzyme was similar to the *E. coli* pyrophosphatase. Both enzymes consist of six identical polypeptides (Wong et al. 1970).

The heat resistance of pyrophosphatase of *A. johnsonii* 210A up to a temperature of 80°C for at least 10 min clearly shows that it belongs to the thermostable enzymes. Resistance to heat treatment has been demonstrated previously for pyrophosphatases isolated from several Gram-negative bacteria (Blumenthal et al. 1967; Howard and Lundgren 1970; Josse 1966; Tominaga and Mori 1977). Storage at -20°C resulted in a degradation of the enzyme into two identical, active parts.

The enzyme required divalent cations for activity. As reported for other pyrophosphatases, Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} were found to be effective (Josse 1966; Howard and Lundgren 1970). Similar to the increase in pyrophosphatase activity of the *E. coli* enzyme at pH 7.5, addition of Zn^{2+} resulted in a relatively strong stimulation (Josse 1966). Mn^{2+} and Co^{2+} affected the enzyme to a lesser extent.

Pyrophosphatase of *A. johnsonii* 210A only used pyro- and triphosphate as a substrate. Other phosphate esters were not hydrolyzed. A similar high specificity has been observed for many other microbial pyrophosphatases (Josse 1966; Hachimori et al. 1975; Tominaga and Mori 1977; Lahti and Niemi 1981). Inhibition by fluoride has been reported previously for pyrophosphatases of *E. coli* (Josse and Wong 1971), *T. thiooxidans* (Tominaga and Mori 1977), *B. stearothermophilus* (Hachimori et al. 1975) and *Ferrobacillus ferrooxidans* (Howard and Lundgren 1970). In contrast to several pyrophosphatases (Josse and Wong 1971; Lahti and Niemi 1981; Tominaga and Mori 1977), the enzyme of *A. johnsonii* 210A was also strongly inhibited by sulfhydryl-reagents indicating a possible participation of cysteine residues in pyrophosphate hydrolysis.

In conclusion, pyrophosphatase of *A. johnsonii* 210A appears to be similar to other bacterial pyrophosphatases with respect to heat stability, metal requirement and substrate specificity. On the other hand, the molecular mass and the effect of sulfhydryl-reagents on activity differ from those of most other pyrophosphatases.

Pyrophosphatase of *A. johnsonii* 210A may be involved in polyphosphate metabolism. Recently, an inhibition of the polyphosphate degrading enzymes of *A. johnsonii* 210A, polyphosphatase and polyphosphate:AMP phosphotransferase, by pyrophosphate was found (Bonting et al. 1991; Bonting et al. 1993). Since the concentration of pyrophosphate depends on the intracellular pyrophosphatase activity, this enzyme may influence polyphosphate degradation in an indirect way by removing the inhibiting pyrophosphate. Pyrophosphate may not only be involved in polyphosphate degradation but may also play a role in polyphosphate formation. In *Rhodospirillum rubrum*, evidence was found for the synthesis of polyphosphate by the use of pyrophosphate as a primer (Oh and Lee 1987). Kulaev postulated a reaction in which activated phosphate is transferred from pyrophosphate to polyphosphate. Removal of pyrophosphate in this way could be a very effective method to control biosynthetic reactions in which pyrophosphate is formed (Kulaev 1979). Therefore, it may be speculated that pyrophosphate can not be regarded only as a by-product of nucleoside triphosphate dependent reactions but also plays a role in cellular polyphosphate metabolism.

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CHAPTER 8

Regulation of polyphosphate metabolism in *Acinetobacter* strain 210A grown in carbon- and phosphate- limited continuous cultures

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Regulation of polyphosphate metabolism in *Acinetobacter* strain 210A grown in carbon- and phosphate-limited continuous cultures

Abstract

The response of *Acinetobacter* strain 210A to low phosphate concentrations was investigated in P- or C- limited chemostat cultures. The organism accumulated poly-ß-hydroxybutyric acid under P-deprivation, at phosphate concentrations ranging from 0.1 to 0.7 mM. The amount of biomass was proportional to the phosphate concentration in the medium and no polyphosphate was formed. When shifting a culture from P- to C-limitation, phosphate was accumulated as polyphosphate. No poly-ß-hydroxybutyrate could be detected in these cells. The amount of polyphosphate in the cell showed a hysteresis. When cultures were shifted from low to high phosphate concentrations, polyphosphate reached a maximum of about 60 mg P per gram of dry weight at about 3 times excess phosphate (ca. 2.5 mM P_i). It decreased to 45 mg P per gram of dry weight at approximately 5 times the phosphate needed for growth (ca. 3.5 mM P_i). In the reverse case (high to low), polyphosphate did never exceed 45 mg P per gram of dry weight. The specific activities of alkaline phosphatase and the phosphate uptake system were induced at residual P_i-concentrations below the detection limit ($< 10\ \mu M$). The specific uptake rate followed also a hysteresis. The specific activities of polyphosphatase and polyphosphate:AMP phosphotransferase increased when polyphosphate formation was possible.

Introduction

Phosphorus is one of the most important nutrients required for growth, and the effect of P_i-limitation on bacterial growth and cellular composition has been investigated in a variety of microorganisms. Phosphate limitation triggered different responses. *Escherichia coli* showed an induction of the synthesis of several proteins such as an outer membrane pore protein, periplasmic binding proteins for P_i, cytoplasmic membrane carriers involved in active transport of phosphate, periplasmic alkaline phosphatase and cytoplasmic polyphosphatase (Tomassen and Lugtenberg 1982). Studies on the chemical composition of the bacterial cell envelope of *Bacillus subtilis* and *Pseudomonas fluorescens* revealed that the P_i-containing cell wall components were replaced by non-phosphorylated compounds when phosphate was limiting (Ellwood and Tempest 1972; Minnikin and Adbolrahimzadeh 1974). In *Alcaligenes eutrophus* large amounts of poly-ß-hydroxybutyric acid were accumulated under phosphate deprivation (Anderson and Dawes 1990; Byrom 1987). Polyphosphate accumulating microorganisms degraded the polymer during phosphate starvation. Subsequent addition of phosphate to these cultures restored the initial

polyphosphate level (Harold 1966; Kulaev 1979). Data on growth and cellular composition of P_i-accumulating microorganisms in continuous cultures with phosphate as the limiting nutrient are scarce. Trotsenko and Shishkina (1990) reported that the amount of polyphosphate in the obligate methanotroph *Methylococcus capsulatus* increased with increasing phosphate concentrations in the growth medium. In the marine bacterium, *Vibrio natriegens*, growing in phosphate-limited chemostats at different dilution rates, polyphosphate synthesis was induced after addition of a P_i- pulse (Nissen et al. 1987).

Acinetobacter strain 210A is known for its ability to accumulate large amounts of phosphate in carbon- or nitrogen-limited chemostat cultures as well as in batch cultures (Van Groenestijn et al. 1989b). The response of this organism to different phosphate concentrations during growth in a chemostat is, however, unknown. The aim of this study was to investigate the partitioning of phosphate over biomass and polyphosphate and the activities of enzymes involved in (poly)-phosphate metabolism during growth at different phosphate concentrations. The paper reports on the effect of phosphate concentration on biomass, cellular composition, phosphate uptake rate and activities of enzymes involved in (poly)phosphate metabolism of *Acinetobacter* strain 210A.

Material and methods

Microorganism and cultivation

Acinetobacter strain 210A was isolated and described by Deinema et al. (1985). It was grown in phosphate-limited chemostat cultures in fermenters with a working volume of 0.9 l at a dilution rate of 0.1 h^{-1}. The temperature was kept at 20°C and the pH was automatically controlled at 7.0 with 1 M HCl. The medium contained per liter: 2.29 g of Na-butyrate, 1 g of NH_4Cl, 0.6 g of $MgSO_4 \cdot 7H_2O$, 0.06 g of $CaCl_2 \cdot 2H_2O$, 0.74 g of KCl, and 0.02 g of EDTA. After autoclaving, 3 ml heat-sterilized trace element solution (Van Groenestijn et al. 1987) and various amounts of NaH_2PO_4 were added to the medium reservoir. The different parameters were determined after at least 10 volume changes, when the cultures had reached a steady-state.

Cell extract preparation and enzyme assays

Cells were harvested by centrifugation at 8,000 x *g* for 10 min at 4°C, washed twice with 50 mM Tris-HCl buffer (pH 7.6) and stored as a pellet at -20°C until use. The frozen pellet was resuspended in 6 ml 50 mM Tris-HCl (pH 7.6) and disrupted at 0°C by sonication (10 x 30 s with 30 s intervals at 40 W; Branson Sonic Sonifier). The broken cells were centrifuged at 4°C for 30 min at 30,000 x *g*. The supernatant is referred to as cell-free extract and was used for enzyme assays.

All enzyme assays were carried out at 30°C.

Polyphosphate:AMP phosphotransferase was measured photometrically at 340 nm by following the reduction of $NADP^+$ as described by Van Groenestijn et al. (1987). The reaction mixture contained: 100 mM Tris-HCl (pH 8.5); 8 mM $MgCl_2$; 5 mM glucose; 0.4 mM $NADP^+$; 1 mM AMP; 0.2 g $\cdot$ l^{-1} polyphosphate; 1 U adenylate kinase; 2 U hexokinase; 1 U glucose-6-phosphate dehydrogenase. The reaction was started by the addition of AMP.

Adenylate kinase (EC 2.7.4.3) was measured by following the reduction of $NADP^+$. The reaction mixture contained: 100 mM Tris-HCl (pH 8.5); 8 mM $MgCl_2$; 5 mM glucose; 0.4 mM $NADP^+$; 2 mM ADP; 1 U hexokinase; 0.5 U glucose-6-phosphate dehydrogenase. The reaction was started by the addition of ADP.

Polyphosphatase (EC 3.6.1.11) activity was

determined by following the formation of phosphate from polyphosphate. The reaction mixture contained: 50 mM Tris-HCl (pH 7.6); 300 mM NH_4Cl; 10 mM $MgCl_2$; 0.4 g · l^{-1} polyphosphate. The reaction was started by the addition of polyphosphate.

Alkaline phosphatase (EC 3.1.3.1) was determined using a modification of the method described by Yasphe et al. (1990). The assay mix (0.5 ml) contained: 50 mM Tris-HCl (pH 8.5); 2 mM $CaCl_2$; 6 mM *p*-nitrophenylphosphate. The reaction was started by the addition of cell-free extract and stopped by the addition of 0.5 ml 0.2 M NaOH. After centrifugation for 2 min in an Eppendorf micro-centrifuge at 13,000 x *g*, the amount of *p*-nitrophenol formed was photometrically determined at 410 nm.

Pyrophosphatase (EC 3.6.1.1) was measured by following the rate of hydrolysis of pyrophosphate. The incubation mix contained: 50 mM Tris-HCl (pH 7.6); 10 mM $MgCl_2$; 2 mM P_2 (pyrophosphate). The reaction was started by the addition of P_2.

Phosphate-uptake measurements

Transport studies were performed as described by Kaback (1971). Cells were washed and diluted to a protein concentration of about 0.5 mg per ml in 20 mM K-Pipes buffer (pH 7.0) containing 10 mM $MgSO_4$ and 50 $\mu g \cdot ml^{-1}$ chloramphenicol. After 5 min of pre-energization in the presence of 20 mM glucose and 2 μM pyrroloquinoline quinone (PQQ), $^{32}P_i$ (0.20-0.30 TBq · mol^{-1}) to a final concentration of 100 μM was added. Initial rates of uptake were measured in duplicate at 30°C.

Analytical methods

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Bacterial dry weight was quantified by centrifuging culture samples at 8,000 x *g* for 10 min, washing the pellets twice with demineralized water and drying them to constant weight at 105°C. Poly-ß-hydroxybutyric acid (PHB) granules were stained by Sudan Black (Burdon 1946) and PHB was quantified according to Braunegg et al. (1978). Polyphosphate granules were stained with Neisser staining (Gurr 1965). Orthophosphate was measured spectrometrically according to the Standard Methods (1976).

For quantifying the phosphorus content of the cells, culture samples were centrifuged at 8,000 x *g* for 10 min and washed twice in 0.01 N HCl. The phosphorus content of the cells was calculated by subtracting the total amount of phosphorus present in the supernatant of each centrifugation step from the amount of phosphorus in the medium reservoir and dividing the difference between these values by the bacterial weight. In this way the phosphorus content can be expressed in mg P · g dry $weight^{-1}$.

Low polymeric and high polymeric polyphosphates were extracted from washed cells by the method of Clark et al. (1986). The last step of this method, the removal of nucleic acids, requires a concentration of at least 0.15 mg of polyphosphate per ml. This amount of polyphosphate in the isolated fraction was only found in organisms grown at phosphate concentrations in the influent exceeding 1 mM. Therefore this step was omitted. The amount of phosphorus in the polyphosphate fractions was spectrophotometrically determined according to the Standard Methods (1976) after boiling 0.1-0.5 ml of each fraction with 5 ml 1 M HCl during 20 min.

Butyrate in culture supernatants was measured by injecting 1 μl sample into a CP 9000 gaschromatograph (Chrompack, Middelburg, The Netherlands) fitted with a chromosorb 101 column. The oven temperature was 175°C and the eluent was nitrogen gas saturated with formic acid.

Electron microscopy

Samples used for electron microscopy were resuspended in 3% glutaraldehyde for 1.5 h. Cells were washed with 0.1 M sodium cacodylatebuffer (pH 7.2), fixed in 1% osmiumtetroxide, dehydrated and embedded in Epon 812. Sections of 50-70 nm were examined with the Philips EM 201 transmission electron microscope after staining with uranyl acetate and lead citrate.

Biochemicals

Polyphosphate glasses were obtained from Sigma Chemical Co. (Amsterdam, The Netherlands). $^{32}P_i$ was purchased from Amersham (Den Bosch, The Netherlands). Enzymes and biochemicals were obtained from Boehringer Mannheim (Almere, The Netherlands). All other chemicals were of analytical grade.

Results

Growth yield and cellular composition

Biomass. In order to study the effect of phosphate on the biomass and cellular composition of *Acinetobacter* strain 210A, the phosphate concentration in the medium reservoir was varied between 0.1 and 5.0 mM. Yields of steady-state cultures of *Acinetobacter* strain 210A as a function of the P_i-concentration in the influent (S_R^P) are shown in Figure 1A. At S_R^P-values below 0.7 mM the yield was proportional to the amount of phosphate in the medium reservoir. At phosphate concentrations above 0.7 mM, the amount of biomass showed a constant value of ca. 1,100 mg dry weight per liter.

Polyphosphate. At S_R^P-values below 0.7 mM the phosphorus content of the cells remained constant at about 15 mg phosphorus per gram of dry weight (Fig. 1B). No polyphosphate granules could be detected by Neisser staining, and the amount of phosphorus in the high polymeric polyphosphate fractions was almost zero (Fig. 1C).

An increase in the phosphate concentration in the medium reservoir from 0.7 to 1.5 mM did not result in an accumulation of phosphate in the culture supernatant although the amount of biomass remained constant. *Acinetobacter* strain 210A was able to take up more phosphate than required for growth. Excess phosphate was accumulated as high polymeric polyphosphate (PP_n, $n > 20$) (Figs. 1 B, C). The amount of low polymeric PP_n ($n < 20$) was always low (about 1 mg P · g dry weight^{-1}) regardless of the P_i-concentration in the influent (not shown). At phosphate concentrations of 1.5 - 2.5 mM, the phosphorus content of the biomass depended on whether the culture was shifted from low (< 1 mM) or from high (> 3 mM) phosphate concentrations to 1.5 - 2.5 mM phosphate. Values of more than 60 mg phosphorus per gram of dry weight were observed by shifting a culture from a low (< 1 mM) to a high (2.5 mM) phosphate concentration (Figs. 1 B, C). Analysis of the culture supernatant showed that in this situation the residual phosphate concentration was still below the detection limit (< 10 μM) (Fig. 1A). A phosphorus content of 45 mg P · dry weight^{-1} was obtained by shifting a culture from more than 3 mM to 2.5 mM phosphate in the influent. At S_R^P-values above 3 mM neither the yield nor the cellular phosphorus content (ca. 45 mg P · g dry weight^{-1}) changed and phosphate accumulated in the supernatant of the culture (Figs. 1 A, B).

Poly-ß-hydroxybutyric acid. At phosphate influent concentrations above 0.7 mM, all butyrate added was taken up by the cells. Surprisingly, even at a S_R^P-value of 0.5 mM, no butyrate could be detected in the culture supernatant although the

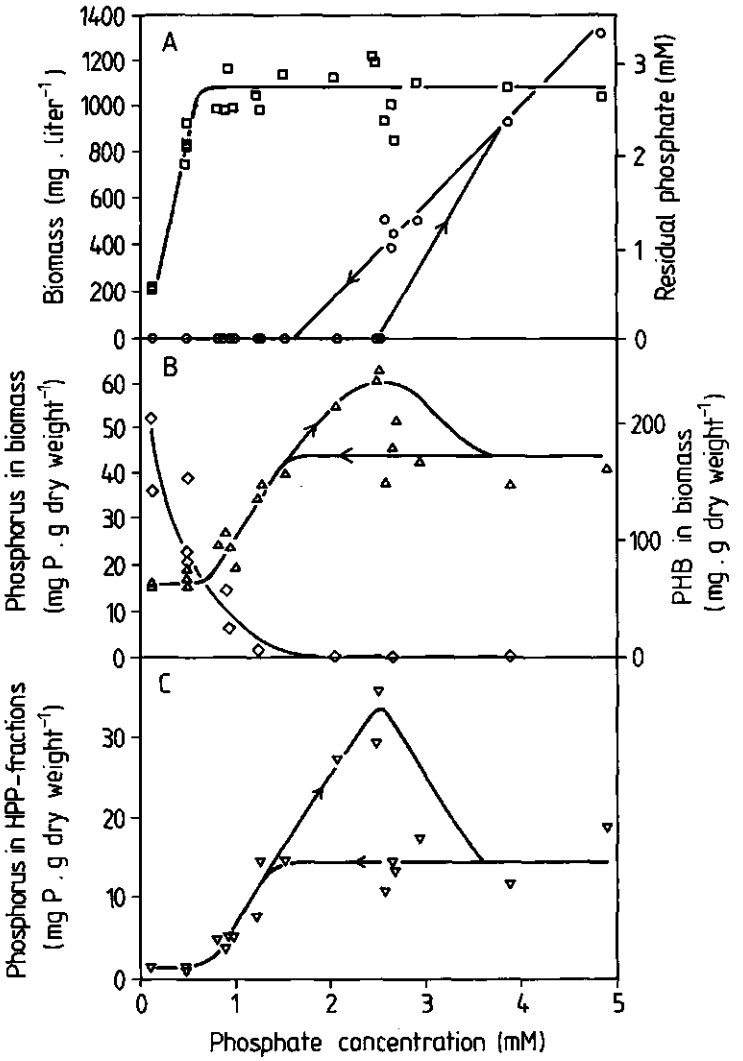

Figure 1. Effect of addition of increasing phosphate concentrations (0.1-5.0 mM) to the medium reservoirs of butyrate-limited (S_R = 20 mM) chemostat cultures of *Acinetobacter* strain 210A at D = 0.1 h^{-1} on a number of parameters: □, biomass; ○, residual phosphate concentrations; ▵, amount of phosphorus in biomass; ◇, amount of PHB in biomass; ▽, amount of phosphorus in high polymeric polyphosphate fractions. Arrows pointing in the direction of higher phosphate concentrations: shifting the chemostat culture from low to high phosphate influent concentrations. Arrows pointing to lower phosphate concentrations: shifting the chemostat culture from high to low phosphate influent concentrations.

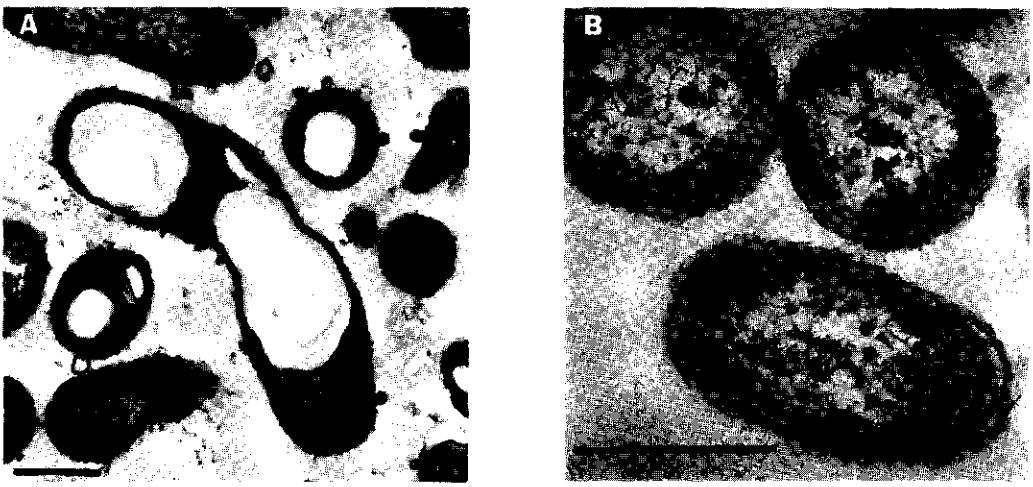

Figure 2. Electron micrographs of thin sections of *Acinetobacter* strain 210A grown in butyrate-limited (S_R = 20 mM) chemostat cultures at D = 0.1 h^{-1} at a phosphate influent concentration of: A) 0.1 mM. Cells contained 14 % (w/w) PHB and 15 mg P · g dry $weight^{-1}$. Bar is 0.5 μm. B) 2.5 mM. Cells contained no PHB but 45 mg P · g dry $weight^{-1}$. Bar is 0.5 μm.

amount of biomass was significantly lower than at higher P_i-concentrations in the medium reservoir (Fig. 1A). Bacteria grown at phosphate influent concentrations below 0.7 mM contained granules with a high refractivity. These inclusions retained Sudan Black, an agent generally used for staining PHB. The electron micrographs showed large electron transparent inclusions (Fig. 2A). At a S_R^P-value above 1.5 mM the electron translucent inclusions were absent (Fig. 2B). Determination of the PHB content of cells grown at different phosphate influent concentrations showed that the amount of PHB gradually decreased with an increasing phosphate concentration in the medium (Fig. 1B).

Phosphate uptake

A high P_i-uptake rate of 150 to 200 nmol · mg^{-1} protein · min^{-1} was observed at residual phosphate concentrations below the detection limit (Fig. 3). By shifting a culture from high (S_R^P > 2.5 mM) to lower P_i-concentrations in the influent, the uptake rate increased at S_R^P-values lower than 1.5 mM. If a culture was shifted from low (S_R^P < 1 mM) to high P_i-concentrations, the uptake rate showed a hysteresis effect between 1.5 and 3 mM (Fig. 3). Low P_i-uptake rates of 10-25 nmol · mg^{-1} protein · min^{-1} were found at S_R^P-values above 3 mM.

Enzyme activities

Orthophosphate is known to be a specific regulator of alkaline phosphatase (Torriani 1960). Its synthesis is phosphate repressed, but is induced if the P_i-concentration in the medium is lowered to about 50 μM (Rosenberg 1987). As shown in Fig. 4A, the alkaline phosphatase activity rapidly increased if the residual phosphate concentration in the growth medium was below the detection limit.

Until now two polyphosphate degrading enzymes have been found in *Acinetobacter* strain 210A, polyphosphate:AMP phosphotransferase and polyphosphatase (Van Groenestijn et al. 1989a).

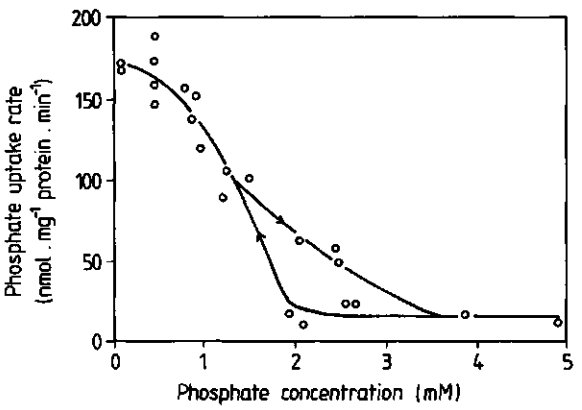

Figure 3. Influence of the phosphate concentration in the growth medium (S_R^P = 0.1-5.0 mM) on the phosphate uptake rate of *Acinetobacter* strain 210A grown in butyrate-limited (S_R = 20 mM) chemostat cultures at D = 0.1 h^{-1}. Arrow pointing in the direction of higher phosphate concentrations: shifting the chemostat culture from low to high phosphate influent concentrations. Arrow pointing to lower phosphate concentrations: shifting the chemostat culture from high to low phosphate influent concentrations.

The activities of these two enzymes were low in the absence of polyphosphate in the cell (S_R^P < 0.7 mM). As soon as polyphosphate was detectable, the specific activity of both enzymes increased by a factor of 3-4 (Figs. 4 B, C). The amount of polyphosphate in the range from 5 to 30 mg P · g dry $weight^{-1}$ did not influence the activity of these enzymes (Figs. 1C, 4B and 4C). The activities of the enzymes pyrophosphatase and adenylate kinase remained at a constant level of 1,200 and 590 nmol · mg^{-1} protein · min^{-1}, respectively, regardless of the phosphate concentration in the medium.

Discussion

Although the ability of *Acinetobacter* strain 210A to accumulate large amounts of polyphosphate is well documented (Van Groenestijn 1988), the influence of phosphate on biomass and polyphosphate formation was unknown. The data presented in this paper show that addition of phosphate to phosphate-starved cells resulted in biomass formation. Polyphosphate was accumulated only after the maximal amount of biomass was attained (Fig. 1). The organism was able to form poly-ß-hydroxybutyrate when grown under phosphate limitation. This finding is in contrast with studies of Baumann et al. (1968) who reported that bacteria of the genus *Acinetobacter* do not accumulate PHB as reserve material. However, recently PHB was found in eight strains of the genus *Acinetobacter*. The amount of PHB was very small. Less than 0.001 % of the dry weight of the cells consisted of this polymer (Vierkant et al. 1990).

An increase in phosphate uptake rates has been observed in numerous microorganisms when grown under phosphate limitation (Rosenberg 1987). The uptake rate of *Acinetobacter* strain 210A increased 10-fold, when the phosphate concentration was lowered (Fig. 3). The stimulation of the phosphate uptake system in this organism is coupled to the stimulation of alkaline phosphatase (Fig. 4A), just as observed in *Escherichia coli* (Tomassen and Lugtenberg 1982; Rosenberg 1987).

The influence of phosphate deprivation on polyphosphate degrading enzyme activities has been studied in *E. coli* and *Aerobacter aerogenes*. In these organisms an increase in polyphosphate degrading enzyme activity could be measured during growth under phosphorus limitation (Nesmeyanova et al. 1974; Harold 1966). Kulaev (1979) concluded that in *E. coli* alkaline phosphatase, triphosphatase and polyphosphatase are regulated by a single metabolite, possess a

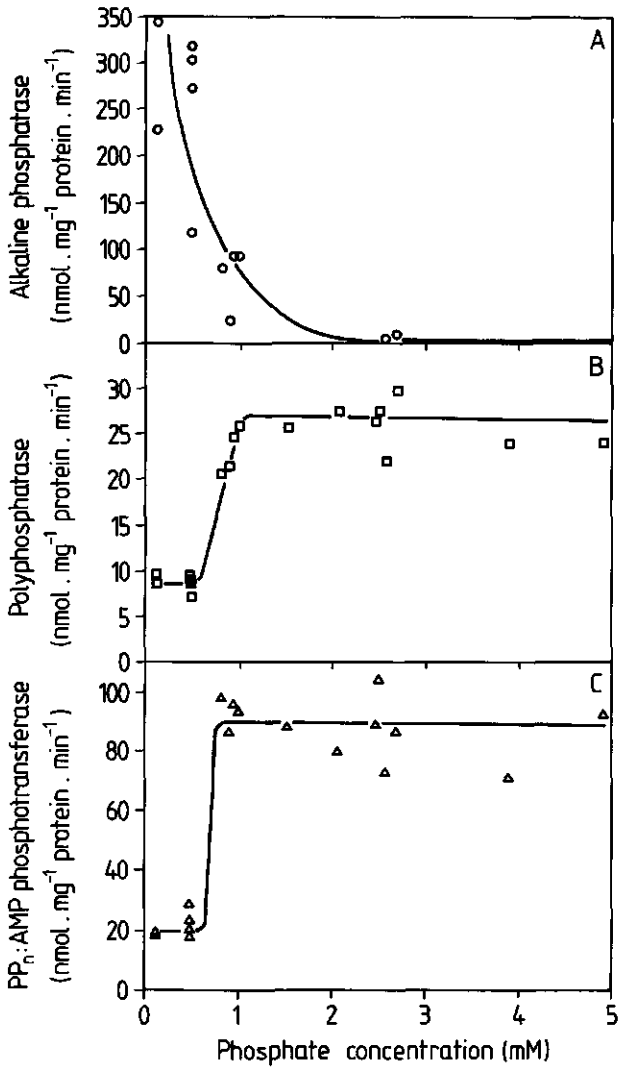

Figure 4. Relation between enzyme activities and phosphate influent concentrations (0.1-5.0 mM) of butyrate-limited (S_R = 20 mM) chemostat cultures of *Acinetobacter* strain 210A at D = 0.1 h^{-1}. ○, alkaline phosphatase; □, polyphosphatase; ▵, polyphosphate:AMP phosphotransferase.

common regulatory gene and constitute a single regulon. Harold (1966) suggested that in *A. aerogenes* polyphosphate kinase, polyphosphatase and alkaline phosphatase share a common regulator gene but do not fall into a single operon. In *Acinetobacter* strain 210A polyphosphatase and polyphosphate:AMP phosphotransferase are, however, not regulated in the same way as the phosphate uptake system and alkaline phosphatase (Figs. 3, 4). The activities of polyphosphatase and polyphosphate:AMP phosphotransferase were low at low S_R^P-values (< 0.7 mM) whereas the activity of alkaline phosphatase and the phosphate uptake rate were high. Polyphosphatase and polyphosphate:AMP phosphotransferase seem not to be regulated by the extracellular concentration of phosphate but rather by the presence of polyphosphate in the cell. As soon as polyphosphate formation is possible, the activities of these enzymes increase.

The correlation of polyphosphatase and polyphosphate:AMP phosphotransferase activity and the presence of polyphosphate in the cell can be interpreted in two ways. Either these enzymes participate in the biosynthesis of polyphosphate or the biosynthesis of these enzymes is activated by its own substrate (polyphosphate). Further investigations on the polyphosphate synthesis in *Acinetobacter* strain 210A are needed to determine which of these two hypotheses is correct.

The uptake rate of phosphate at S_R^P-values between 1.5 and 2.5 mM depends on whether the culture was shifted from low to higher or from high to lower phosphate concentrations in the influent (Fig. 3). By shifting a culture from low (< 1 mM) to high (2.5 mM) phosphate concentrations, relatively high uptake rates and increased amounts of high polymeric polyphosphate were observed (Figs. 1C, 3). The shifting effect may be regarded as a hysteresis, a feature recently also reported in a study on the micro-aerobic growth of *Saccharomyces cerevisiae* in continuous cultures (Grosz and Stephanopoulos 1990). The hysteresis effect may be either due to the regulation of the P_i-uptake system or to the regulation of the polyphosphate synthesis or to both.

The data presented in this paper show that the partitioning of phosphate over biomass and polyphosphate is regulated very strictly. At very low phosphate concentrations in the influent (0.1 - 0.7 mM), phosphate is used exclusively for biomass production and the flux to polyphosphate formation is zero. Only if enough phosphate is present to attain maximal cell yield, polyphosphate is synthesized. As soon as polyphosphate formation is possible, the activities of the polyphosphate degrading enzymes increase.

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CHAPTER 9

Summary and outlook

Summary and outlook

Since the seventies, there is a growing interest in the process of biological phosphate removal in which microorganisms able to accumulate large amounts of phosphate play a central role. Over the years many bacteria have been isolated from sludge systems showing enhanced biological phosphate removal, either to investigate the bacterial population or to obtain pure cultures which were used as model organisms in studies on enhanced biological phosphate removal. Often, bacteria of the genus *Acinetobacter* were found. Therefore, it was suggested that these organisms probably play an important role in biological phosphate removal. In 1980, *Acinetobacter* strain 210A was isolated from sludge of a wastewater treatment plant in Renkum. This organism was able to accumulate large amounts of phosphorus in the form of polyphosphate (Van Groenestijn, 1988). The aim of this study was to examine various aspects of the metabolism of polyphosphate in this organism. This thesis describes next to the identification of *Acinetobacter* strain 210A, properties of bacterial polyphosphate, degradation and *in vivo* synthesis of polyphosphate and the regulation of polyphosphate metabolism by external phosphate.

Acinetobacter strain 210A was identified as *Acinetobacter johnsonii* by using a combination of biochemical and genetic properties. The organism was able to synthesize two polymers, polyphosphate and poly-ß-hydroxybutyric acid. Polyphosphate was formed during growth at excess phosphate whereas poly-ß-hydroxybutyric acid was synthesized when phosphate was the limiting nutrient. Intact cells were able to oxidize a variety of monosaccharides in the presence of PQQ (pyrollo-quinoline quinone). This ability was also found in other *Acinetobacter* strains and was used in the examination of the bacterial population of two activated sludge types showing enhanced biological phosphate removal. It was found that the bacterial community structure of these sludges differed strongly. Therefore, it was suggested that the presence of *Acinetobacter* sp. in activated sludge systems showing enhanced biological phosphate removal depends on process design and influent composition of the treatment system (Chapter 2).

Polyphosphate synthesized by *Acinetobacter johnsonii* 210A was localized in the cytoplasm mostly complexed in one or two large and several small granules. Electron microscopy and energy dispersive X-ray micro-analysis were used to examine the elemental composition of the large polyphosphate granules in unfixed and unstained intact cells of *A. johnsonii* 210A. When the organism was grown in standard medium, the granules were composed of phosphorus, magnesium and potassium. By modifying the amount of Ca^{2+} and Mg^{2+} in the medium, the intracellular concentration of Ca^{2+} and Mg^{2+} as well as the elemental composition of the polyphosphate granules could be changed. A high Mg/Ca-ratio in the medium resulted in a high Mg/Ca-ratio in the cytoplasm and in the presence of Mg as counterion in the polyphosphate granule. Ca became the major cation in the polyphosphate bodies during growth in a medium

with a low Mg/Ca-ratio. These results were at variance with previous studies dealing with the elemental composition of large polyphosphate granules which revealed Ca as the dominant counterion in the large polyphosphate bodies (Buchan 1981; Buchan 1983). This discrepancy could be ascribed to fixation and embedding procedures. Fixation of cells in glutaraldehyde and embedding in EPON have a profound effect on the elemental composition of polyphosphate granules (Chapter 3). Polyphosphate accumulated by *A. johnsonii* 210A consisted of about 700 P-residues as determined by gelelectrophoresis. *In vivo* ^{31}P-NMR was used to follow polyphosphate formation in intact cells. The amount of polyphosphate synthesized, correlated positively with the intracellular ATP concentration, suggesting an involvement of ATP in polyphosphate formation. In contrast to polyphosphate synthesis, polyphosphate degradation is a slow process (Chapter 4). Two polyphosphate degrading enzymes, polyphosphate:AMP phosphotransferase and polyphosphatase, have been found in *A. johnsonii* 210A.

Polyphosphate:AMP phosphotransferase phosphorylates AMP to ADP with polyphosphate: PP_n (polyphosphate) + AMP → PP_{n-1} + ADP. The enzyme was purified more than 1,500-fold from *A. johnsonii* 210A by streptomycin sulfate precipitation, and by Mono-Q, Phenyl Superose, and Superose column chromatography. Kinetic studies showed apparent K_m values of 0.26 mM for AMP and 0.8 μM for polyphosphate with an average chain length of 35 P-groups. The highest activities were found with polyphosphate molecules of 18 to 44 phosphate residues. The polyphosphate chain was degraded completely to ADP via a processive mechanism. No activity was obtained with ortho-, pyro-, tri-, and tetraphosphate. The enzyme was inhibited by pyro-, tri-, and tetraphosphate (Chapter 5).

Chapter 6 describes the purification and characterization of polyphosphatase of *A. johnsonii* 210A. This enzyme hydrolyzes polyphosphate to P_i: $PP_n + H_2O \rightarrow PP_{n-1} + P_i$. It was purified 77-fold by Q-Sepharose, hydroxylapatite and mono-Q column chromatography. The enzyme was specific for high polymeric polyphosphates and showed no activity towards pyrophosphate and organic phosphate esters. Analysis of kinetic properties revealed an apparent K_m-value for polyphosphate with an average chain length of 64 residues of 30 mg polyphosphate per liter and for tetraphosphate of 1.2 mM. Polyphosphate chains were degraded to short chain polymers via a processive mechanism. The enzyme was inhibited by iodoacetamide and, in the presence of high Mg^{2+}-concentrations, by pyro-and triphosphate. The activating effect of Mg^{2+} on polyphosphatase was enhanced by K^+ and NH_4^+.

Pyro-and triphosphate were hydrolyzed by pyrophosphatase, an enzyme which is in contrast to the polyphosphate degrading enzymes, widely distributed in nature. It was purified from *A. johnsonii* 210A and showed except against pyro-and triphosphate no activity towards polyphosphates and a wide variety of organic phosphate esters. The enzyme is composed of 6 identical subunits of 23 kDa, giving a molecular mass of 141 kDa for the native enzyme. Mg^{2+} was required for activity. The enzyme was heat-stable and inhibited by fluoride and iodoacetamide. The apparent K_m value for pyrophosphate was estimated to be 0.26 mM (Chapter 7).

The effect of varying phosphate concentrations on biomass, cellular composition, phosphate uptake rate and activities of enzymes involved in (poly)phosphate metabolism of *A. johnsonii* 210A was investigated in P-or C-limited chemostat cultures. The organism accumulated poly-ß-hydroxybutyric acid under P-deprivation, at phosphate concentrations ranging from 0.1 to 0.7 mM. The amount of biomass was proportional to the phosphate concentration in the medium and no polyphosphate was formed.

When shifting a culture from P-to C-limitation, phosphate was accumulated as polyphosphate. No poly-ß-hydroxybutyric acid could be detected in these cells. As soon as polyphosphate synthesis was possible, the specific activities of polyphosphate:AMP phosphotransferase and polyphosphatase increased about four fold. The specific activities of alkaline phosphatase and the P-uptake system were induced at residual phosphate concentrations below the detection limit. The effect of phosphate on the cellular polyphosphate content and on the P-uptake rate showed a hysteresis behaviour. When chemostat cultures were shifted from low to high phosphate concentrations, polyphosphate reached a maximum of about 60 mg P per gram of dry weight at a phosphate influent concentration of 2.5 mM. In the reverse case (high to low), polyphosphate did never exceed 45 mg P per gram of dry weight (Chapter 8).

To optimize the process of biological phosphate removal, a thorough understanding of all processes involved in polyphosphate metabolism of bacteria present in activated sludge is needed. These processes comprise: polyphosphate synthesis, polyphosphate degradation, and phosphate transport. In this thesis, properties of polyphosphate and polyphosphate degradation in *A. johnsonii* 210A were examined extensively. However, polyphosphate synthesis and phosphate transport were not clarified. From the positive correlation between intracellular ATP levels and polyphosphate synthesis, it was suggested that polyphosphate kinase may be involved in the process of polyphosphate formation. However, in cell extracts of *A. johnsonii* 210A, no polyphosphate kinase activity could be detected. This discrepancy can be interpreted in two ways. Either the polyphosphate kinase enzyme looses its activity during extract preparation or other polyphosphate synthesizing enzymes are involved in the formation of polyphosphate in *A. johnsonii* 210A. Two enzyme systems different from the polyphosphate kinase can be hypothesized: (1) a system in which a phosphorylated compound other than ATP is the P-donor or (2) a membrane bound proton translocating enzyme system. However, no positive indications for either of these two systems were found. Recently, the transport of phosphate in *A. johnsonii* 210A was characterized. Two transport systems could be demonstrated, (i) an inducible, ATP-driven, binding protein-dependent system, and (ii) a constitutive, low-affinity uptake system. It was suggested that the low-affinity system may be involved in the anaerobic metabolism of *A. johnsonii* 210A. Metabolic energy could be conserved by the generation of an electrochemical gradient across the cytoplasmic membrane, when phosphate is excreted together with ions (Van Veen et al. 1993). A more detailed knowledge of polyphosphate synthesis and phosphate transport would not only enlarge the insight in polyphosphate metabolism of phosphate accumulating bacteria, but would be of major importance for a better understanding of the process of biological phosphate removal. A better understanding would allow the optimization of its application based on information on physiological and biochemical mechanisms.

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CHAPTER 10

Samenvatting en discussie

Samenvatting en discussie

Sinds de zeventiger jaren is er een toenemende belangstelling voor het proces van biologische defosfatering waarin micro-organismen die in staat zijn grote hoeveelheden fosfaat op te nemen, een centrale rol spelen. Om de samenstelling van de bacteriële flora in biologisch defosfaterend slib te kunnen bepalen en om reincultures in handen te krijgen die als model organisme voor het bestuderen van biologische fosfaatverwijdering kunnen dienen, zijn vele bacteriën uit dergelijke slibsystemen geïsoleerd. Vaak werden bacteriën van het geslacht *Acinetobacter* gevonden wat leidde tot de veronderstelling dat deze organismen een belangrijke rol spelen in het verwijderen van fosfaat in actief slib. In 1980 werd een bacteriestam uit slib geïsoleerd, welke geïdentificeerd werd als een *Acinetobacter* sp. Dit organisme, *Acinetobacter* stam 210A, werd het model organisme voor het onderzoek aan polyfosfaat. Nadat uit het werk van Van Groenestijn (1988) bleek dat deze bacterie in staat was grote hoeveelheden fosfaat op te nemen en in de cel als polyfosfaat op te slaan, werd dit onderzoek gestart met als doel de kennis met betrekking tot de fysiologie van polyfosfaat vormende bacteriën te vergroten. Dit proefschrift beschrijft naast de identificatie van de model bacterie *Acinetobacter* stam 210A tot op soortsniveau, eigenschappen van het bacteriële polyfosfaat, de afbraak en *in vivo* synthese van polyfosfaat en de regulatie van het polyfosfaat metabolisme door extern fosfaat.

Met behulp van biochemische en genetische technieken werd het micro-organisme *Acinetobacter* stam 210A geïdentificeerd als een *Acinetobacter johnsonii*. Het organisme bleek in staat behalve polyfosfaat ook poly-ß-hydroxybutyraat te vormen indien het gekweekt werd onder P-limiterende condities. Het vermogen van dit organisme om monosacchariden te oxideren tot de overeenkomstige zuren in aanwezigheid van de cofactor PQQ (pyrollo-quinoline quinone) was ook in andere *Acinetobacter* stammen gevonden. Deze eigenschap werd gebruikt om de bacteriële samenstelling van twee defosfaterende slibsoorten te bestuderen. De bacterie populatie bleek zeer verschillend te zijn, wat leidde tot de veronderstelling dat procescondities en influent samenstelling van invloed zijn op de microbiële flora van defosfaterende slibsystemen (Hoofdstuk 2).

Het polyfosfaat dat door *A. johnsonii* 210A gevormd wordt tijdens groei in aanwezigheid van overmaat energie en fosfaat, werd uitvoerig onderzocht. Polyfosfaat komt in deze bacterie vooral voor in grote polyfosfaat granules. Deze granules bevatten naast het element P ook de elementen Mg, K of Ca. Dit is niet zo verwonderlijk gezien de sterk negatieve lading van het polyfosfaat molecuul. Deze lading moet gecompenseerd worden door positief geladen tegenionen. Mg^{2+}, K^{+} en Ca^{2+} bleken als zodanig te kunnen dienen. Zowel de aanwezigheid van Mg en Ca in de polyfosfaatbol als de concentratie van deze metaalionen in het cytoplasma bleek te worden beïnvloed door de concentratie van deze kationen in het groeimedium. Een hoge Mg/Ca-ratio in het medium leidde tot een grote

Mg/Ca-ratio in het cytoplasma en tot de aanwezigheid van uitsluitend Mg als tegenion in de polyfosfaatgranule. Werd *A. johnsonii* 210A gekweekt in een medium met weinig Mg^{2+} en veel Ca^{2+}, dan bleek de Ca^{2+}-concentratie in het cytoplasma sterk toe te nemen terwijl de cytoplasmatische Mg^{2+}-concentratie juist duidelijk afnam. Onder deze condities was Ca het tegenion van de polyfosfaatketens in de polyfosfaatgranules. Deze resultaten bleken niet in overeenstemming te zijn met de literatuur waarin Ca als dominant kation werd gevonden (Buchan 1981; Buchan 1983). Dit verschil bleek een gevolg te zijn van het effect van fixatie en inbedding op de samenstelling van de polyfosfaat granule (Hoofdstuk 3). *A. johnsonii* 210A bleek uitsluitend hoog polymere polyfosfaten te bevatten. De ketenlengte van deze polymeren werd met behulp van gelelectroforese geschat op 700 P-groepen. Met *in vivo* ^{31}P-NMR werd een correlatie gevonden tussen het intracellulaire ATP niveau en de polyfosfaat synthese. Dit duidt op een mogelijke betrokkenheid van ATP in de polyfosfaat vorming (Hoofdstuk 4). In tegenstelling tot de relatief snelle polyfosfaat synthese, verloopt de polyfosfaat afbraak zeer traag. Voor deze polyfosfaat degradatie zijn twee enzymen verantwoordelijk, polyfosfaat:AMP fosfotransferase en polyfosfatase.

Polyfosfaat:AMP fosfotransferase katalyseert de reactie PP_n (polyfosfaat) + AMP → PP_{n-1} + ADP. Het enzym werd 1500 maal opgezuiverd uit *A. johnsonii* 210A en bleek een goede affiniteit te hebben voor de substraten AMP (K_m = 0.26 mM) en hoog polymeer polyfosfaat (K_m = 3 mg polyfosfaat/liter, n = 35). Het hoog polymere polyfosfaat werd via een 'processive' mechanisme afgebroken met ADP als eindproduct. Kleine polyfosfaten werden door dit enzym niet als substraat gebruikt maar bleken de activiteit te remmen (Hoofdstuk 5).

Hoofdstuk 6 beschrijft de zuivering en karakterisering van het andere polyfosfaat afbrekende enzym van *A. johnsonii* 210A, polyfosfatase. Dit enzym katalyseert de reactie $PP_n + H_2O \rightarrow PP_{n-1} + P_i$. Het werd 77 maal opgezuiverd en bleek in staat hoog polymere polyfosfaten te hydrolyseren (K_m = 30 mg polyfosfaat/liter, n = 64). Pyro-en trifosfaat werden door dit enzym niet afgebroken. Ook de activiteit van dit enzym werd door deze kleine polyfosfaten geremd. Maximale activiteit vertoonde het enzym indien naast Mg^{2+}, ook hoge concentraties K^+ of NH_4^+ aanwezig waren. Volledige remming vond plaats in aanwezigheid van 2 mM jodoaceetamide. Afbraak van hoog polymere polyfosfaten door polyfosfatase leidde tot de vorming van kleinere polyfosfaten via een 'processive' mechanisme.

Pyro-en trifosfaat werden gehydrolyseerd door een enzym welke niet specifiek is voor polyfosfaat accumulerende micro-organismen namelijk pyrofosfatase. Dit enzym werd uit *A. johnsonii* 210A geïsoleerd en gezuiverd tot homogeniteit. Het bleek te bestaan uit zes identieke subunits van 23 kDa. Het enzym had Mg^{2+} nodig voor activiteit, was hitte stabiel en werd geremd door jodoaceetamide en fluoride (Hoofdstuk 7).

De invloed van extern fosfaat op de polyfosfaat afbrekende enzymen en cellulaire samenstelling werd onderzocht door *A. johnsonii* 210A te kweken in P-en C-gelimiteerde continu cultures. Tijdens P-limitatie bleek het organisme geen polyfosfaat maar poly-ß-hydroxybutyraat te accumuleren, was de opnamesnelheid van fosfaat hoog en de activiteit van zowel het polyfosfaat:AMP fosfotransferase als het polyfosfatase laag. Zodra de fosfaat concentratie in het medium hoog genoeg was om polyfosfaat synthese mogelijk te maken, werden de activiteiten van de polyfosfaat afbrekende enzymen 3 à 4 maal zo hoog en nam de fosfaat opnamesnelheid af. Gekweekt onder C-limitatie (P-overmaat) bleek de cel geen poly-ß-hydroxybutyraat maar polyfosfaat te bevatten (45 mg P/ gram droog gewicht), waren de activiteiten van polyfosfaat:AMP fosfo-

transferase en polyfosfatase maximaal (90 en 25 nmol/min mg eiwit, respectievelijk) en de fosfaat opnamesnelheid zeer laag. De fosfaat opnamesnelheid en celsamenstelling vertoonden tijdens dit experiment een hysterese effect. Cellen gekweekt bij lage fosfaat concentraties (< 1 mM) bleken 60 mg P/gram droog gewicht te kunnen accumuleren bij een fosfaat influent concentratie van 2.5 mM, terwijl cellen gekweekt bij een hoge fosfaat concentratie (> 3 mM) slechts 45 mg P/gram droog gewicht bevatten indien de fosfaat concentratie in het influent verlaagd werd tot 2.5 mM (Hoofdstuk 8).

Voor het optimaliseren van de biologische fosfaatverwijdering is een gedegen kennis nodig van het polyfosfaat metabolisme van bacteriën die aanwezig zijn in actief slib. Drie processen zijn hierbij van belang: polyfosfaat synthese, polyfosfaat afbraak en fosfaat transport over de cytoplasmatische membraan. In dit proefschrift worden eigenschappen van polyfosfaat en de polyfosfaat afbraak uitgebreid behandeld. De polyfosfaat synthese en het fosfaat transport werden echter niet opgehelderd. Met behulp van *in vivo* ^{31}P-NMR werd op indirecte wijze aangetoond dat ATP mogelijk betrokken is bij de vorming van polyfosfaat. Echter, in een celvrij extract kon geen activiteit van het enzym polyfosfaatkinase worden aangetoond. Dit enzym katalyseert de overdracht van de γ-P groep van ATP naar polyfosfaat. Voor het ontbreken van polyfosfaatkinase activiteit zijn twee mogelijke oorzaken aan te wijzen. Óf het enzym verliest zijn activiteit tijdens de vorming van het extract óf polyfosfaat wordt met behulp van andere enzymen gesynthetiseerd. Twee enzym systemen, verschillend van polyfosfaatkinase, zijn mogelijk: (1) een systeem waarin niet ATP maar een andere gefosforyleerde verbinding de P-donor is of (2) een membraan gebonden, proton motive force gedreven polyfosfaat synthetiserend enzym. Voor de aanwezigheid van deze enzym systemen werden echter geen positieve aanwijzingen gevonden. Onlangs werd de opname en afgifte van fosfaat door *A. johnsonii* 210A gekarakteriseerd. Er bleken twee transport systemen aanwezig te zijn: (1) een induceerbaar ATP-gedreven systeem en (2) een constitutief opname-systeem met een lage affiniteit voor fosfaat. Het laatst genoemde systeem is mogelijk betrokken bij het conserveren van energie tijdens de polyfosfaat afbraak indien de afgifte van fosfaat samengaat met het transport van ionen (Van Veen et al. 1993). Een gedegen kennis van polyfosfaat synthese en fosfaat transport zou niet alleen kunnen bijdragen tot een beter inzicht in het polyfosfaat metabolisme van fosfaat accumulerende bacteriën maar is tevens van groot belang voor een beter begrip en sturing van de biologische fosfaatverwijdering.

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curriculum vitae

De auteur van dit proefschrift werd op 30 maart 1961 geboren te Stadskanaal alwaar hij de lagere en middelbare school doorliep. In 1979 werd het Atheneum-B diploma behaald. Vijf jaar later, in augustus 1984, studeerde hij af als 2e graads leraar Biologie en Scheikunde aan de lerarenopleiding 'Ubbo Emmius' te Groningen. In datzelfde jaar werd met het doorstroomprogramma van de studie Biologie van de Rijksuniversiteit Groningen begonnen. Het doctoraalpakket bestond uit het hoofdvak Microbiële Fysiologie en de bijvakken Plantenfysiologie en Scheikunde. Tevens werd voor zowel Biologie als Scheikunde een 1e graads onderwijsbevoegdheid verkregen. Na het afstuderen in februari 1988, werd in maart van dat jaar begonnen met het onderzoek aan het polyfosfaatmetabolisme in *Acinetobacter johnsonii* 210A aan de vakgroep Microbiologie van de Landbouwuniversiteit Wageningen. De resultaten van dit promotie-onderzoek staan beschreven in dit proefschrift.

nawoord

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