SYNOPSIS

Tyrosine phosphorylation and dephosphorylation of proteins was, for long, thought to be a process which is restricted to the eukaryotic kingdom. However, in the last decade biochemical and genome analysis studies have established that tyrosine phosphorylation occurs in all the three kingdoms of life viz., the Eukarya, Archaea and Bacteria. In eukaryotic kingdom, tyrosine phosphorylation has been shown to be involved in the regulation of important cellular events, such as development, differentiation and control of the cell cycle. The physiological significance of tyrosine phosphorylation in bacteria though mostly unknown seems to be emerging gradually, and it appears that majority of the membrane located bacterial protein tyrosine kinases (PtkS) are involved in regulation of extracellular polysaccharide synthesis and/or virulence in case of pathogenic bacteria.

The psychrotrophic Antarctic bacterium *Pseudomonas syringae* Lz4W was earlier shown to have a tyrosine phosphorylated 66 kDa protein in the cytosol. However, neither was it known about the role of the tyrosine phosphorylated protein, nor anything was known about the protein tyrosine kinase (Ptk) and protein phosphotyrosine phosphatase (Ptpase) which might regulate such process. The present biochemical study was, therefore, undertaken (a) to understand the similarities and dissimilarities of the bacterial tyrosine kinase with the eukaryotic enzymes, (b) to identify various phosphorylated proteins, including other tyrosine phosphorylated proteins, (c) to study the nature of bacterial protein tyrosine phosphatase (Ptpase) which, together with Ptk, regulates the cellular modification of proteins at tyrosine residue, and (d) to study the expression of Ptk and Ptpase activity in the cells of *P. syringae* during the changes in environmental temperatures and/or other metabolic process (Chapter 1).

Chapter 2 describes a biochemical characterisation of the Ptk activity from *P. syringae*. The biochemical characteristics of the Ptk were analysed by two methods namely in vitro phosphorylation of the endogenous 66 kDa protein with $\gamma^{32}$P [ATP], and a non radioactive
anti-phosphotyrosine antibody based method, which uses an exogenous eukaryotic substrate peptide (PKS1, corresponding to amino acids 6-20 of cell division kinase p34<sup>cdc2</sup>) for phosphorylation. The phosphorylation studies were carried out both with the cytosolic extracts and a partially purified Ptk fraction in presence of kinase and phosphatase inhibitors. Standard protein tyrosine kinase inhibitors such as staurosporine, genistein, dimethyl aminopurine (DMAP) did not have any effect either on the 66 kDa phosphorylation or on the phosphorylation of the exogenous substrate PKS1. Since, the staurosporine, genistein and dimethyl aminopurine are the competitive inhibitors of the ATP binding site of eukaryotic Ptk it was speculated that the ATP binding site of the P. syringae tyrosine kinase might be different from the eukaryotic enzyme. Surprisingly, phosphatase inhibitors like vanadate, molybdate and zinc sulphate also inhibited the phosphorylation of the cytosolic 66 kDa protein. Therefore, a regulation of the Ptk activity by an associated protein phosphatase was suspected. It was also observed that piceatannol which is a peptide substrate-binding site competitive inhibitor of eukaryotic Ptks, could inhibit the phosphorylation of the 66 kDa protein and PKS1 peptide, suggesting that the prokaryotic tyrosine kinase substrate-binding site is similar to its eukaryotic counterpart.

Since the 66 kDa protein tyrosine phosphorylation was inhibited (IC<sub>50</sub> = 20 µM) by sodium orthovanadate which is a known protein tyrosine phosphatase (Ptpase) inhibitor, it was postulated that the inhibition could be via a tyrosine phosphatase mediated mechanism. Such a possibility cannot be ruled out since there are recent reports of the bacterial Ptpase which could dephosphorylate protein tyrosine autokinase.

Chapter 3 describes the attempts to purify and characterise the cytosolic Ptpase from <i>P. syringae</i>. In order to investigate the nature of Ptpase the cytosolic acid phosphatases were thought to be useful candidates to look into, since various studies had earlier suggested that the acid phosphatases, especially the ones with low molecular mass, have the in vitro Ptpase activity. Accordingly, two acidic phosphatases (P1 and P2) were purified by ammonium sulphate fractionation, gel filtration and ion exchange chromatography from the cytosolic extract of <i>P. syringae</i>. 
syringae. During the purification steps, para-nitrophenyl phosphate (PNPP) was used as a substrate for assaying the phosphatase activity. Both the phosphatases had optimal activity at pH 4.5, and the activities could be enhanced by Zn^{2+}. The P1 phosphatase had an apparent molecular mass of 43 kDa on SDS-PAGE. The phosphatase however could not dephosphorylate the 66 kDa phosphoprotein and was considered a non-specific acid phosphatase of P. syringae.

The second phosphatase (P2) had an apparent molecular mass of 56 kDa on SDS-PAGE. The phosphatase was inhibited by various inhibitors that were tested viz., vanadate, molybdate, N-ethyl maleimide, iodoacetic acid and sodium fluoride. Interestingly, the P2 phosphatase could dephosphorylate the cytosolic 66 kDa phosphoprotein of P. syringae. It could also dephosphorylate specifically the tyrosine phosphorylated substrates including the phosphorylated poly(Glu-Tyr) and phosphotyrosine. The P2 phosphatase could not dephosphorylate other substrates such as ATP, phosphoserine, phosphothreonine and Ser/Thr phosphorylated protein (e.g., phosphorylated phosphorylase b). Thus, it appears that the P2 phosphatase is a phosphotyrosine specific phosphatase (Ptpase) present in the cytoplasm of P. syringae. Since P2 phosphatase could dephosphorylate the endogenous tyrosine phosphorylated 66 kDa protein, it might be important for cellular physiology of P. syringae. But, whether the P2 phosphatase regulates the phosphorylation of the 66 kDa protein in vivo is still a conjecture.

Attempts were also made to identify other phosphorylated proteins from cytosolic fractions of P. syringae (Chapter 4). A new 20 kDa protein was found to be phosphorylated in a semi-purified fraction of the cytosol. In freshly isolated 60-100% ammonium sulphate fraction of the cytosol, the 20 kDa protein was predominantly phosphorylated at cold temperature (e.g., 0°C, on ice) under in vitro conditions. However, upon further purification steps and/or ageing of the fraction this temperature specificity was lost. It was also observed that the 20 kDa protein phosphorylation is not due to adenylation but due to kinase mediated reaction.

The 20 kDa protein was phosphorylated in presence of Mn^{2+} and Ca^{2+} but not with Mg^{2+} in crude cytosolic extract. In the semi-purified cell extracts, the 20 kDa protein was also found to
be phosphorylated better in presence of Ca\(^{++}\) and Mn\(^{++}\) rather than with Mg\(^{++}\). The time kinetics of phosphorylation of the 20 kDa protein suggested that the protein is phosphorylated and dephosphorylated at a fast rate. This could either be due to a high catalytic turnover of ATP during phosphorylation, or due to a phosphatase present in the cell extract.

The phosphoamino acid analysis and the pH stability of the 20 kDa phosphoprotein revealed that the protein is phosphorylated on the tyrosine residue. Western blot analysis using a phosphotyrosine specific monoclonal antibody also supported the observation. Interestingly, the tyrosine phosphorylation of the 20 kDa and 66 kDa protein (described in Chapter 2) differed in their reactions. For example, the 66 kDa phosphorylation inhibitory piceatannol could not inhibit the 20 kDa phosphorylation.

The N-terminal sequence analysis of the proteins from the 20 kDa region of the SDS-PAGE, by Edman reaction showed the presence of two distinct sequences. The major protein sequence (ARVAKDGVKVPAEGV) had homology with the large subunit ribosomal L6 protein of *Haemophilus influenzae*, while the minor sequence (MVSLVPADDLPLADV) showed no homology with any known proteins in the NCBI protein data bank. The expression studies indicated that, in room temperature grown cells the 20 kDa phosphorylation activity in the cytoplasm decreased as the cell density increased, thus suggesting that the phosphorylation of 20 kDa protein might have some physiological role during logarithmic growth phase of *P. syringae*.

The phosphotyrosine specific monoclonal antibody also identified another cytoplasmic protein of molecular mass 43 kDa, which was possibly phosphorylated on tyrosine residue. This protein however, could not be phosphorylated by radioactively labelled \([\gamma \text{-} 32\text{P}]\) ATP, *in vitro*.

The N-terminal sequence analysis of the cytosolic 66 kDa phosphoprotein was carried out. The sequence (SLEVQLIKT) showed no homology with any known proteins in the NCBI protein data bank.
The histidine phosphorylated 30 kDa protein of the cytosol was identified as the α-subunit of succinyl CoA synthetase of the bacterium.

The experiments reported in Chapter 5 were attempted to find out the physiological importance of Ptk and Ptpase in the cellular physiology of *P. syringae*. The strategy was to find out the level of expression of the Ptk and Ptpase activities in the cytoplasm of the bacterium, at various phases of growth and at low (4°C) and high (22°C) temperature. The expression of Ptk, as evidenced by the phosphorylation of the endogenous 66 kDa protein and the exogenous peptide substrate PKS1 phosphorylation activity, was highest in the stationary phase than in the exponential phase of growth of the cells. The expression was also found to be higher in the high (22°C) temperature grown cells than in the low temperature (4°C) grown cells. The expression of acidic phosphatases which included the P2 Ptpase activities were also found to increase with the cell density during growth at both temperatures, similar to the expression of the tyrosine kinase in the bacterium.

Since the Ptk and Ptpase of *P. syringae* were found to be inhibited by sodium orthovanadate, studies were carried out to examine the effect of vanadate *in vivo* on the growth of *P. syringae*. The vanadate was found to inhibit the growth of the bacterium at both low (4°C) and high (22°C) temperature. The inhibition by vanadate was more pronounced at the lower temperature of growth (4°C) and at lower cell densities. It is however still not known as to whether the vanadate effect on growth is mediated through the inhibition of the 66 kDa protein phosphorylation, or due to the effect of vanadate on any other components of the growth machinery. Similarly, the effect of piceatannol on the growth of *P. syringae* were assessed at both low (4°C) and high (22 °C) temperatures. The piceatannol inhibited bacterial growth at both the temperatures but the effect on the generation time of the bacterium was more pronounced at the higher temperature (22 °C).
In conclusion:

1. The nature of Ptk of *P. syringae* was biochemically characterised. The major Ptk seems to have an altered ATP binding site and a conserved peptide substrate-binding site when compared with the properties of eukaryotic tyrosine kinases.

2. A Ptpase, which could dephosphorylate the tyrosine phosphorylated 66 kDa protein of the cytoplasm, was purified and characterised. The Ptpase (56 kDa) is an acidic phosphatase with phosphotyrosine specificity.

3. A new 20 kDa protein that gets phosphorylated on tyrosine residue was also identified in the cytoplasm of *P. syringae*. The nature of the 20 kDa protein phosphorylation activity was different from the 66 kDa protein phosphorylation activity. The amount of 20 kDa protein phosphorylation activity was higher in the exponential growth phase of the bacterium.

4. The studies on the expression of Ptk and Ptpase suggested that the levels of expression of these enzymes were dependent on the temperature of growth, and cell density (i.e., growth phase) of the bacterium. Both Ptk and Ptpase were produced more at higher temperature and at stationary phase of growth of *P. syringae*. Vanadate and piceatannol which were found to inhibit the cytosolic Ptk activity had also inhibitory effect on the growth of the bacterium both at low and high temperatures.