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Perera, Ganegodage Shyama, M.S.

University of Nevada, Las Vegas, 1993

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MYCELOID CELL FORMATION IN

Arthrobacter globiformis

DURING OSMOTIC STRESS

by

G. Shyama Perera

A thesis submitted in partial fulfillment
of the requirements for the degree of

Master of Science

in

Biology

Department of Biological Sciences
University of Nevada, Las Vegas
December 1993

The Thesis of G. Shyama Perera for the degree of Master of Science in Biology is approved.

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University of Nevada, Las Vegas

December 1993

ABSTRACT

Arthrobacters exhibit a dimorphic growth cycle where exponential phase cells appear as irregular bacilli and stationary phase cells as cocci. *Arthrobacter globiformis* was found to form myceloids when subjected to osmotic stress. Addition of known osmoprotective compounds did not relieve this effect. Since myceloid formation may result from altered penicillin-binding proteins (PBPs), PBP profiles of stressed and unstressed *A. globiformis* were studied. Eight PBPs were identified, ranging in molecular mass from 91,000 to 30,000 daltons. Myceloids appeared to lack PBP 2a (84 kD) and PBP 2b (82 kD) in all growth conditions. The myceloids were 12-20 times more sensitive to beta-lactam antibiotics than the normal bacteria and 320 times more resistant to streptomycin. Myceloids were more sensitive to heat than the coccal forms and more resistant to long term starvation in a buffer lacking a nitrogen source. These results indicate that myceloid formation in *A. globiformis* may result from the altered synthesis of specific PBPs.

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ACKNOWLEDGEMENT

I like to express my sincere gratitude to Dr. Charles Deutch for letting me work in his lab and for his valuable advise and guidance at all times despite his tight schedule of work. His knowledge in Microbiology and his dedication to his work are remarkable and he will always inspire me.

I like to extend my thanks to Dr. Amy, Dr. Neuman and Dr. Carper for their valuable suggestions and comments on my work and being very supportive at all times.

My sincere thanks to Ananda, without whose support and constant encouragement this would have been impossible. Finally, my thanks to my parents to whom this thesis is dedicated and my brother who constantly supports me even across thousands of miles.

CHAPTER 1

GENERAL INTRODUCTION

Arthrobacters thrive well in desert habitats and their characteristic dimorphic life cycle enhances their value as a model for morphogenesis. The ability of arthrobacters to metabolize a variety of organic compounds also qualifies them as a primary candidate in bioremediation of desert habitats. Because arthrobacters do well in desert habitats, I examined the characteristics of Arthrobacter globiformis under osmotic stress in this project.

1.1 General characteristics of arthrobacters

Arthrobacters belong to the family Corynebacteriaceae (Breed et al. 1957). They are closely related to the other coryneform genera Aureobacterium, Cellulomonas, Curtobacterium and Microbacterium, and more distantly related to the genus Brevibacterium (Jones & Keddie 1992). According to 16S rRNA cataloging (Stackebrandt et al. 1980; Stackebrandt and Woese 1981), arthrobacters belong to the high GC "actinomycete" branch of Gram positive eubacteria (Fig.

1.1). They have a 59-70 mol% G+C content in their DNA and are obligate aerobes with an optimum growth temperature of 25-30 °C (Keddie et al. 1986). Arthrobacters are chemoheterotrophs and metabolize a wide variety of organic compounds (Stevenson 1967; Hagedorn & Holt 1975b). Arthrobacter globiformis (ATCC 8010) is the type species and the type strain of the genus Arthrobacter (Conn & Dimmick 1947).

The characteristic feature of the arthrobacter life cycle (Fig. 1.2) is a changing cell morphology. During early exponential phase, the bacteria are rod shaped, but sometimes have irregular rudimentary branches. These branched forms are called myceloids. During exponential growth, the bacteria often appear "V" shaped, and are bent or curved at sites of septation. Arthrobacters later become small cocci 0.6-1.0 µm in diameter as growth ceases and they enter stationary phase. The ability of arthrobacters to undergo morphogenesis makes them a valuable tool in understanding the processes that determine the shape of a bacterium. Both the rod and the coccal forms are Gram positive in wall structure but readily lose color and often show Gram negative staining characteristics.

Corynebacteria are an economically important group in the commercial production of amino acids and nucleotides. Arthrobacters in particular are important

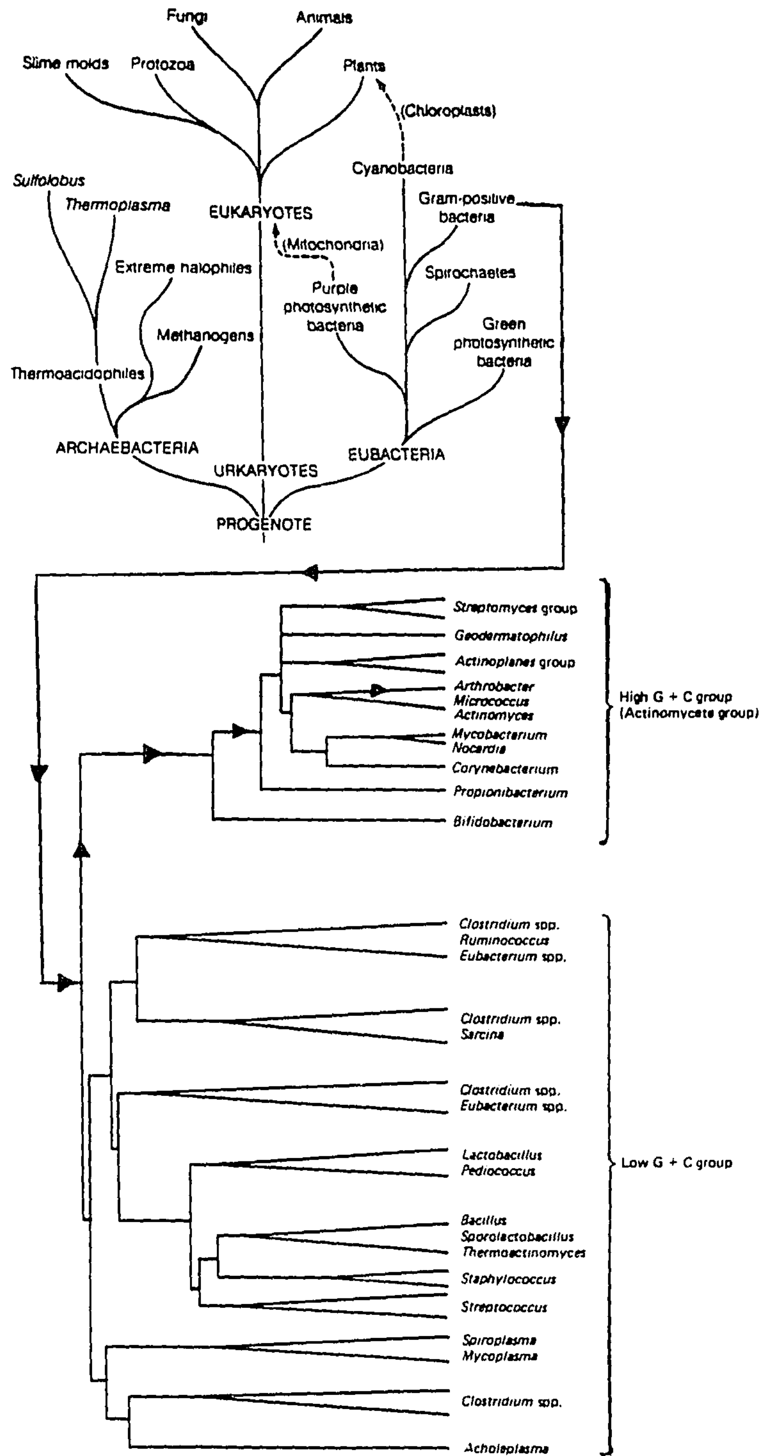


Fig. 1.1 Position of Arthrobacter globiformis in the high GC "actinomycete" branch of Gram positive eubacteria.

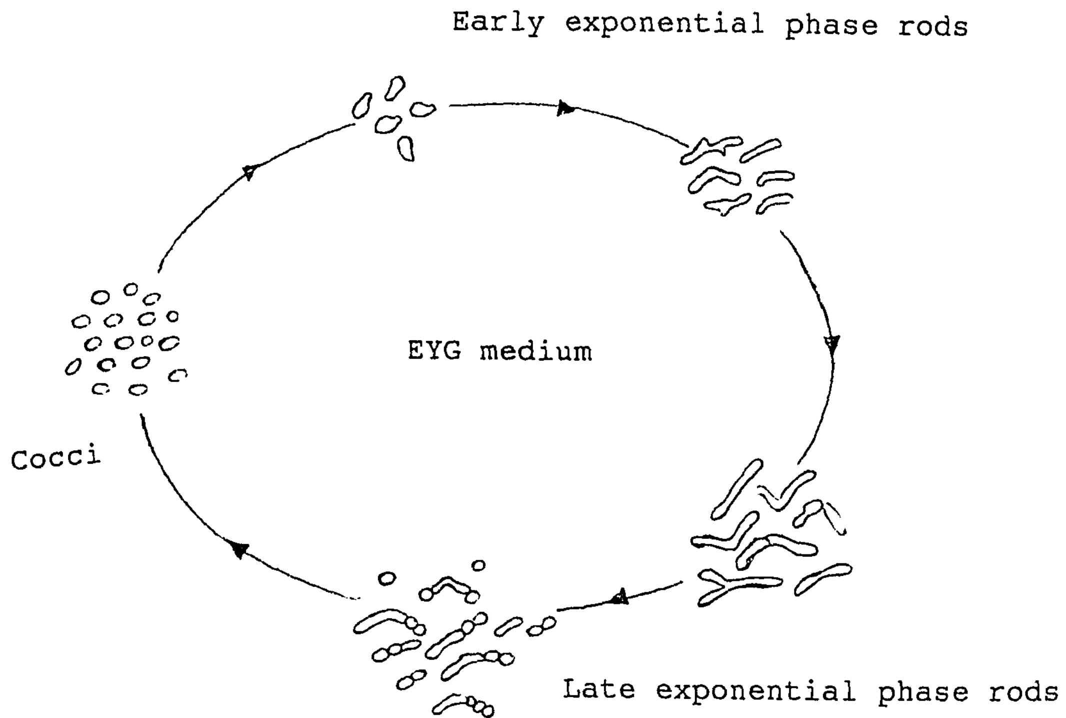


Fig. 1.2 Life cycle of <u>Arthrobacter globiformis</u> in EYG medium.

in that they represent the most dominant species of soil bacteria encountered on aerobic plate counts (Hagedorn & Holt 1975b; Holm & Jensen 1972; Lowe & Gray 1972; Mulder & Antheunisse 1963; Skyring & Quadling 1969; Soumare & Blondeau 1972). Arthrobacter habitats include, but are not limited to, subterranean cave silts (Gounot 1967), glacier silts (Moiroud & Gounot 1969), 200-700 m deep oil brines (Iizuka & Komagata 1965), aerial plant surfaces (Wallace & Gates 1986; Forni et al. 1989,1990; Petro & Gates 1987) and sea water (Sieburth 1964).

Arthrobacters are also very resistant to drying (Boylen 1973; Chen & Alexander 1973; Labeda et al. 1976; Mulder & Antheunisse 1963; Robinson et al. 1965) and can withstand long periods of starvation (Boylen & Ensign 1970; Boylen & Mulks 1978; Zevenhuizen 1966). A. crystallopoietes in particular is resistant to total starvation and extreme desiccation. Exponential phase rods and cocci of A. crystallopoietes have remained fully viable for a period up to one month, and the viability dropped only by 35% in 60 days (Ensign 1970). No difference in viability was found between the rod and the coccal forms, and both could remain viable up to 6 months in sand (Boylen 1973). These properties may qualify them as suitable candidates for the bioremediation of desert soils.

1.2 Effects of Osmotic Stress on Bacteria

Bacteria are subject to two types of osmotic stress. Hyperosmotic stress leads to efflux of water while hypoosmotic stress leads to influx of water. Hypoosmotic stress generally leads to a minor increase of the cytoplasmic volume (Stock et al. 1977). This is because bacterial cell walls can withstand pressures up to 10 MPa (Carpita 1985). Hyperosmotic stress can result from either desiccation or high salt concentrations. It usually results in a decrease in the cytoplasmic volume or plasmolysis. The result is reduced water activity (Csonka 1989), which in turn may affect functions which are essential to the cell such as deoxyribonucleic acid (DNA) replication (Meury 1988) and nutrient uptake (Roth et al. 1985a; 1985b; Walter et al. 1987). Hyperosmotic stress may also lead to an increase in the levels of specific enzyme inhibitors (Atkinson 1969) and have a toxic effect on the cell by the elevation of ion concentrations (Walderhaug et al. 1987). Increased adenosine triphosphate (ATP) levels have been reported to occur with sudden plasmolysis (Ohwada & Sagisaka 1988) and this is thought to result from impaired macromolecular biosynthesis (Csonka 1989).

Organisms undergoing hyperosmotic stress generally increase the concentrations of specific solutes, which

help them counteract cytoplasmic volume changes. These solutes are termed compatible solutes (Fig.1.3), and include 1) K^+ ions; 2) amino acids such as glutamate, glutamine, proline, gamma aminobutyrate, and alanine; 3) glycine betaine and other fully N-methylated amino acid derivatives; and 4) sugars like trehalose (α-D-glucopyranosyl-α-D-glucopyranoside). Compatible solutes may be accumulated by de novo synthesis or by transport into the cell from the culture medium (Csonka 1989).

Only a few studies have investigated the osmotic responses of corynebacteria. Brevibacterium ammoniagenes accumulates pipecolic acid at high osmolalities (Gouesbet et al. 1992) while Brevibacterium linens accumulates ectoine (Bernard et al. 1993). There are similarities between bacteria and plants in their response to osmotic stress and accumulation of pipecolic acid in these bacteria is one example (Goas et al. 1976; Stewart & Larher 1980; Gouesbet et al. 1992).

1.3 Morphogenesis of bacteria

The idea that biological form reflects the action of physical forces was first stated by D'Arcy Thompson (1917) in the book, On Growth and Form. There he expresses his point of view: "The form...of any portion of matter, whether it be living or dead, and the

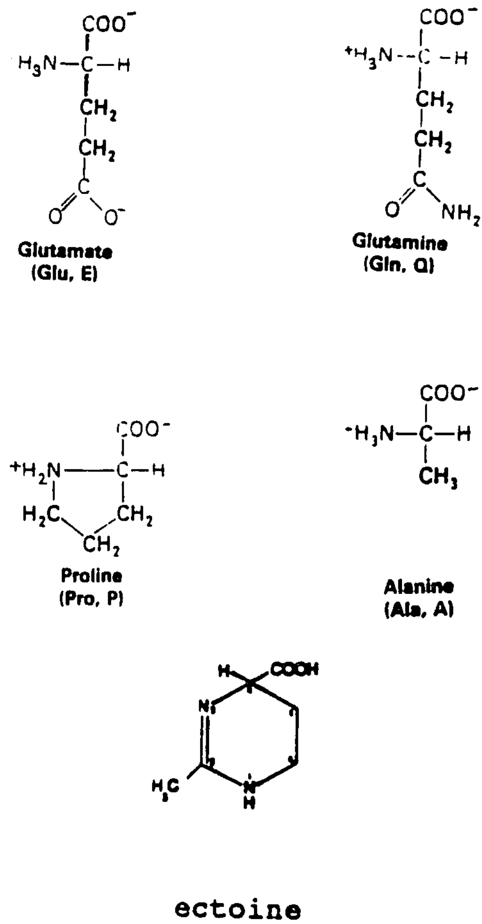


Fig. 1.3 Osmoprotective compounds

changes of form which are apparent in its movements and its growth, may in all cases alike be described as due to the action of force. In short, the form of an object is a 'diagram of forces' in this sense, at least, that from it we can judge or deduce the forces that are acting or have acted upon it" (Thompson 1961; Harold 1990).

A principle of morphogenesis which is widely accepted by most biologists is self assembly. This principle states that the shape of a structure is specified by the spontaneous association of its component parts. At the subcellular level, the assembly of subunits determines the shape of a structure. A few examples of this principle are the formation of bacterial flagella and ribosomes, the creation of nuclear membrane pore complexes, the assembly of microtubules from tubulin monomers, and the assembly of microfilaments from actin (Harold 1990).

Another principle of morphogenesis is that in objects governed by surface tension, shape is achieved by minimizing surface area compared to volume (Thompson 1961). Biological forms are compatible with this law of minimal surface area (Harold 1990). Although the surface tension of biological membranes is insufficient to determine shape (Thompson 1961), membrane tension, which arises due to contractile forces acting on the

cell, operate to shrink the surface area. The cell thus obtains the minimum surface area for any given volume and mechanical constraints (Harold 1990).

The shape of a bacterial cell cannot be accounted for by the model of self-assembly (Daneo-Moore & Shockman 1977; Henning 1975; Shockman et al. 1974). The shape of a bacterial cell is determined by its cell wall, which is not a self-assembling structure. Rather, the wall is a stress-bearing fabric whose form is determined by how the wall is laid down as cells grow and divide (Koch 1988a). The surface stress theory proposes that hydrostatic pressure, about 0.5 MPa in a Gram negative cell and about 2 MPa in a Gram positive cell, provides the driving force for surface enlargement. This force counteracts the cohesive forces that hold the components of the wall together as a unit. Cells respond to these forces by controlled expansion of the wall at particular sites (Koch 1983; 1985; 1988b). The shape of bacterial cells is determined by this surface expansion within the limitations of the law of minimal surface area (Harold 1990). This can be expressed mathematically using the energy conservation equation: $PdV=TdA$, where P and V are gas pressure and volume, respectively; dA is the newly-made surface area and T the surface tension. This relationship is true for bacterial cells as well as

soap bubbles. The surface stress theory applies to the rod and the coccal forms of bacteria (Koch 1983; 1985) and also to prosthecate bacteria (Koch 1988a). Although this model does not provide any information in the molecular sense, it is the only one that has so far been able to explain how the bacteria obtain their form.

The efflux of water under elevated osmolalities results in a decrease of turgor pressure that acts on the wall (Csonka 1989). According to the surface stress theory the decrease in the turgor pressure in a growing cell should affect the expansion of the surface area at particular sites. This would be expected to give rise to different morphological forms, while maintaining the minimum surface area to the cell volume.

1.4 Structure of the Bacterial Cell Wall

The Gram positive cell wall (Fig. 1.4A) is a 20-50 nm thick amorphous layer which consists mainly of peptidoglycan (murein) and one or more non-peptidoglycan polymers like teichoic acids, teichuronic acids, and/or other polysaccharides (Shockman & Barrett 1983). The cell wall provides the characteristic shape (rod, coccal, etc.) to the species and can be isolated from mechanically disrupted bacteria as an insoluble residue true to its

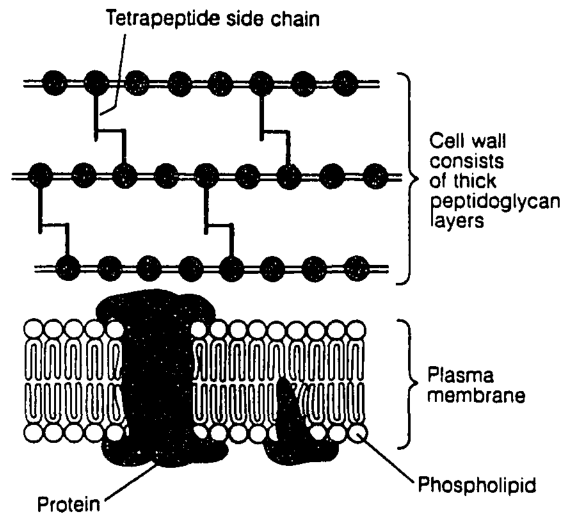


Fig. 1.4A Gram positive cell wall

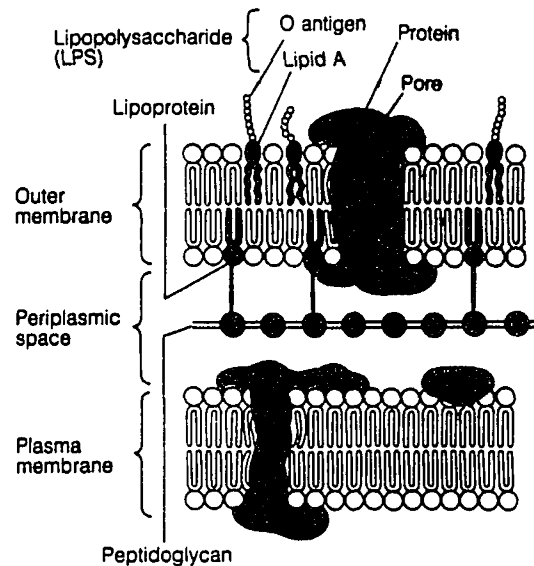


Fig. 1.4B Gram negative cell wall

characteristic morphology (Daneo-moore & Shockman 1977; Ghuysen 1977; Ghuysen & Shockman 1973; Rogers et al. 1980; Tipper & Wright 1979). The Gram positive cell wall is made up of several layers of peptidoglycan.

The typical Gram negative cell wall (Fig. 1.4B) consists of a relatively thin peptidoglycan (murein) sacculus and a bilayered outer membrane. The outer layer of this outer membrane contains a unique substance called bacterial lipopolysaccharide (LPS) not found elsewhere in nature. The LPS is made of three components, 1) a glycolipid called lipid A, 2) a core usually containing keto-deoxyoctonoic acid and a heptose, and 3) the O antigen made of long carbohydrate chains which is unique to each bacterial strain. The inner layer of the outer membrane resembles the cytoplasmic membrane.

The insoluble peptidoglycan polymer (Fig. 1.5) is made of repeating carbohydrate units, namely, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). These are linked to each other by beta-1,4 linkages. The NAM units are linked by a tetrapeptide, which usually consists of L-alanine, D-glutamic acid, a dibasic amino acid, and D-alanine. In most Gram-negative bacteria and a few Gram-positive bacteria, the dibasic amino acid is meso-diaminopimelic acid. In Gram-positive cells such as Streptococcus and in

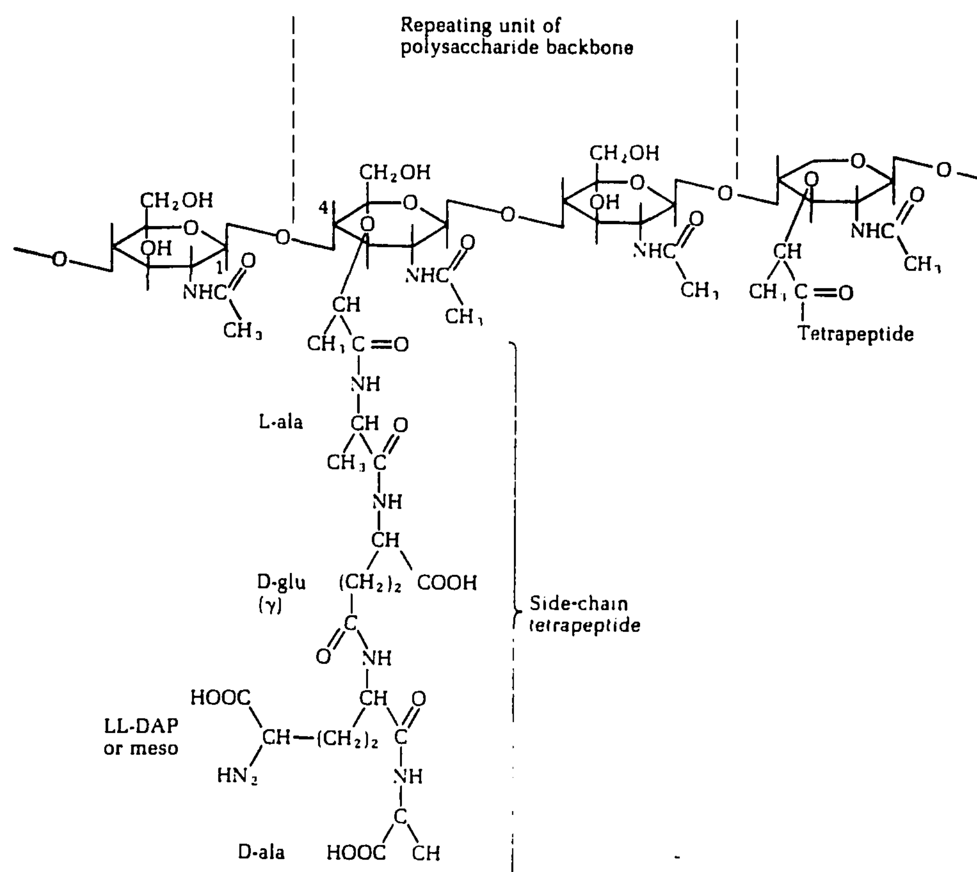
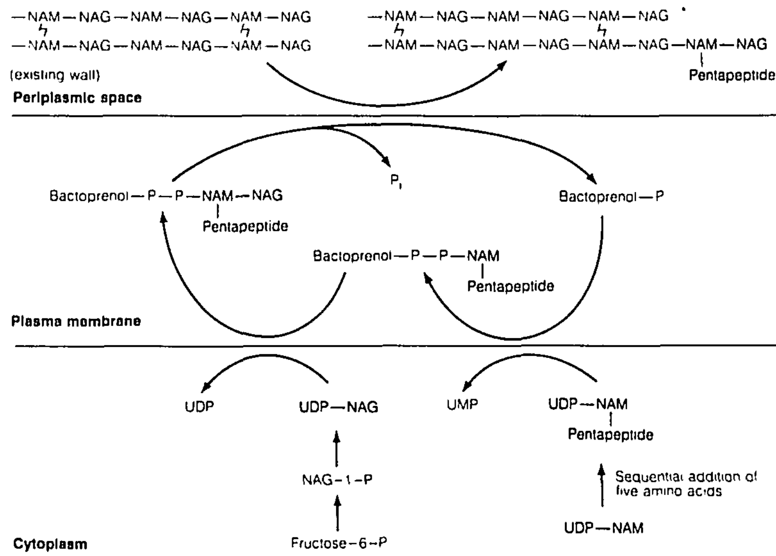


Fig. 1.5 Peptidoglycan repeating unit

arthrobacters, the dibasic amino acid is lysine (Fiedler et al. 1970; Yamada & Komagata 1972b; Schleifer & Kandler 1972; Keddie & Cure 1977; Keddie & Cure 1978). The cell wall structure gains mechanical strength due both to the cross-links between the third and fourth amino acids of the adjacent tetrapeptides, to the layered sheet configuration of the alternating D and L amino acids in the tetrapeptide and the beta-1,4 linkages between the repeating NAM and NAG units.

There are three phases in the assembly of the bacterial cell wall (Fig. 1.6). These occur in the cytoplasm, in the membrane, and in the environment external to the membrane. The UDP-N-acetylmuramyl-pentapeptide is synthesized in the cytoplasm by a series of addition reactions. The NAM-pentapeptide is then transferred to a lipid carrier (undecaprenylpyrophosphate) in the cytoplasmic membrane which is able to carry the NAM-pentapeptide through the phospholipid membrane. At this stage, the NAM-lipid complex is linked to UDP-N-acetylglucosamine. Further modifications of the polymer may take place depending on the bacterium. Polymerization of the disacharide-peptide units occurs by a headward elongation mechanism (Lipmann 1968) and is thought to occur on the exterior face of the cytoplasmic membrane (Ward & Perkins 1973). The new glycan chains are inserted to the pre-existing



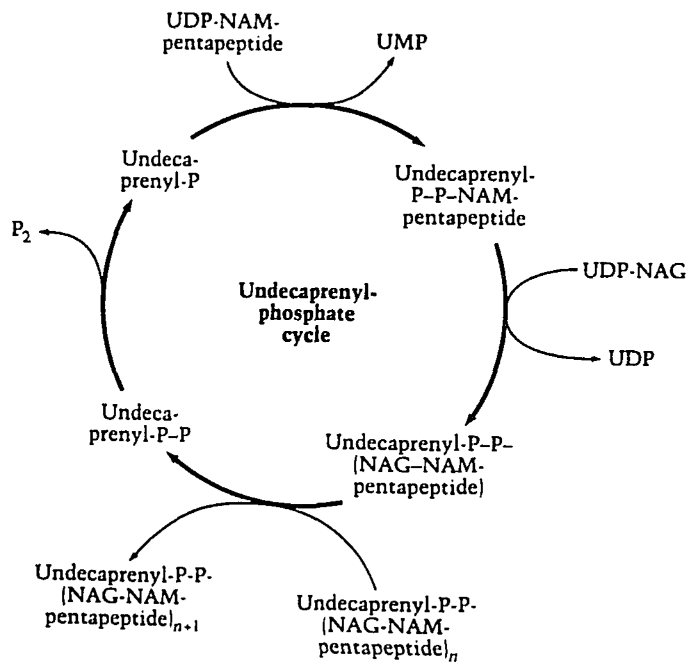


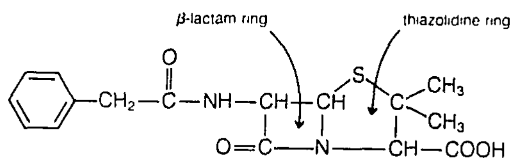
Fig. 1.6 Peptidoglycan synthesis

cell wall by transglycosylation and transpeptidation. A terminal D-alanine is cleaved by D-alanine carboxypeptidase either from the new or pre existing units (Hammes 1976; Hammes & Kandler 1976). The transpeptidation reaction is inhibited by beta-lactam antibiotics, while transglycosylation is sensitive to moenomycin and related drugs (Ishino & Matsuhashi 1981; Ishino et al. 1980; Van & Van 1980).

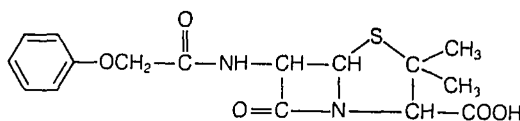
1.5 Penicillin Binding Proteins (PBPs) of Bacteria

Beta-lactam antibiotics like penicillin (Fig. 1.7) are used against a variety of bacterial infections due to their low toxicity to humans. The target of penicillin activity is the bacterial cell wall (Duguid 1946; Gardner 1940; 1945). The enzymes responsible for catalyzing reactions involved in the extension, cross-linking, and septation of the peptidoglycan sacculus bind penicillin covalently and are defined as penicillin-binding proteins (PBPs). The antibacterial effect of the beta-lactams is due to their ability to bind to these PBPs, thus inhibiting their activity in cell wall formation.

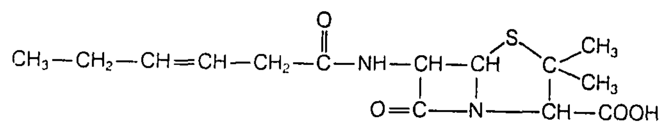
PBPs can be detected by incubation of bacterial membranes with [14C]penicillin G or [3H]penicillin G followed by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis and autoradiography (eg. Blumberg &



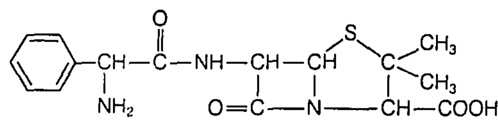
Penicillin G



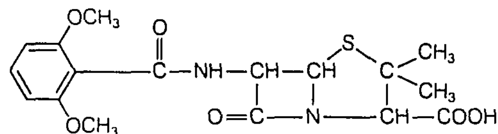
Penicillin V



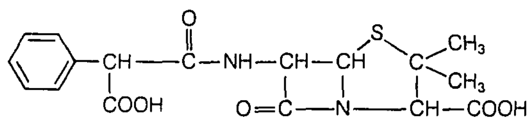
Penicillin F



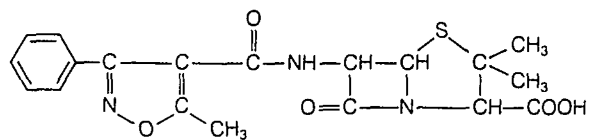
Ampicillin



Methicillin



Carbenicillin



Oxacillin

Fig. 1.7 Beta-lactam antibiotics

Strominger 1972; Spratt 1977). PBPs usually have apparent molecular masses of 140,000-40,000 (Waxman & Strominger 1983) although molecular masses around 25,000 also have been reported (Dusart et al. 1981). There are two categories of PBPs: relatively abundant low molecular PBPs (40,000-50,000), which are less sensitive to penicillins, and less abundant high molecular weight PBPs (60,000-140,000), which are more sensitive to penicillins. All eubacteria studied contains 3-8 PBPs (Waxman & Strominger 1983). The PBPs can be purified by covalent penicillin affinity chromatography (Blumberg & Strominger 1972). The low molecular mass PBPs (CPases) catalyze nucleophilic attacks at the carbonyl carbon of the D-alanine residue of cell wall pentapeptide-like compounds.

1.6 PBPs of *Escherichia coli*

The Gram-negative bacterium *Escherichia coli* has served as a model for the study of PBPs. There are six PBPs consistently found in the envelope of *E. coli*; two others are less frequently detected (Spratt 1977). The properties of these PBPs are summarized in Table 1.1. Spratt (1975) showed that the varying effects of beta-lactam antibiotics on cell division, cell elongation, and cell shape in *E. coli* were due to three distinct PBPs. A PBP with an apparent molecular mass of 66,000

Table 1.1 Properties of penicillin-binding proteins of _E. coli_

PBP	Molecules per cell	Enzyme activities	possible function
1a/1b	100 each	Transglycolase-transpeptidase	Murein synthesis during cell elongation
2	20	Transpeptidase	Growth in rod shape; cell elongation
3	50	Transglycolase-transpeptidation	Murein synthesis during septation
4	110	DD-endopeptidase, DD-carboxypeptidase	Cross-link hydrolysis in cell elongation
5	1,800	DD-carboxypeptidase	Destruction of unutilized pentapeptide
6	600	DD-carboxypeptidase	Destruction of unutilized pentapeptide
1C,7,8	Not known	Not known	Not known

Source: Adapted from Neidhardt et al. 1990, originally from Park (1987b).

(PBP 2) was responsible for the production of ovoid cells as a result of beta-lactam treatment. Cell division was affected by beta-lactams that preferentially bound to a protein with a molecular mass of 60,000 (PBP 3). Cell elongation was preferentially inhibited and the cells were lysed by preferential binding of penicillin to a protein with an apparent molecular mass of 91,000 (PBP 1). All three PBPs are peptidoglycan synthetases which act together in catalyzing the duplication of the peptidoglycan network (Nakagawa et al. 1982; Ishino et al. 1980; 1981; 1982; Matsuhashi et al. 1981; 1982).

PBP 2 is thought to be a bifunctional enzyme catalyzing both transglycolase and transpeptidase reactions (Ishino et al. 1986). PBP 1A, PBP 1B, and PBP 3 also appear to be bifunctional (Matsuhashi et al. 1981; 1982; Ishino et al. 1980; 1981). PBP 4 has a secondary transpeptidase activity in peptidoglycan biosynthesis (Curtis et al. 1980; dePedro & Schwarz 1981) as well as carboxypeptidase activity and endopeptidase activities (Park 1987b). PBP 5 & 6 both show carboxypeptidase activity. PBP 5 is also viewed as a cell-shape gene (Waxman & Strominger 1983), since overproduction of this D-alanine CPase leads to the production of osmotically stable ovoid cells. These cells show abnormal peptidoglycan biosynthesis similar

to that obtained after inactivating PBP 2 (Markiewicz *et al*. 1982). PBP 7 of *E. coli* is a protein with an apparent molecular mass of 32,000 but its function is unknown.

Genes for most of the reactions needed for peptidoglycan synthesis in *E. coli* are known (Begg & Donachie 1985), as well as genes required for the maintenance of its rod shape (Donachie *et al*. 1984). Genetic analysis shows that there are at least 12 genes in *E. coli* specifically required for cell division (Donachie & Robinson 1987; Holland & Darby 1976; Reeve *et al*. 1970; Ricard & Hirota 1973; Slater & Schaechtler 1974). Temperature-sensitive mutants at these loci (fts A-H) stop division and grow into long filaments when transferred to restrictive temperatures (Spratt 1977). Numerous chemicals also result in filamentation of bacterial cells; two that act on division are penicillins and cephalosporins (Blumberg & Strominger 1974; Burdett & Murray 1974; Schwarz *et al*. 1969; Spratt 1975). The isolation of mutants (sp258, sp63) with thermolabile PBP 3 has indicated that PBP 3 is specifically required for division in *E. coli*. It is the target at which penicillin interacts to inhibit cell division and cause filamentation (Spratt 1977).

1.7 Effects of beta-lactams on *Escherichia coli*

In *Escherichia coli*, the two growth processes of septation and elongation are differentiated by the response of the cells to various doses of penicillin (Schwarz *et al*. 1969). Low doses (10-50 U/ml) of penicillin block cell division and causes the formation of bulges in the middle of the cell where the cross wall forms. Bulge formation is thought to be due to the action of hydrolytic enzymes (autolysins) after the transpeptidation reaction is inhibited (Schwarz *et al*. 1969). Here, the elongation of the cell continues, but higher doses of penicillin (100 U/ml or more) totally inhibit mucopeptide synthesis.

Mecillinam (6-b aminodinopenicillanic acid), also designated FL 1060 (Lund & Tybring 1972), acts on a target to affect the shape of *E. coli*, and at its lowest concentrations, causes the conversion of *E. coli* rods into ovoid shaped cells (Lund & Tybring 1972; Park & Burman 1973). Normally, low concentrations of beta-lactam antibiotics specifically inhibit cell division in *E. coli*. The atypical effects of FL 1060 may be due to competition between a minor PBP or inhibition of an enzyme in the cell envelope (Spratt & Pardee 1975). The penicillin precursor 6-aminopenicillanic acid (6-APA) also results in the production of ovoid cells, but does not specifically inhibit cell division like most other

beta-lactam antibiotics.

Addition of FL 1060 to exponential and synchronous cultures of E. coli B/r results in a block of a very early event of the cell cycle (beginning of the C period). This event seems to be essential for normal cell elongation in the rod configuration (James et al. 1975). In E. coli, a FL 1060-sensitive event initiates the onset of the C period of the cell division cycle and is responsible for the normal cell elongation. Mecillinam is considered an ideal probe for cell shape since it doesn't initially inhibit cell division or cause typical penicillin lysis. Table 1.2 summarizes the effects of beta-lactam antibiotics on growth of E. coli (Spratt 1975).

1.8 PBPs of Gram positive bacteria

PBPs of a few Gram positive bacteria have been studied. The first mechanism to explain the effect of penicillin was that inhibition of the transpeptidase reaction led to absence of cross linkages in newly-formed peptidoglycan of Staphylococcus aureus (Tipper & Strominger 1965; Wise & Park 1965).

The susceptibility of Streptococcus faecium to penicillin is heavily influenced by the physiological status of the cells (Fontana et al. 1983). S. faecium shows different PBP labeling patterns at different

Table 1.2 Morphological Effects of beta-lactam antibiotics

Relative affinities of PBP 1, 2 and 3 for a Beta-lactam antibiotic*	Morphological effects produced by three arbitrary concentrations of a Beta-lactam antibiotic		
	Low	Medium	High
1>2 or 3	lysis	lysis	lysis
2>1>3	ovoid cells	lysis	lysis
2>3>1	ovoid cells	filaments'	lysis
2>1 or 3	ovoid cells	ovoid cells	ovoid
3>1>2	filaments	lysis	lysis
3>2>1	filaments	filaments'	lysis

* 1=cell elongation; 2=cell shape; 3=cell division
filaments'= filaments with bulges

Source: Spratt (1975)

temperatures when grown in a chemically defined medium. With the fastest growing cells, the target for penicillin most likely to be responsible for growth inhibition is PBP 3 (Fontana et al. 1980).

Some Streptococcus species, like S. pyogenes, S. pneumoniae, and S. mitis, are sensitive to beta-lactams (Masten 1972; Moellering & Krogstad 1979; Faber et al. 1983) but other species and strains are resistant (e.g., Escribano et al. 1990; Goldfarb et al. 1984). Penicillin resistant streptococci show alterations in the binding affinity of one or more PBPs (Faber et al. 1983; Massida & Moore 1988; Zighelboim & Tomasz 1981; Zeto & Moore 1988). S. mitis (NCTC 10712) has 7 PBPs with apparent molecular masses of 49-82 kDa. The PBP profiles of penicillin-susceptible and penicillin-resistant S. mitis vary even at the same minimal inhibitory concentrations (Potgieter et al. 1992). In transformation experiments with penicillin-resistant clinical isolates of S. mitis and S. pneumoniae as the donors and S. mitis (NCTC 10712) as the recipient, transformants with a minimum inhibitory concentration 33 times higher than the original were obtained. These transformants showed reduced penicillin-binding affinities of PBPs 2, 3, 4, 5, and 6 depending on the donor DNA. The level of resistance and PBP 3 (74 kDa) was altered in the transformants with the increased

resistance (Potgieter *et al*. 1992).

Hartman and Tomasz (1984) showed that methicillin resistance in *Staphylococcus aureus* is also associated with alterations in PBPs. Methicillin resistance was shown to be pH dependent. A PBP (PBP-2a, molecular mass 78,000 daltons) was detected in methicillin resistant staphylococci grown at pH 7.0 while it was absent in cultures grown at a pH of 5.2, supporting the fact that PBP functions vary depending on the physiological status of the cell.

In the following chapters myceloid cell formation in *Arthrobacter globiformis* will be discussed in terms of their penicillin-binding protein (PBP) profiles and sensitivity to extreme conditions. Chapter 2 will look at the phenomenon of myceloid formation during osmotic stress; chapter 3 at the PBP profile of *A. globiformis* and how it varies in myceloids and normal cells; chapter 4 at the difference in sensitivity of the myceloids and the normal cells to heat, starvation, and desiccation. This will be followed by a general discussion of the project as a whole, and a general bibliography.

CHAPTER 2

Myceloid Cell Formation in *Arthrobacter globiformis* during Osmotic Stress

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2.1 ABSTRACT

Arthrobacter globiformis was grown in a semi-defined liquid medium containing added solutes to determine the effects of osmotic stress on its reproduction and cell morphology. There was a progressive reduction in the specific growth rate during exponential phase as the concentration of NaCl was increased, although the final yields of the cultures during stationary phase were not affected. Clusters of branching myceloid cells rather than the typical bacillary forms predominated during exponential phase. These myceloids did not undergo complete septation and persisted into stationary phase. Similar responses were observed with potassium sulfate as the exogenous solute but less dramatic morphological effects were found with added polyethylene glycol or sucrose. The myceloids formed in response to osmotic stress could not be disrupted mechanically but were more sensitive than normal cells to lysozyme, particularly during stationary phase. Addition of osmoprotective compounds such as proline, glutamate, glycine betaine, or trehalose to the growth medium did not significantly relieve the effects of osmotic stress on growth rate or morphology. A. simplex also formed myceloid cells during osmotic stress but A. crystallopoietes did not. These results indicate

arthrobacters exhibit characteristic responses to osmotic stress and suggest these bacteria may contain novel osmoprotective compounds.

2.2 INTRODUCTION

Bacteria of the genus Arthrobacter have been found in many different terrestrial habitats (Hagedorn & Holt 1975a; Vollmer et al. 1977; Keddie & Jones 1987; Shivaji et al. 1989). These aerobic chemoheterotrophs can metabolize a wide range of organic compounds (Stevenson 1967; Hagedorn & Holt 1975b), including herbicides (Pipke & Amrhein 1988), chlorinated phenols and alkanes (Stanlake & Finn 1982; Scholtz et al. 1987), and complex aromatic compounds (Tomasek & Crawford 1986). Because arthrobacters are also resistant to dessication (Boylen 1973) and long-term starvation (Boylen & Ensign 1970), they may be particularly useful for bioremediation in dry desert soils.

A characteristic feature of arthrobacters is a dimorphic growth cycle in which exponential-phase cells appear as irregular bacilli and stationary-phase cells as cocci (Cure & Keddie 1973; Clark 1975). Both forms have a Gram-positive wall structure but are readily decolorized during the Gram-staining reaction and often appear to be Gram-negative (Keddie & Jones 1987). When

nongrowing cocci are added to fresh medium, a rod-shaped structure emerges from each cell and elongates. The bacteria then may go through a transient myceloid stage in which some rudimentary branching is observed. More commonly, the cells directly enter exponential phase where they grow as V-shaped bacilli bent at the division septa. Coccal cells are formed again when the bacteria cease growth and go into stationary phase (Sundman 1958; Stevenson 1961; Duxbury & Gray 1977).

This dimorphic cycle is under genetic control (Achberger & Kolenbrander 1978) but can be modified by nutritional conditions in two ways. First, *A. crystallopoietes*, but not most other species, can be grown through exponential phase as cocci in a minimal medium with glucose as the carbon source. The bacillary form can be restored by addition of specific amino acids or other carbon sources to this medium (Ensign & Wolfe 1964; Lucas & Clark 1975). *A. crystallopoietes* also exhibits coccal growth in carbon-limited chemostat cultures, suggesting that morphology is determined in part by growth rate (Luscombe & Gray 1971). Second, when arthrobacters are deprived of nutrients such as biotin (Chan *et al*. 1973), vitamin B12 (Chaplin & Lochhead 1976), or manganese (Germida & Casida 1980), stable myceloid cells are formed during exponential phase and persist into stationary phase. Morphology

thus is also determined by the availability of essential nutrients.

We have begun to study the effects of osmotic stress on the morphology and metabolism of arthrobacters with the long-term goal of constructing strains with enhanced biodegradative properties in desert habitats. Although the physiology of osmoregulation in Gram-negative bacteria such as Escherichia coli and Salmonella typhimurium has been investigated in detail (Csonka 1989), less is known about the effects of osmotic stress on Gram-positive bacteria and no prior studies on arthrobacters have been published. Measures (1975) reported that members of the generic groups Bacillus, Lactobacillus, Micrococcus, Staphylococcus, and Streptococcus contained elevated amounts of L-proline, γ-aminobutyric acid, and L-glutamate when grown in high concentrations of NaCl. Killham & Firestone (1984) later showed that in Streptomyces griseus, the cellular pools of proline, glutamine, and alanine rose as a result of increased biosynthesis during growth in the presence of NaCl. However, if proline was added to the medium, its biosynthesis was reduced and proline transport was increased instead. Whatmore et al. (1990) found that the cellular concentrations of potassium ions and proline in Bacillus subtilis increased dramatically

during osmotic stress, although proline synthesis was suppressed in the presence of glycine betaine. In none of these cases was an alteration in cell morphology observed as a consequence of osmotic stress. In this paper, we report that growth in the presence of exogenous solutes has a dramatic effect on the morphology of Arthrobacter globiformis.

2.2 MATERIALS AND METHODS

Bacteria and growth conditions

Arthrobacter globiformis (ATCC 8010), A. simplex (ATCC 6946), and A. crystallopoietes (ATCC 15481) were obtained from the American Type Culture Collection and maintained on Tryptic Soy Agar (Difco Laboratories) supplemented with 0.2% (w/v) yeast extract (TSYE agar). The bacteria were routinely grown in 300 ml nephelometer flasks containing 25 ml of liquid minimal medium E supplemented with 0.1% (w/v) yeast extract and 0.1% (w/v) D-glucose (EYG medium, Cure & Keddie 1973). Solutes and other compounds were added to this medium as specified in each experiment. In some cases, the yeast extract was omitted from the EYG medium to make EG medium. Cultures were incubated at 30°C in a Lab-Line environmental chamber and shaken at 250 rpm. Optical densities were determined with a Klett-

Summerson colorimeter (no. 66 filter) or with a Shimadzu UV-160 UV-visible spectrophotometer.

Photomicrography

Bacteria from liquid exponential- or stationary-phase cultures were spotted on glass slides coated with a thin layer of the same type of medium in which they had been grown that had been solidified with 1% (w/v) agar. The sample was covered with a glass coverslip and photographed through a 100X oil immersion objective with a Nikon Optiphot phase contrast microscope fitted with a UFX-II photographic system. Kodak Technical Pan black and white film (ASA 100) was used in all cases.

Other assays

Bacterial dry weights were determined by filtering 10 ml samples of cultures through tared nitrocellulose filters (Millipore Corporation, Type HA). The filters then were dried to a constant weight in a 37°C oven. In most cases, filters with 0.45 µm pores were used for dry weight determinations, but for one experiment, filters with 1.2 µm, 3.0 µm, or 8.0 µm pores were employed as well. Viable cell counts were performed by serially diluting bacteria in the same medium in which they had been grown and by spreading the cell suspensions on TSYE agar plates. The colonies were

counted after two days of incubation at 30°C. Protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin (Sigma Chemical Company) as a standard.

2.2 RESULTS

Growth and morphology of Arthrobacter globiformis during osmotic stress

To study the effects of osmotic stress on A. globiformis (ATCC 8010, the bacteria were grown in liquid EYG medium containing increasing concentrations of NaCl. There was a linear decrease in the specific growth rate during exponential phase as the solute concentration was raised (Fig.2.1). The arthrobacters were quite tolerant of osmotic stress, however, and growth occurred even in the presence of 1.5 M NaCl. The final yields of the cultures in stationary phase were not affected by the presence of the added solutes.

When cells from cultures without added NaCl were examined by phase-contrast microscopy, bacillary and coccal forms typical of Arthrobacter were observed during exponential phase and stationary phase, respectively (Figs. 2.2A and 2.2B). When bacteria from cultures with added NaCl were examined in the same way, myceloid cells rather than bacillary forms predominated

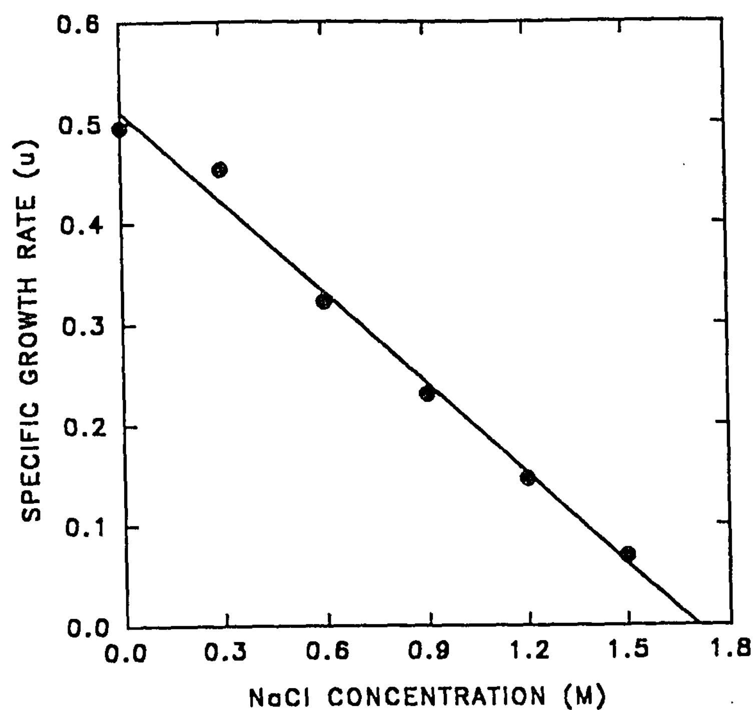

Fig. 2.1 Specific growth rates of A. globiformis during osmotic stress. A. globiformis (ATCC 8010) was grown at 30°C with continuous aeration in 300 ml nephelometer flasks containing 25 ml of liquid EYG medium supplemented with different concentrations of NaCl. Optical densities were measured periodically during exponential phase with a Klett-Summerson colorimeter (no. 66 filter), and the specific growth rates calculated as u = 0.693/apparent generation time in hours.

during exponential phase (Fig. 2.2C). These myceloids could be seen at NaCl concentrations as low as 0.3 M but were most obvious at 0.6 M or 0.9 M. Higher salt concentrations did not alter cell morphology further. The myceloid cells had a marked tendency to aggregate and often formed clusters containing 10 to 20 organisms. As the bacteria entered stationary phase, normal septation did not occur and the clusters of myceloids persisted even though the cells became smaller (Fig. 2.2D). A. globiformis could be maintained in the myceloid form indefinitely by transfer to fresh medium containing added NaCl. The formation of myceloid cells by A. globiformis was most dramatic in liquid medium. While myceloids were sometimes observed after growth on EYG agar containing 0.9 M NaCl, most of the bacteria appeared normal.

Quantitative effects of myceloid formation

The quantitative values of optical density per mg of dry weight and protein content per mg of dry weight were compared for bacteria from cultures with or without 0.9 M NaCl. There were no significant differences between the cultures for either exponential- or stationary-phase cells. The number of colony forming units per mg of dry weight also was similar for exponential-phase bacteria from cultures

Fig. 2.2 Morphology of A. globiformis during osmotic stress. Bacterial cultures were grown as described in Fig. 1 to either exponential phase (about 100 Klett Units) or stationary phase (about 250 Klett Units). Panels A and B show exponential-phase and stationary-phase cells grown in EYG medium without added NaCl; panels C and D show exponential-phase and stationary-phase cells grown in EYG medium containing 0.9 M NaCl. The scale bar in each figure indicates 2 μm.

with or without added NaCl, although the variance for samples containing myceloids was much greater. However, the number of colony forming units per mg of dry weight decreased from 2.56 x 10^7 for stationary-phase cells from unstressed cultures to 1.99 x 10^6 for stationary-phase cells from cultures containing 0.9 M NaCl. This was consistent with the presence of clusters of myceloids in the cultures containing added NaCl rather than single cocci.

The presence of myeloids in cultures with 0.9 M NaCl was also reflected in the filtration properties of the cells. This was demonstrated by measuring the dry weights of the bacteria after filtration through nitrocellulose filters with different pore sizes. When exponential-phase bacteria from stressed and unstressed cultures were passed through filters with 0.45 µm, 1.2 µm, 3.0 µm, or 8.0 µm pores, there was little decrease in dry weight as the pore size increased. When stationary-phase cocci from cultures without added NaCl were passed through these filters, the dry weight on filters with 8.0 µm pores was only 11% of that found on filters with 0.45 µm pores. By contrast, when stationary-phase myceloids from cultures containing 0.9 M NaCl were passed through these filters, the dry weight on filters with 8.0 µm pores was 87% of that found on filters with 0.45 µm pores. This was again

consistent with the formation of larger aggregates during osmotic stress.

Characteristics of myceloids

To determine whether the myceloid cells were permanently altered in morphology, A. globiformis was grown to exponential phase in liquid EYG medium containing 0.9 M NaCl, harvested by centrifugation, washed, and resuspended in EYG medium lacking added NaCl. There was no immediate change in morphology, indicating that the myceloids were not simply transiently-deformed bacteria. However, as A. globiformis grew in EYG medium without added NaCl, the myceloid cells gradually divided and separated. When the culture reached stationary phase, almost all of the bacteria exhibited the coccal morphology typical of untreated cells.

The myceloids formed in cultures containing added NaCl were readily decolorized during Gram-staining reactions and showed the same pink color as the bacillary and coccal forms. However, both the myceloids and the normal cells gave a negative result in the KOH test (Gregersen 1978), indicating they have a Gram-positive wall structure. The stress-induced mycleoids could not be disrupted by mechanical agitation with a Vortex mixer or a "bead-breaker." They exhibited the

same sensitivity as unstressed cells to sonication and were not degraded by treatment with a nonspecific protease.

However, the myceloids formed in response to osmotic stress were more susceptible than normal cells to degradation by lysozyme, particularly during stationary phase (Fig. 2.3). Sensitivity was demonstrated by the formation of spherical protoplasts that underwent lysis to produce a decrease in optical density. Exponential-phase cells were quite sensitive to lysozyme treatment and the optical density decreased more than 60% in one hour. Bacteria grown in EYG medium containing 0.9 M NaCl were somewhat more sensitive to lysozyme treatment than those from unstressed cultures. Stationary-phase cells from EYG medium without added NaCl were quite resistant to lysozyme treatment. On the other hand, stationary-phase myceloids from EYG medium containing 0.9 M NaCl remained sensitive to the enzyme as indicated by a marked decrease in optical density.

Effects of growth conditions on myceloid formation

Arthrobacters have been previously found to form myceloids when deprived of nutrients such as biotin (Chan et al. 1973), vitamin B12 (Chaplin & Lochhead 1976), or manganese (Germida & Casida 1980). To determine whether the formation of myceloids during

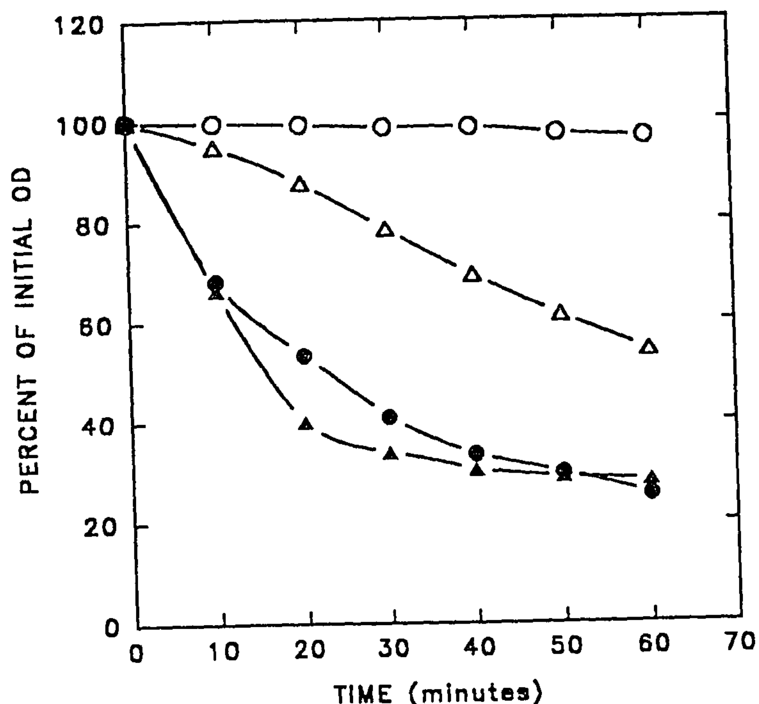

Fig. 2.3 Sensitivity of A. globiformis to lysozyme. Bacteria were grown as described in Fig. 2.1 to exponential phase (about 100 Klett Units) or stationary phase (about 250 Klett Units) in EYG medium or EYG medium containing 0.9 M NaCl. The bacteria were harvested by centrifugation and suspended in EYG medium containing 0.9 M NaCl and 1 mg/ml of lysozyme (Sigma). The suspensions then were incubated at 30°C and the optical densities determined periodically at 420 nm in a Shimadzu UV-visible spectrophotometer. The figure shows the results for exponential-phase cells from EYG medium without added NaCl (●), stationary-phase cells from EYG medium without added NaCl (O), exponential-phase cells from EYG medium containing 0.9 M NaCl (▲), and stationary-phase cells from EYG medium containing 0.9 M NaCl (△).

osmotic stress was due to a limitation of any of these compounds, the bacteria were grown in EYG media containing 0.9 M NaCl and 1000-fold additions of each these nutrients. Myceloid cells again were formed during exponential phase and persisted into stationary phase.

To test whether chemicals that act as osmoprotectants in other bacteria (Csonka 1989) could relieve the effects of osmotic stress on _A. globiformis_, bacteria were grown in liquid EYG media containing 0.9 M NaCl and 10 mM L-proline, 10 mM L-glutamate, 10 mM L-glutamine, 10 mM glycine betaine, 10 mM choline, 10 mM trehalose, or 10 mM γ-aminobutyric acid. None of these supplements increased the growth rate of the bacteria significantly or prevented the formation of myceloid cells during exponential phase (Fig. 2.4A). However, cells grown in the presence of proline or glutamate did show partial septation in stationary phase (Fig. 2.4B). The myceloid cells began to divide so that chains and clusters of cocci were gradually formed over the next 2-3 days; single cocci were occasionally observed. Similar but less dramatic effects were found in cultures containing choline or trehalose.

Because the EYG medium used in the preceding studies contained yeast extract as a possible source of

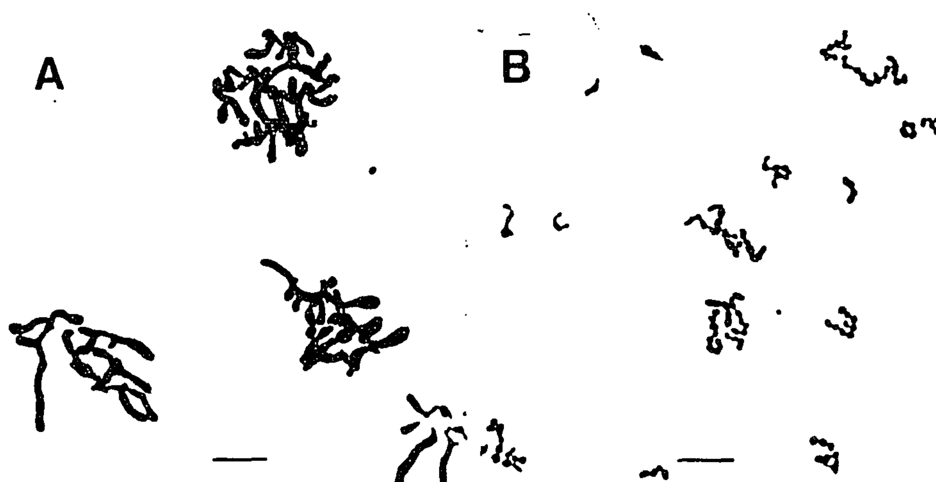

Fig. 2.4 Effect of exogenous proline on the morphology of A. globiformis during osmotic stress. Bacteria were grown to exponential phase (Panel A) or stationary phase (Panel B) in EYG medium containing 0.9 M NaCl and 10 mM L-proline. The scale bar in each figure indicates 2 μm. Control cultures in EYG medium lacking added NaCl but containing 10 mM L-proline exhibited the same morphologies as shown in Figs. 2.2A and 2.2B.

osmoprotective compounds, the experiments were repeated with a similar medium lacking yeast extract (EG medium). Bacteria grown in EG medium without added NaCl exhibited the typical arthrobacter morphologies shown in Figs. 2.2A and 2.2B. Bacteria grown in EG medium containing 0.9 M NaCl formed very well-defined myceloids during exponential phase which persisted into stationary phase. Again, however, addition of proline, glycine betaine, trehalose, and other osmoprotective compounds did not prevent the formation of myceloid cells.

Since the myceloids formed in response to osmotic stress showed an increased sensitivity to lysozyme, experiments were also performed in which amino acids found in the peptidoglycan (Keddie & Jones 1987) were added to cultures containing 0.9 M NaCl. D-alanine, D-glutamate, or L-lysine had no effect on the growth rate of the bacteria and did not prevent the formation of myceloids during exponential phase. After 5 days of incubation, however, the myceloids from cultures containing D-alanine or L-lysine showed partial septation and chains or clusters of coccal cells were observed similar to those shown in Fig. 2.4B.

Finally, to see whether these effects of osmotic stress were specific to NaCl, similar experiments were conducted with potassium sulfate, polyethylene glycol

(average molecular weight of 200), and sucrose as the exogenous solute. A progressive decrease in growth rate occurred in liquid cultures with increasing concentrations of each solute. With potassium sulfate, the morphological response was very similar to that observed with NaCl. Myceloid cells were formed during exponential phase and persisted into stationary phase. However, growth was not observed at potassium sulfate concentrations greater than 0.9 M. With polyethylene glycol, bloated or irregular bacilli and cocci were observed during exponential phase. No growth occurred at concentrations greater than 0.6 M and the bacteria seemed particularly sensitive to this compound. With sucrose, some elongation and branching were observed during exponential phase in the presence of 0.6 to 0.9 M sucrose; short rods rather than cocci were found in stationary phase.

Myceloid formation in other arthrobacters

To determine whether these effects of osmotic stress were unique to *A. globiformis*, similar growth and morphology studies were performed with *A. simplex* (ATCC 6946) and *A. crystallopoietes* (ATCC 15481). *A. simplex* showed responses comparable to those of *A. globiformis*, although growth was not observed at NaCl concentrations greater than 0.6 M. At 0.3 or 0.6 M NaCl, however,

pronounced myceloid cell formation occurred during exponential phase and the bacteria failed to divide as they entered stationary phase. On the other hand, A. crystallopoietes remained generally rod-shaped throughout exponential phase and few myceloids were observed. However, the bacteria did not convert to cocci as they entered stationary phase and retained a bacillary form.

2.5 DISCUSSION

These experiments indicate for the first time that exposure of A. globiformis to osmotic stress leads to characteristic changes in growth rate and morphology. The effects appear to be a general reaction to this environmental factor since similar responses were observed with different solutes. All of the exogenous solutes led to decreased growth rates, but ionic solutes produced the most dramatic morphological effects. None of the amino acids or other compounds tested was found to reverse the morphological changes completely. Although A. simplex also formed myceloid cells in response to osmotic stress, A. crystallopoietes did not. This is consistent with previous studies indicating the latter species is quite different from other arthrobacters (Lucas & Clark

1975).

Two aspects of this work warrant further investigation. The first concerns the mechanism of myceloid formation. Electron micrographs of myceloids induced by nutrient limitation indicate they consist of chains of cells in which septation is incomplete (Chan et al. 1973; Germida & Casida 1980). The myceloid cells formed in response to osmotic stress appear to arise in the same way, but the relationship between osmotic stress and deprivation of biotin, vitamin B12, or manganese is not obvious. It is possible myceloid formation is an adaptive response to certain environmental conditions and these multicellular forms have an enhanced chance of survival. The observation that the myceloids formed in response to osmotic stress were more sensitive to lysozyme treatment than normal cells suggests they may be altered in the formation of the peptidoglycan layer. Although penicillin-binding proteins that might be involved in wall synthesis have not been described in arthrobacters, an analysis of such proteins and the effects of osmotic stress on their synthesis and activity would be very useful. The second aspect worth pursuing is the physiology of osmoregulation in arthrobacters. The addition of proline or other well-characterized osmoprotectants to cultures containing added NaCl did not increase the

growth rate of A. globiformis or prevent the formation of myceloid cells. This suggests other osmoprotective compounds may be formed endogenously during osmotic stress. A complete analysis of the effects of osmotic stress on the intracellular solutes of A. globiformis by [13C]nuclear magnetic resonance spectroscopy thus would be important.

One implication of these experiments is that arthrobacter morphology may be useful in monitoring osmotic stress in terrestrial habitats. When indigenous populations of these bacteria are subjected to this condition, they too may form myceloid cells rather than bacillary or coccal forms. In situ observations of arthrobacters using fluorescently-labelled antibodies to surface components (Bohlool & Schmidt 1980) thus may document the occurrence of stress in the habitat of interest. A second implication of these experiments is that formation of myceloid cells in response to osmotic stress may affect the use of arthrobacters in bioremediation. We have not yet determined whether myceloid cells differ qualitatively from the bacillary forms in their metabolism. However, the addition of exogenous solutes to liquid medium provides a convenient system for testing the effects of dehydration on metabolic function.

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CHAPTER 3

Penicillin-binding proteins in salt-induced myceloids of *Arthrobacter globiformis*

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3.1 ABSTRACT

Bacteria of the genus Arthrobacter exhibit a dimorphic growth cycle in which exponential-phase cells appear as irregular bacilli and stationary phase cells as cocci. Exposure of Arthrobacter globiformis to osmotic stress leads to the formation of clusters of branching myceloid cells during exponential phase, which then persist into stationary phase. To determine whether these changes in morphology are related to the presence or absence of specific penicillin-binding proteins, membrane fractions from unstressed and salt-stressed bacteria were exposed to [14C]benzylpenicillin and the proteins separated by SDS-polyacrylamide gel electrophoresis. Eight penicillin-binding proteins (PBPs) were identified, ranging in molecular mass from 91,000 to 30,000 daltons. The PBP profile varied with salt concentration and growth state. During exponential phase, normal rods contained PBP 1b (90 kD), 1c (89 kD), 2a (84 kD), 2b (82 kD), 4 (51 kD) and 5 (30 kD) while salt-induced myceloids contained only 1b (90 kD), 1c (89 kD), 4 (51 kD) and 5 (30 kD). During stationary phase, normal coccal form had 1a (91 kD), 1b (90 kD), 1c (89 kD), 2b (82 kD), 3 (63 kD), 4 (51 kD) and 5 (30 kD) while salt-induced myceloids had PBP 1a (91 kD), 1b (90 kD), 1c (89 kD), 3 (63 kD), 4 (51 kD) and 5 (30 kD). Myceloids arising from osmotic stress

appeared to lack PBP 2a (84kD) and PBP 2b (82 kD) under all growth conditions tested, suggesting these proteins are involved in septation. The myceloids were 12 to 20 times more sensitive to beta-lactam antibiotics than the normal bacteria, and while there was no difference in tetracycline sensitivity, the myceloids were 320 times more resistant to streptomycin. Mutanolysin, a hydrolytic enzyme which degrades peptidoglycan, caused breakage of the myceloids into short rods and cocci without significant lysis. These results indicate that myceloid formation in A. globiformis may result from the altered synthesis of specific penicillin-binding proteins.

3.2 INTRODUCTION

Arthrobacters are Gram positive, aerobic chemoheterotropic bacteria that are abundant in a wide variety of habitats. They thrive well in dry soils, qualifying them as candidates for bioremediation of contaminated desert sites. Arthrobacters have a characteristic rod/coccus life cycle in which the exponential phase cells take on an irregular bacillary form and the stationary-phase cells a coccal form (Cure & Keddie 1973; Clark 1975). When subjected to osmotic stress, Arthrobacter globiformis does not divide normally. Rather, it forms clusters of long, branching

myceloids that persist into stationary phase (Deutch & Perera 1992).

Penicillin-binding proteins (PBPs) are predominantly found in the bacterial cell membrane and can covalently bind penicillin (Blumberg & Strominger 1974; Waxman & Strominger 1983). Most PBPs have molecular masses in the range of 140,000-40,000 daltons (Waxman & Strominger 1983), but PBPs with molecular masses as low as 25,000 have been reported (Dusart et al. 1981). These proteins are important in determining cell morphology, since treatment of bacteria with penicillin often changes their morphology and mutants with altered morphology often differ in their PBP profile (Spratt 1975; Markiewicz et al. 1982; Schuster et al. 1990).

Pucci et al. (1986) found that treatment of Streptococcus faecium ATCC 9790 with N-formimidoyl thienamycin and methicillin blocked the cell division cycle prior to completion of the chromosome replication, giving rise to "dumbbell" shaped cells; cefoxitin and cephalothin treatment induced a block later in the cell cycle, producing "lemon" shaped cells. The dumbbell-shaped cells were thought to result from the specific inhibition of PBP 3 and the lemon shaped cells from inhibition of PBP 2 (90 kD). In Streptococcus pneumoniae, mutants with altered PBP 3

(43 kD) were found to grow as irregular enlarged spheres; septa formation was unevenly distributed at multiple sites and the peptidoglycan layer was variable in thickness (Schuster et al. 1990). In Staphylococcus aureus, mutants lacking PBP 4 (46 kD) grew as enlarged spheres (Curtis et al. 1980; Wyke et al. 1981). Although Bacillus subtilis was the first species in which multiple PBPs were identified (Suginaka et al. 1972; Blumberg & Strominger 1972), no dramatic morphological changes were observed with selective binding of beta-lactams to particular PBPs (Frere & Joris 1985). However, mutants lacking PBP 1a and 1b showed a reduced diameter, suggesting it has a role in septum formation (Waxman & Strominger 1983).

Since myceloid formation in A. globiformis seemed to result from a defect in septation, we hypothesized that these cells might have an altered penicillin binding protein profile. No prior studies on the PBPs of arthrobacters have been published. Since arthrobacters have a characteristic rod/coccus life cycle, identifying and characterizing their PBPs will provide valuble information about cellular morphogenesis in prokaryotic cells. In this paper, we report differences in the protein profiles of unstressed and salt-stressed bacteria. We also describe the relative sensitivities of myceloids and normal

cells to beta-lactam antibiotics and peptidoglycan-degrading enzymes.

3.3 MATERIALS AND METHODS

Bacterial growth and culture conditions

Arthrobacter globiformis (ATCC 8010) was obtained from the American Type Culture Collection and maintained on Tryptic soy agar (Difco) supplemented with 0.2% (w/v) yeast extract (TSYE agar). The bacteria were grown in EYG medium (Cure & Keddie, 1973) as described previously (Deutch & Perera, 1992). Minimum inhibitory concentrations (MICs) for antibiotics were determined by a tube dilution method. *A. globiformis* was grown in EYG medium overnight and exponential phase cells diluted 1:25 into 1 x 10 cm glass tubes containing 5 ml of EYG medium or EYG medium containing 0.9 M NaCl. Serially diluted filter-sterilized solutions of penicillin-G (benzyl-penicillin), methicillin, ampicillin, streptomycin, or tetracycline were added and the cultures incubated at 30°C in a Lab-Line environmental chamber and shaken at 250 rpm. Optical density was determined periodically using a Klett-Summerson colorimeter (no. 66 filter) and morphology monitored with a Nikon phase contrast microscope. The cultures were incubated for 20 hours and the minimum

inhibitory concentration (MIC) was defined as the concentration at which four or fewer doublings took place.

Treatment with peptidoglycan degradating enzymes

Achromopeptidase, lysostaphin, mutanolysin, and lysozyme were obtained from Sigma Chemical Company. Arthrobacter globiformis was grown in EYG medium or EYG medium containing 0.9 M NaCl and exponential phase or stationary phase cells were harvested by centrifugation in a TOMY microcentrifuge. The bacteria were resuspended in a 5 mM sodium phosphate buffer, pH 7, and the above enzymes were added to a final concentration of 3.0 µg/ml. The suspensions were incubated at 37°C for 60 minutes, and the turbidity measured periodically.

Membrane preparations

Membranes were prepared as described by Coyette et al. (1977). Bacteria were grown in EYG medium or EYG medium containing 0.9 M NaCl to mid-exponential or stationary phase, and 10 ml aliquots harvested by centrifugation at 10,000 rpm in a TOMY centrifuge. The cells were washed twice in a 5 mM sodium phosphate buffer, pH 7 containing 1 mM $MgCl_2$, and treated for 20 minutes at 37°C with lysozyme, DNase (pancreatic) and RNase at

final concentrations of 0.5 mg/ml, 2 μg/ml and 1 μg/ml respectively. The membranes were recovered by centrifugation for 10 minutes at 10,000 rpm, washed twice in the 5 mM phosphate buffer, and stored at -20°C in 0.5 ml aliquots in 40 mM sodium phosphate buffer/5% glycerol, pH 7. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin (Sigma) as a standard.

[14C]Benzylpenicillin labelling

[14C]benzylpenicillin, with the radioactive label on the C=O substituent, was purchased from the Amersham Corporation; specific activity was 57 Ci/mol. Frozen membrane fractions were thawed at room temperature and 4-10 μl aliquots incubated with 1 μl of [14C]benzylpenicillin (10 μM final concentration) for 20 minutes at 37°C. The reactions were terminated by adding 1 μl of 0.1 M non-radioactive benzylpenicillin and 12 μl of a sample denaturing buffer.

SDS polyacrylamide gel electrophoresis and autoradiography

Membrane fraction proteins were analyzed in 10% polyacrylamide slab gels (10% acrylamide-0.13% bis-acrylamide) as described by Laemmli (1970) with 4% stacking gels. The samples were boiled for one minute

and 10 µl aliquots subjected to electrophoresis at a constant voltage of 200 V in a discontinuous buffer system. The gels were stained with Coomassie Brilliant Blue R (Fairbanks et al. 1971) and destained with a 40% methanol/10% acetic acid solution. The destained gels were soaked in a 3% glycerol/30% methanol solution overnight, and dried in a conventional vacuum gel dryer at room temperature for at least two hours. The dried gels were then placed in paper cassettes with Kodak X-OMAT RT X-ray film and stored in a -70°C freezer for 4.5 months prior to development.

3.4 RESULTS

Penicillin binding proteins of A. globiformis

The protein profiles of unstressed bacilli and cocci and of salt-induced myceloids revealed numerous differences (Fig. 3.1). For example, three bands with molecular masses of 66 kD, 59 kD and 55 kD were apparently more intense in the exponential phase normal rods than in exponential phase myceloids while a 47 kD band was more intense in the exponential phase myceloids. The stationary phase myceloids showed a very intense band at 130 kD while the corresponding band was less intense in the cocci. A 100 kD protein seemed to

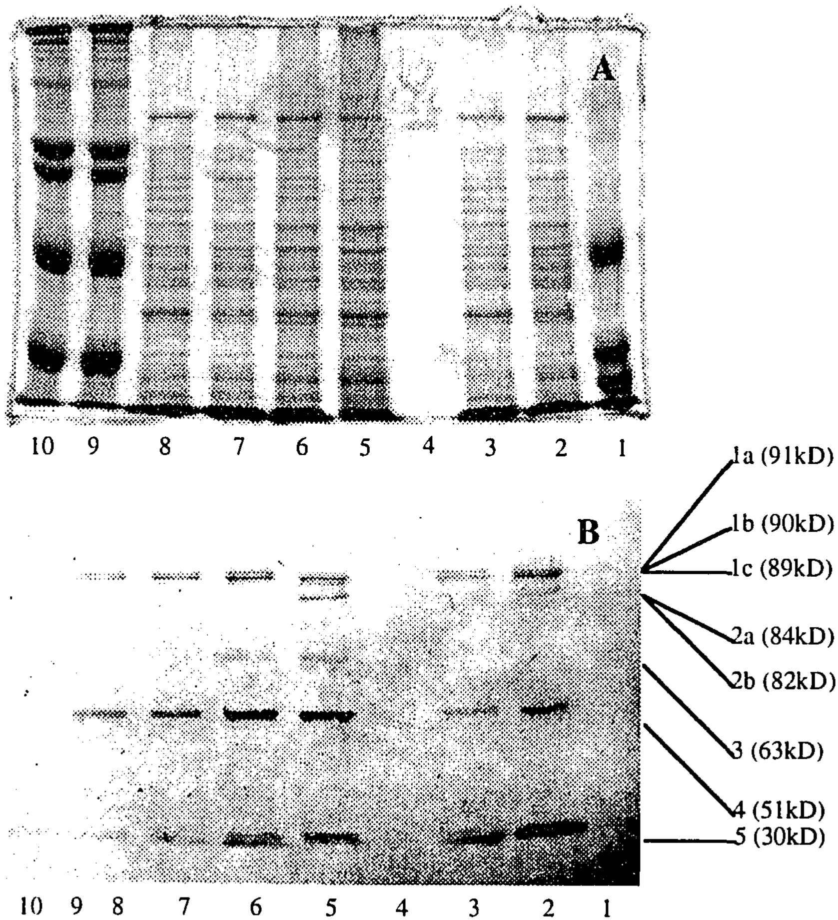

Fig.3.1 Protein and penicillin-binding protein (PBP) profiles of A. globiformis. Membrane fractions prepared as described in Materials and Methods were incubated with 10 µM [14C]benzylpenicillin at 37°C for 20

minutes; the reactions were terminated with 0.1 M non radioactive penicillin, and after staining with coomassie blue, the dried gel exposed to X-OMAT x-ray film for 4.5 months. Panel A shows coomassie blue stained gel and panel B shows the penicillin-binding protein profile of A. globiformis. Low molecular weight standards (lane 1), High molecular weight standards (lanes 9 & 10), exponential phase cells in EYG medium (lanes 2 & 8), exponential phase cells in EYG medium containing 0.9 M NaCl (3 & 7), stationary phase cells in EYG medium (lane 5), and stationary phase cells in EYG medium containing 0. 9 M NaCl (lane 6) are shown. Lane 4 was left blank. Lanes 2, 3, 5 & 6 contain 10 µM, and lanes 7 & 8 1 µM [14C]benzylpenicillin respectively.

be absent in the myceloids while a 34 kD band was very intense in the cocci.

Autoradiograms of radioactively labelled proteins indicated that A. globiformis contains as many as eight penicillin binding proteins. The PBPs were numbered in order of decreasing molecular mass (Waxman & Strominger 1983). Four to seven bands could be identified depending on the growth medium and the stage of growth (Table 3.1). Two high molecular mass proteins, PBP 1b (90 kD) and PBP 1c (89 kD) and two low molecular mass proteins, PBP 4 (51 kD) and PBP 5 (30 kD) were present in all growth stages irrespective of the medium. PBP 1a (91 kD) and PBP 3 (63 kD) appeared to be formed only during stationary phase (lanes 5 & 6, Fig. 3.1B) since they were absent in the exponential-phase cells (lanes 2 & 3, Fig. 3.1B). PBP 2a (84 kD) and PBP 2b (82 kD) were absent in the myceloids from the EYG medium containing NaCl (lanes 3, 6, & 8, Fig. 3.1B). These may be associated with septation in arthrobacters.

Effects of beta-lactam antibiotics on Arthrobacter globiformis

When beta-lactam antibiotics were added to exponential phase cultures of A. globiformis grown in EYG medium, many morphological aberrations could be observed. With ampicillin, some cells lysed and others appeared as

PBP	Exp-phase	Exp-phase	Sta-phase	Sta-phase
Molecular mass # (kD)	EYG	EYG + 0.9 M NaCl	EYG	EYG + 0.9 M NaCl
1a-91	-	-	+	+
1b-90	+	+	+	+
1c-89	+	+	+	+
2a-84	+	-	-	-
2b-82	+	-	+	-
3-63	-	-	+	+
4-51	+	+	+	+
5-30	+	+	+	+

Table 3.2 The PBPs of exponential and stationary phase cells of A. globiformis grown in EYG medium and EYG medium containing 0.9 M NaCl.

large protoplasts; rod-shaped cells with large bulges in the middle were not uncommon. Cell clusters and cells with tiny side branches were also apparent and a significant amount of cellular debris was present. The same was true for the other beta-lactam antibiotics tested (benzylpenicillin and methicillin). When these beta-lactam antibiotics were added to exponential-phase myceloids from EYG medium containing 0.9 M NaCl, the myceloid branches appeared bloated and portions of the branches were observed to lyse.

There were major differences in the minimum inhibitory concentrations (MICs) of these different antibiotics depending on the growth medium (Table 3.2). The myceloids were more sensitive to the beta-lactam antibiotics than normal cells, and the MICs were 12 to 20 times lower. By contrast, the myceloids did not differ from normal cells in their sensitivity to tetracycline. Surprisingly, the MIC for streptomycin was about 320 times higher for the myceloids than for normal cells.

Sensitivity to degradative enzymes

A number of hydrolytic enzymes including mutanolysin, achromopeptidase and lysostaphin act on the bacterial peptidoglycan. When mutanolysin (3 μg/ml) was added to the exponential phase myceloids grown in EYG medium

Minimum inhibitory concentrations (μM)

Medium	Pen	Amp	Met	Tet	Str
EYG	3	3	6	0.20	0.25
EYG + 0.9 M NaCl	0.06	0.25	0.40	0.20	80

Table 3.2 Antibiotic sensitivities of myceloids grown in EYG containing 0.9 M NaCl and normal cells grown in EYG. Abbreviations: Pen-Penicillin, Amp-Ampicillin, Met-Methicillin, Tet-Tetracycline, Str-Streptomycin.

containing NaCl, there was a significant drop in absorbance, which was much more rapid than that found with cells from medium lacking NaCl (Figure 3.2). Myceloids from stationary phase cultures were also sensitive to mutanolysin, but the drop in absorbance was not as dramatic as during the exponential phase. The stationary phase cocci, on the other hand, were not affected by the mutanolysin treatment.

The exponential phase normal rods and stationary phase myceloids were very easily fragmented to short rods and cocci when treated with mutanolysin (Figure 3.3). The myceloids began to break up within 10 minutes and only short rods and cocci were observed at the end of a 60 minute treatment. With achromopeptidase and lysostaphin, there was no significant change in absorbance or fragmentation of the myceloids during either exponential phase or stationary phase

3.5 DISCUSSION

These experiments demonstrate for the first time the penicillin binding protein profile of Arthrobacter globiformis (ATCC 8010). These proteins range in molecular mass from 90,000 to 30,000. PBPs 1a (91 kD) and 3 (63 kD) are absent from normal exponential phase cells but present in normal stationary phase cocci. PBP 2a may play a major role in maintaining the bacillary

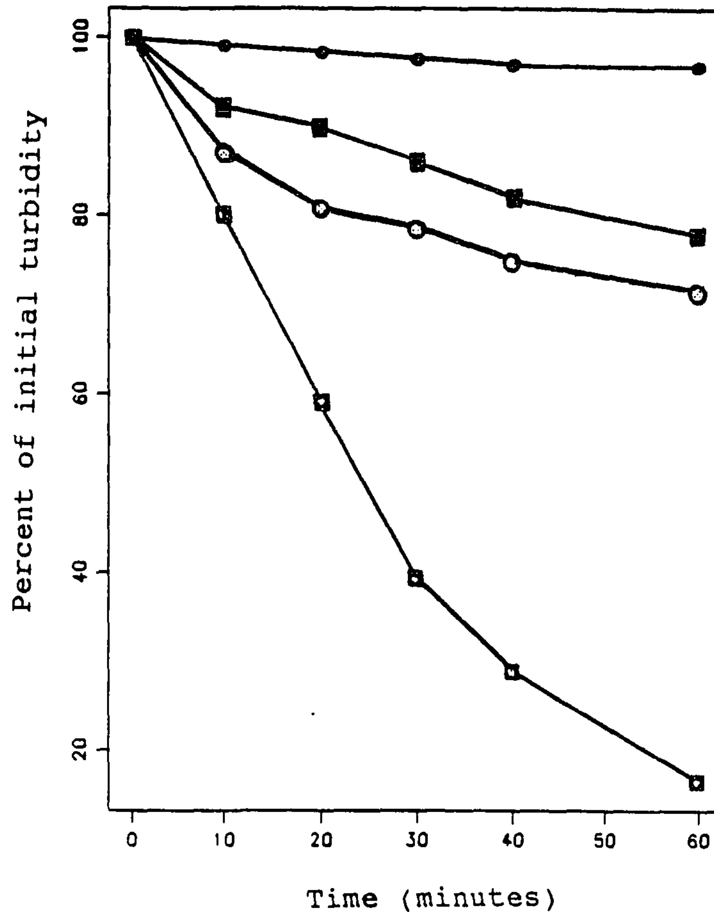

Fig.3.2 Effect of mutanolysin on myceloids and normal rods and cocci. <u>A. globiformis</u> was grown in EYG medium or EYG medium containing 0.9 M NaCl to mid exponential phase (70 Klett units) and stationary phase (200 Klett units). The cells were harvested, washed twice in a phosphate buffer, and treated with 3 μg/ml mutanolysin. The cells were then incubated at 37°C and turbidity was measured periodically. The figure shows the results for exponential phase myceloids (□), normal baccilli (O), stationary phase myceloids (■), and cocci (●).

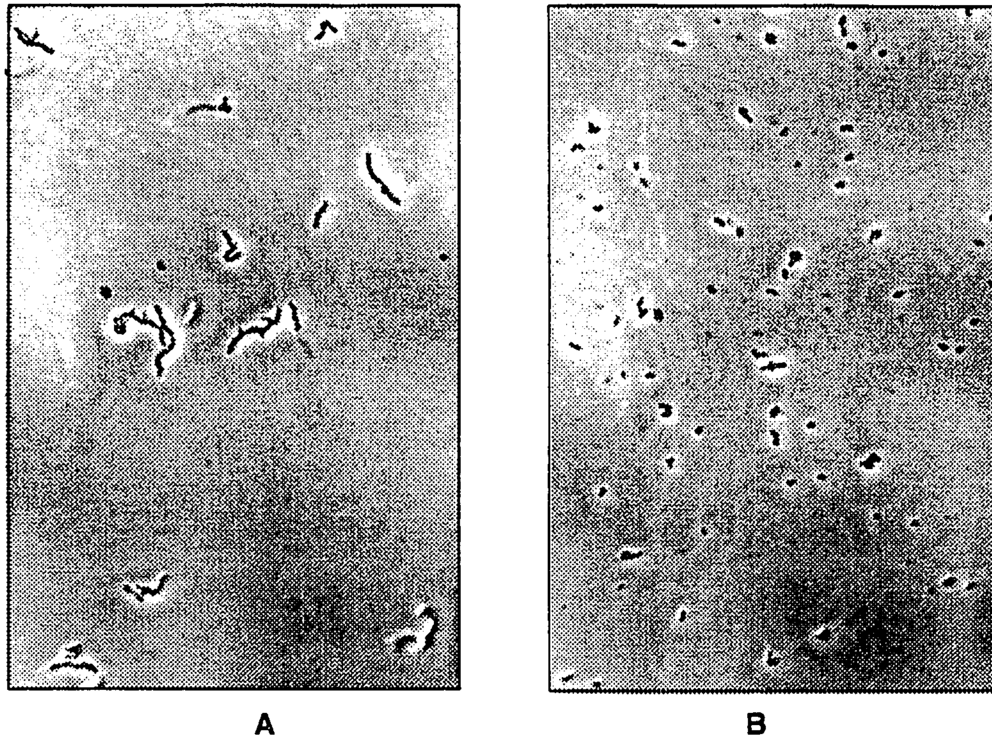

Fig. 3.3 Morphology of A. globiformis before and after treatment with mutanolysin. Panel A shows stationary phase A. globiformis cells grown in EYG medium containing 0.9 M NaCl (myceloids) and panel B shows A. globiformis cells after 60 minutes of mutanolysin treatment.

form since it is the only one absent in the PBP profile of the normal stationary phase cocci. PBP 2a and PBP 2b may be associated with septation since they are both absent in exponential and stationary phase myceloids. Since myceloids form as a result of osmotic stress (Deutch & Perera 1992) it is likely that osmotic stress in some way inhibits the synthesis of these PBPs.

The myceloids were 12-20 times more sensitive to penicillin, ampicillin and methicillin when compared to the normal bacilli. The most common morphological abnormality observed with the beta-lactam treated cells was the formation of bulges. It was also interesting that while there was no difference in the MICs for tetracycline the myceloids were 320 times more resistant to streptomycin than normal cells. Of the hydrolytic enzymes tested, only mutanolysin was able to trigger division the myceloids into smaller cellular units. It is interesting that only the exponential phase myceloids showed significant lysis. Mutanolysin consists of three enzymes; one proteolytic and two lytic enzymes (Yokogawa et al. 1974). If myceloids were the result of incompletely septated cells sticking together, then the separation of cells by treating with the enzyme should not lead to a significant loss of absorbance. The drop in optical density on the other hand implies that a considerable amount of lysis takes

place with mutanolysin treatment at this stage.

Streptomycin is an aminoglycoside that interacts with the 30S ribosomal subunit and often causes misreading of the messenger RNA (Garrod et al. 1981). While it is unclear why osmotic stress leads to streptomycin resistance, this phenotype may be very useful for isolating mutants of A. globiformis that form myceloids in the absence of added salt. Further studies of each of the PBPs of A. globiformis should provide insight into their role in myceloid formation and the morphogenesis of these bacteria.

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CHAPTER 4

Sensitivity of myceloids and cocci of *Arthrobacter globiformis* to heat, starvation, and desiccation

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4.1 ABSTRACT

Arthrobacter globiformis forms myceloids during growth in media containing increased concentrations of NaCl. Viability studies were carried out to compare the sensitivity of stationary-phase myceloids and normal cocci to elevated temperature, starvation, and desiccation. The coccal form maintained a 1000-fold higher viability than the myceloids after being treated for one hour at 45°C. The myceloids were more resistant than stationary-phase coccal forms to long term starvation in a buffer containing 0.9 M NaCl and lacking a nitrogen source. In the absence of a carbon source, the coccal form survived better than the myceloids. In the absence of both a carbon and a nitrogen source, there was no difference in survival between the two morphological forms. There was no significant difference in viability when the two forms were subjected to desiccation over a three week period. These results indicate that the myceloids may be more tolerant to nitrogen starvation while the coccal form seem to tolerate heat and carbon starvation better than the myceloids.

4.2 INTRODUCTION

Arthrobacters are coryneform chemoheterotrophs that can utilize a variety of organic substrates (Stevenson, 1967: Hagedorn and Holt, 1975). They exhibit a characteristic dimorphic life cycle where the exponential phase cells appear as bacilli, sometimes with rudimemtary branches, and the stationary phase cells appear as cocci. Arthrobacters are found in many different habitats and are most numerous in soil (Conn, 1948; Clark, 1975). These bacteria should exhibit specific adaptations to limiting conditions found in the soil. Morris (1960) suggested that the ability of the arthrobacters to utilize a variety of compounds at low concentrations as well as their simple nutritional requirements may be contributing factors to their presence in soils poor in organic nutrients.

Several studies have examined the resistance of arthrobacters to extreme conditions like starvation and desiccation (Robinson et al. 1965; Ensign, 1970; Boylen, 1973). Exponential-phase rods and cocci of A. crystallopoietes maintained 100% viability during starvation for a period up to one month. Viability dropped only by 35% after 60 days (Ensign, 1970). No difference was observed for the rod and the coccal forms. The bacteria metabolized intracellular substrates such as glycogen or poly-beta-hydroxybutyric

acid during prolonged starvation (Boylen & Ensign 1970). Ribonucleic acid (RNA) as well as proteins were also substrates for endogenous metabolism, while deoxyribonucleic acid (DNA) and lipids were not metabolized. The protein turn-over rates of starving bacteria are much higher than in growing cells (Dawes & Sutherland 1992). Both rod and coccal forms of A. crystallopoietes were resistant to extreme desiccation and 50% of the cells remained viable for a period of 6 months in sand (Boylen, 1973). A. globiformis, on the other hand, seems to be more variable in its ability to withstand desiccation (Robinson et al. 1965).

We have reported that A. globiformis forms clusters of branching rods or myceloids when subjected to osmotic stress (Deutch & Perera 1992). We have also shown that the penicillin binding protein (PBP) profiles of normal cells and myceloids are different and that two particular PBPs are lacking in the myceloid form. To determine if the myceloid form has an adaptive advantage over the stationary phase coccal form, we have now compared the sensitivity of the two forms to heat, starvation and desiccation.

Because myceloids are aggregates of cells, they might have an advantage over the single cells in producing a colony. Viable count comparisons of myceloids and single cocci could therefore be

misleading. Because of this, we compared the viable counts of myceloids separated prior to each treatment with the hydrolytic enzyme mutanolysin to those of nomal cocci and untreated myceloids. We have previously found that mutanolysin, separates the myceloids into short rods and cocci (Perera & Deutch, unpublished data).

4.3 MATERIALS AND METHODS

Growth and culture conditions

Arthrobacter globiformis (ATCC 8010) was obtained from the American Type Culture Collection and grown as described previously (Deutch & Perera 1992). Bacteria grown to stationary phase in EYG medium (cocci) or in EYG containing 0.9 M NaCl (myceloids) were harvested by centrifugation in a high speed microcentrifuge at 10,000 rpm for 10 min. The cells were washed with 5 mM sodium phosphate buffer, pH 7 to remove any nutrients and diluted into test solutions as required.

Heat treatment of myceloids and cocci

Stationary phase myceloids and cocci were heated at 45° C for up to one hour. Portions were aseptically removed periodically, serially diluted in 0.1 M NaCl, and plated on tryptic soy agar (TSA) medium. Duplicate

plates were counted after 2 days at 30°C.

Starvation of A. globiformis

Cells were diluted 1:25 into mineral base E containing 0.9 M NaCl (Cure & Keddie, 1973) but without an added carbon source (E-C), an added nitrogen source (E-N), or without both a carbon and a nitrogen source (E-C-N). Cell suspensions were incubated at 30°C in a Lab-Line environmental chamber and shaken at 250 rpm. Samples (1 ml) were taken out at 24 hrs, and weekly to one month and at the end of two months. Serial dilutions were done in 0.1 M NaCl prior to plating on tryptic soy agar medium for viable cell counts. Duplicate plates were scored after 2 days at 30°C.

Desiccation of A. globiformis

Aliquots (100 µl) of stationary phase myceloids and normal cocci were transferred to sterile dry sand in loosely screw capped glass vials. The cell suspension was mixed well into the sand using a sterile spatula. The vials were placed in a sealed desiccator containing 50 g of lithium chloride, which was replaced weekly. The dry weights of the vials were also recorded. Viable counts were prepared periodically for up to three weeks. E buffer (5 ml) was added to each vial and the suspension shaken vigorously to free any cells attached

to sand particles. The soil was allowed to settle one minute, the supernatant serially diluted in 0.1 M NaCl and viable counts prepared in duplicate on TSA plates as described above.

4.4 RESULTS

Sensitivity of myceloids to heat

Stationary-phase myceloids rapidly lost viability at 45° C while stationary phase cocci were not greatly affected by this temperature (Fig. 4.1). Twenty percent of the stationary phase cocci, and 0.07 percent of the myceloids remained viable after 60 minute at 45° C.

Sensitivity of myceloids to starvation

During starvation in E-N medium containing D-glucose as the carbon source but no nitrogen source, myceloids maintained viability better than cocci (Fig. 4.2). After one week, 78% of the myceloids were viable while only 27% of the cocci were viable. The viability of both myceloids and cocci then dropped and after two weeks only about 2% of cocci and 4% of myceloids remained viable. At the end of the third week only 0.07% cocci remained viable, but there was no further change in viability of the myceloids. The myceloids separated into shorter cellular units by treatment with

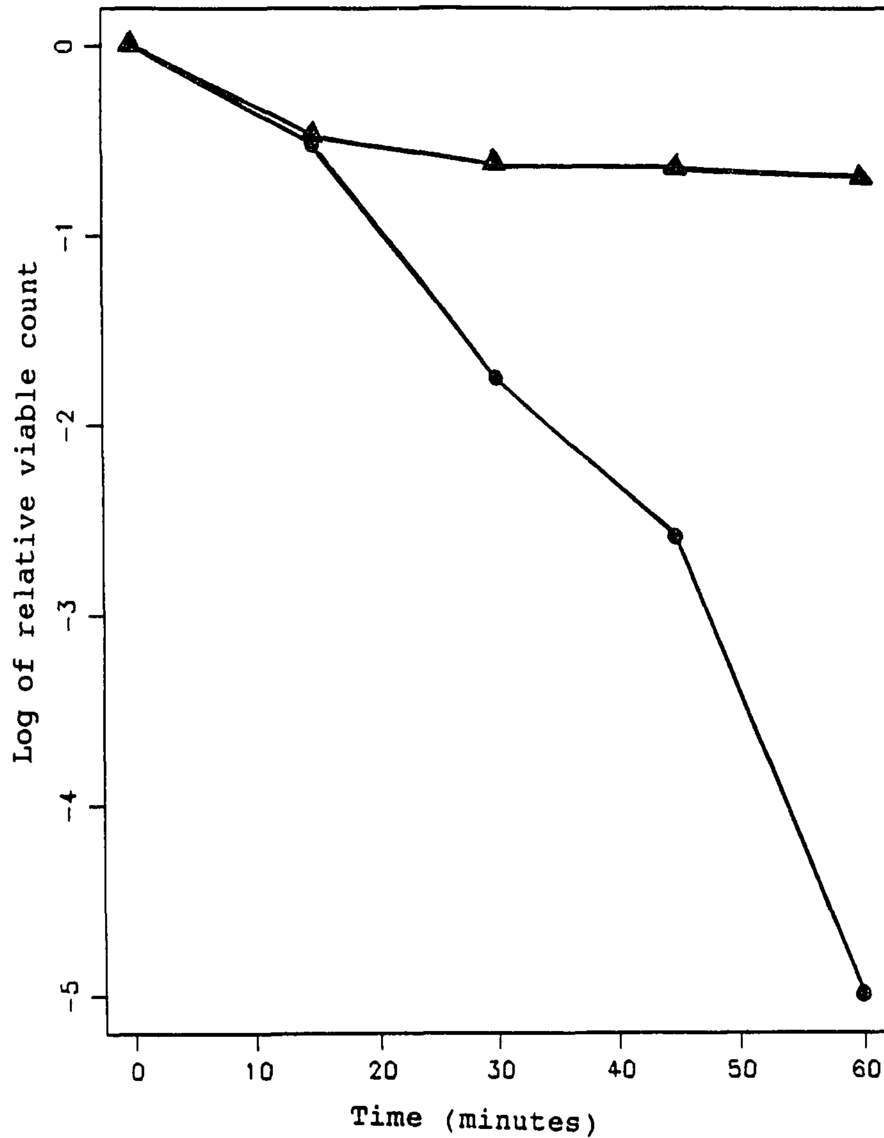

Fig. 4.1 Sensitivity of myceloids to heat. Stationary phase myceloids (●) and cocci (▲)of A. globiformis were treated at 45°C upto one hour. Aliquots were periodically removed and serially diluted in a 0.1 M NaCl solution and plated on tryptic soy agar medium.

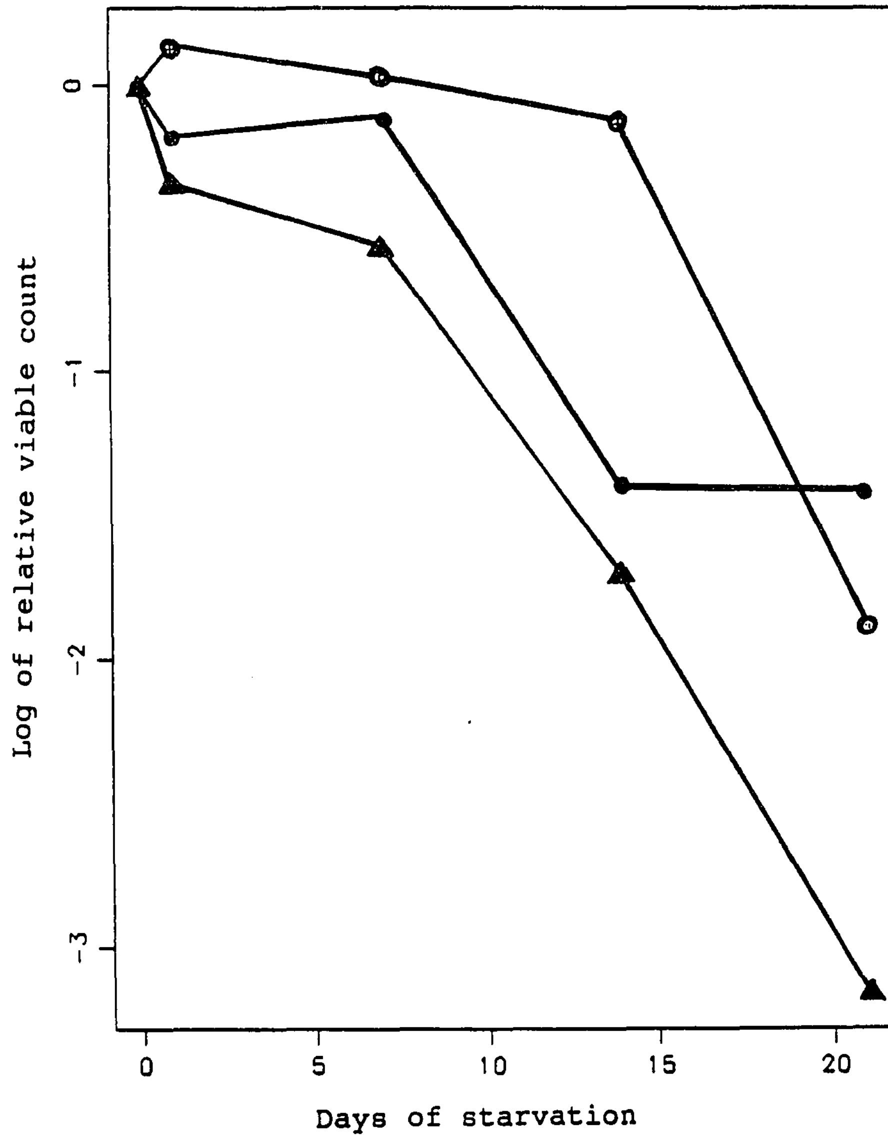

Fig. 4.2 Sensitivity of A. globiformis to starvation in a E-N medium. This medium contained D-glucose as the carbon source and no nitrogen source. Myceloids (●), cocci (▲) and myceloids separated to smaller units with mutanolysin (o) are shown.

mutanolysin maintained 75% viability up to two weeks. Viability then fell to 1.3% of the initial level at the end of a three week period.

During starvation in E-C medium containing ammonium sulphate but no carbon source, the cocci were more resistant to starvation than the myceloids (Fig. 4.3). The myceloids initially maintained a higher viability up to one week and then the viability fell to 1.6% by the end of one month. The cocci and the separated units of myceloids maintained about 27% viability at the end of a one month period.

During starvation in a medium lacking both carbon and nitrogen (E buffer), both forms remained viable. The myceloids maintained greater than 70% viability up to one month (Fig. 4.4). The cocci lost viability more rapidly and at the end of a month, 17% of the cells were still viable. The separated units of myceloids gradually lost viability and 37% remained viable at the end of one month.

Cell size decreased dramatically over time in all the starvation media. No apparent resting stage was observed. Both the cocci and the myceloids remained phase dark while a few protoplast like spheres were observed at the latter stages in the starving media. Attempts to maintain the myceloids in the defined starvation media in the absence of salt were

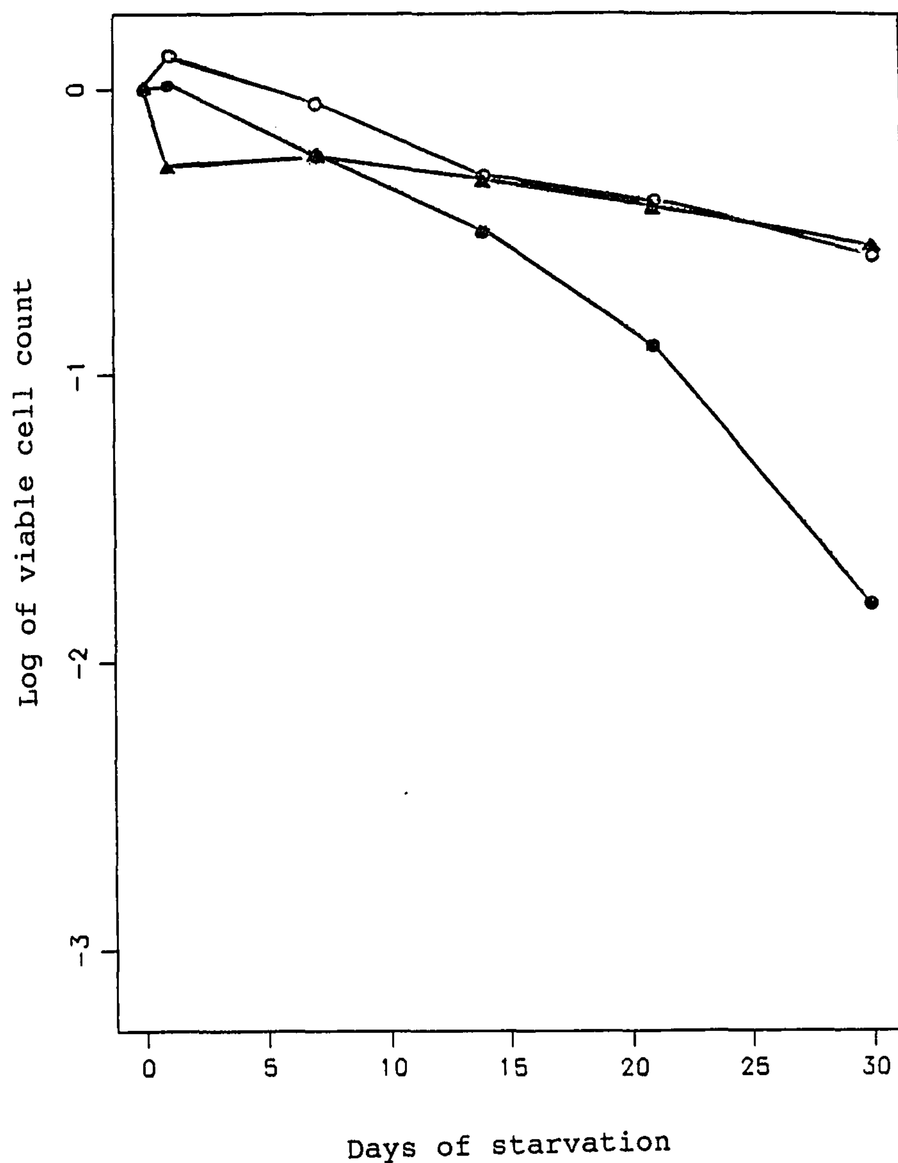

Fig. 4.3 Sensitivity of A. globiformis to starvation in a E-C medium. This medium contained ammonium sulphate but no D-glucose. Myceloids (●), cocci (▲) and myceloids separated to smaller units with mutanolysin (o) are shown.

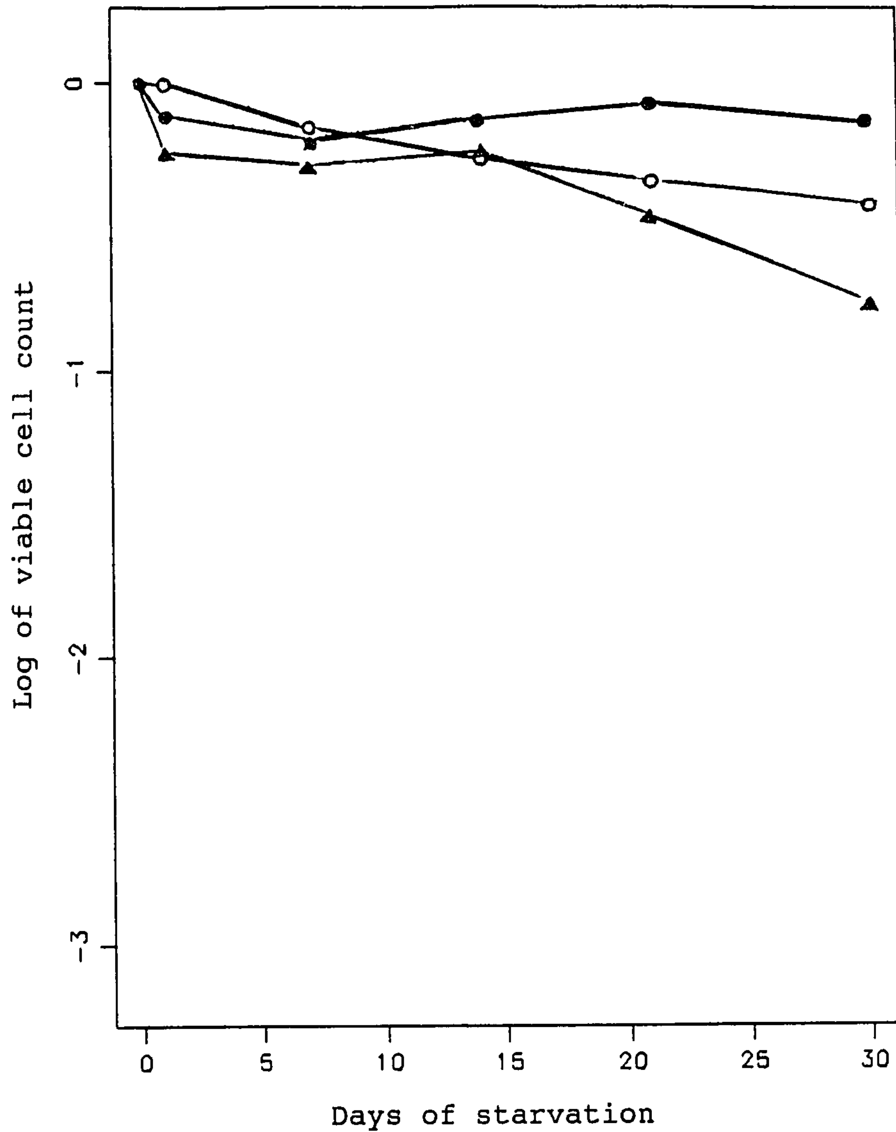

Fig. 4.4 Sensitivity of A. globiformis to starvation in a E-C-N medium. This medium is a non-nutrient buffer (E buffer) lacking both ammonium sulphate and D-glucose. Myceloids (●), cocci (▲) and myceloids separated to smaller units with mutanolysin (○) are shown.

unsuccessful. After six hours the myceloids started to divide and formed shorter fragments.

Sensitivity of myceloids and cocci to desiccation

Both the myceloids and cocci appeared equally sensitive to desiccation. After one week the viable counts of treated and untreated myceloids and cocci fell about 1000 fold. By the end of the third week there were no viable cells in either case.

4.5 DISCUSSION

These results indicate that stationary phase salt-induced myceloids and stationary phase cocci differ in their sensitivities to potential environmental stresses. The myceloids were more sensitive to heating at 45°C than the cocci. However, there was not much difference between the myceloids and the cocci in terms of extreme desiccation. There were no viable cells of either form after three weeks in sterile sand. The two forms varied in their sensitivity to starvation. The myceloids seemed to survive better under nitrogen limiting conditions while the cocci survived better under carbon starvation. There was little difference between the two forms under both carbon and nitrogen

limiting conditions. Both forms exhibit a higher level of viability than during either nitrogen or carbon starvation. The ability of myceloids to survive better under nitrogen starvation could be due to myceloids being an aggregation of cells. Lysis of parts of a myceloid could provide necessary nutrients to the remaining portions of the myceloids, thus maintaining their viability. This would be an example of cryptic growth.

Salt-induced myceloids can be broken into short rods and cocci by the treatment of mutanolysin. These smaller units of the myceloids maintained their viability better than the cocci in E-N and E-C-N media. In E-C medium, the disrupted myceloids more closely resembled the cocci. This suggests that the resistance of myceloids to starvation in E-N or E-C-N medium is not a property of their morphology *per se*. Rather, it may be a physiological consequence of poor growth in medium of high osmolality.

A potential problem encountered in viability studies is the possibility of viable but non-culturable cells (Byrd *et al*. 1991). Certain cells may be viable, but non-culturable at different points in these studies, thus leading to an under estimation of the percent viability. In order to understand viability, therefore, it is important to think of the percent of

cells referred to as "viable" as the percent that are "culturable".

A. globiformis cells grown in carbohydrate-rich media are known to accumulate large amounts of α,α-trehalose and the amount of trehalose is believed to increase upto 1300 µg/mg protein under osmotic stress (Zevenhuizen, 1992). Non-growing cells of *A. globiformis* maintain a constant level of trehalose while the glycogen level depends on the carbon supply of the medium. The starvation resistance of *Arthrobacter* cells in E-C or E-C-N medium may be due to the presence of high contents of trehalose in these cells. It would be of value to analyze the glycogen and trehalose contents of the coccal and myceloid forms under nutrient starving conditions. Another interesting aspect would be to compare the normal cocci and the myceloids formed in response to osmotic stress in terms of their protein turnover rate, RNA metabolism, and trehalose metabolism. Myceloids were somewhat more resistant to nitrogen starvation and therefore may differ in their metabolism of N-containing compounds.

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CHAPTER 5

GENERAL DISCUSSION

5.1 Growth, morphology, and osmotic stress in *A. globiformis*

The unique nature of the arthrobacter lifecycle makes it a valuable tool in understanding the nature of the cell cycle and morphology. The ability of *Arthrobacter globiformis* to form myceloids when subjected to osmotic stress enhances its value as a tool in morphogenesis.

Arthrobacter globiformis shows characteristic responses to osmotic stress: the specific growth rate drops linearly with increasing solute concentrations and myceloids are formed at concentrations as low as 0.3 M NaCl. The myceloids are larger with more branches as the solute concentration increases to 0.6 or 0.9 M NaCl. Known osmoprotectants do not prevent the formation of myceloids or relieve the osmotic effect completely, although proline and glutamate have some effect in initiating partial septation of the myceloids (Deutch & Perera 1992).

Other studies have shown that myceloids may also arise due to the lack of certain nutrients such as

biotin (Chan et al. 1973), vitamin B12 (Chaplin & Lockhead 1956), or manganese (Germida & Casida 1980). Morphology thus is also determined by the availability of essential growth factors. Since myceloids arise either due to lack of certain nutrients or due to osmotic stress, it is possible that osmotic stress in some way leads to deprivation of certain nutrients to the cell. If the above were true, addition of these nutrients should lead to a normal cell cycle. However, I found that addition of these nutrients in 1000 fold or addition of D-alanine, D-glutamate or L-lysine did not relieve myceloid formation. Therefore, it does not seem likely that lack of these growth compounds are responsible for myceloid formation under osmotic stress.

5.2 Penicillin binding proteins and myceloid cell formation during osmotic stress

The PBPs of *A. globiformis* were identified after incubating the membranes with 10 µM final concentration of [14C]benzylpenicillin. At high concentrations of [14C]benzylpenicillin (e.g., 3000 µM, 2000 µM, 300 µM, 200 µM etc.) there was a lot of nonspecific binding as indicated by the blurry black blotches on the auto-radiogram. At low concentrations (e. g., 10 µM and 1 µM) eight clear bands were observed and identified as

the PBPs.

Myceloids seem to arise as a result of alterations in peptidoglycan synthesis during osmotic stress. The salt-induced myceloids cannot be disrupted mechanically but peptidoglycan degradative enzymes like lysozyme and mutanolysin can break down the myceloids. Moreover, the myceloids are 12-20 times more sensitive to beta-lactam antibiotics than normal cells. PBPs are associated with altered morphological forms in both Gram negative and Gram positive bacteria (eg. Schuster et al. 1990; Pucci et al. 1986). In Streptococcus faecium, Streptococcus pneumoniae and Staphylococcus aureus, PBPs are associated with altered morphological forms (Pucci et al 1986; Schuster et al. 1990; Curtis et al. 1980; Wyke et al. 1981). Several penicillin binding proteins have transglycolase and transpeptidase activity (eg. PBPs 1, 2 and 3 of Escherichia coli, PBP 4 of S. aureus and high molecular PBPs of Bacillus) These proteins have been shown to be involved in generating morphological forms other than the characteristic form (Spratt 1975; Waxman & Strominger 1983; Curtis et al. 1980). This implies that these PBPs play a role in determining the shape of a bacterium.

This seems to be true for arthrobacters as well. I found that the PBP profile of the myceloids varies from that of the cocci. Myceloids seem to arise as a result

of lacking PBP 2a (84 kD) and PBP 2b (82 kD) although other unknown proteins may also be involved. Exponential phase myceloids as well as stationary phase myceloids lack PBP 2a and PBP 2b. Therefore, these PBPs seem to play a significant role in septation of A. globiformis. These proteins may have transglycolase and/or transpeptidase activity that is inactivated in a hyperosmotic environment. This would be consistent with Koch's surface stress theory. However, it is also possible that osmotic stress causes changes in cell volume that lead to altered patterns of peptidoglycan synthesis. Since PBP 2a and 2b are totally missing from myceloids, it seem more likely that osmotic stress leads indirectly to an altered pattern of PBP synthesis. It is the absence of PBP 2a and PBP 2b that then leads to defects in septation. These proteins may have transpeptidase or transglycosylase activities required for the formation of peptidoglycan specifically at division septa.

At physiological pH DNA remain as a charged anion and therefore attracts many cations. Binding of proteins to DNA displaces cations from the surface area of the DNA. Hence, DNA-protein binding alterations can occur as a result of fluctuations in the ion concentrations in the cytoplasm due to the influx of cations (e.g. K+) during hyperosmotic conditions. If

this is true, increasing K+ concentration in the cell may affect binding of RNA polymerase to the DNA causing changes in the transcription of many genes including the ones that code for specific PBPs.

5.3 Physiological significance of myceloid cells

In an unfavorable environment, arthrobacters may benefit by the ability to form myceloids, which are a collection of many cells. This may increase their chance of remaining viable. From my observations, myceloids seem to tolerate nitrogen starvation better than the coccal form. Myceloids however, did not seem to have an advantage over cocci in tolerating heat, carbon starvation, or desiccation. Myceloids survived better than the cocci in a 0.1 M NaCl and 0.9 M NaCl solution (data not given). This implies that the bacterium forms myceloids in order to survive in a hyperosmotic environment or as a result of stress. Ability of myceloids to tolerate nitrogen starvation may be an advantage to a bacterium since nitrogen is limiting in most environments.

5.4 Suggestions for further work

Other corynebacteria accumulate ectoine and pipecolic acid at high osmolalities (Gouesbet et al. 1992; Bernard et al. 1993). To understand if

arthrobacters accumulate novel compatible solutes it would be worthwhile to analyse their osmotically compatible solutes. The intracellular solutes can be extracted using ethanol and analysed and purified by paper chromatography and paper electrophoresis. Further analysis of the intracellular solutes can be done by [13C]nuclear magnetic resonance spectroscopy.

Purification of the PBPs and establishing mutants of each PBP also may prove to be very useful in understanding not only how the myceloids form but also in understanding morphogenesis in Gram positive bacteria. PBPs can be purified by covalent penicillin affinity chromatography (Blumberg & Strominger 1972). Mixtures of PBPs will be obtained by this method. Individual PBPs can be purified by varying the Beta-lactam ligand in the Sepharose column. To understand the affinity of beta-lactam antibiotics for the PBPs, a competition experiment can be carried out. Membrane samples can be incubated with a variety of beta-lactam antibiotics (e.g., 1 nM to 1 mM), prior to supplementing with [14C]benzylpenicillin. Once the beta-lactam antibiotics showing a high affinity to certain PBPs are identified, these can be used as ligands in the Sepharose column to isolate the specific PBPs. Moreover, it would be interesting to study PBPs in myceloids formed as a result of nutrient deficiency.

Another possibility is to study the PBP patterns of arthrobacters when subjected to osmotic upshocks or downshocks.

Electron micrographs of myceloids at each stage of formation will provide great insight into how the murein layer is laid down in myceloids. A complete analysis of the cell wall composition and structure on the other hand will be very useful to fully understand the nature of the myceloid peptidoglycan component. Isolation of spontaneous or chemically induced mutants for streptomycin resistance also will be very useful in understanding the phenomenon of myceloid formation in arthrobacters.

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