Investigating the role of chemotaxis operon genes in agrobacterium tumefaciens

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Investigating the role of chemotaxis operon genes in *Agrobacterium tumefaciens*

By:

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In accordance to the requirements for the degree of PhD

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For my father, my late mother, my wife and

also my little girl, Sana

Declaration

I declare that the work contained within this thesis submitted by me for the degree of PhD is my own work, except where otherwise stated, and has not been submitted previously for a degree at this or any other universities.

The role of chemotaxis operon genes in *Agrobacterium tumefaciens*

Behrouz Harighi

PhD 2003

Abstract:

A 1.7 Kb chromosomal DNA of *A. tumefaciens C58C1* downstream of chemotaxis operon was sequenced completely in both directions. The comparison of this sequence with sequence databases revealed one open reading frame with strong sequence identity to *MCP* gene in other bacteria. The sequencing of chromosomal DNA of *A. tumefaciens C58* confirmed that this open reading frame has similarity with cytoplasmic domain of *McpA*.

Four mutants of *A. tumefaciens C58C1* (C1/delY1, C1/delY2, C1/delB and C1/delR) were created by in-frame deletion mutagenesis in *cheY1*, *cheY2*, *cheB* and *cheR* using pK18mobsacB. Some phenotypic properties of mutants were studied. The *cheY2*, *cheR* and *cheB* mutants showed impaired chemotactic capabilities in both swarming and chemotaxis assays. Deletion of *cheY1* appeared to have no significant effect on chemotaxis, under the conditions studied.

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Abbreviation	
A. tumefaciens	*Agrobacterium tumefaciens*
Amp	Ampicillin
A.S.	3'-5' dimethoxy-4'-hydroxy aceto phenone (Acetosyringone)
Asp	Aspartic acid
ATP	Adenosine tri phosphate
Bp	Base pair
BSA	Bovine serum albumin
CI	Chemotaxis index
CCW	Counter clock wise
CW	clock wise
dH_2O	Distilled water
dNTP	Deoxy nucleotide triphosphate
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
EtOH	Ethanol
FAD	Flavin- adenine dinucleotide
HAP	Hook- associated protein
hr	hour
kDa	Kilo dalton
MCPs	Methyl accepting chemotaxis proteins
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PTS	Phosphotransferase sugar
Rnase	Ribonuclease
Rif	Rifampicin
SDS	Sodium dodecyl sulphate
SSC	Saline sodium citrate
TM	Transmembrane
Tris	[2-amino-2-(hydrosymethyl) propane-1-3-diol]

T-DNA	Transfer DNA
Ti-plasmid	Tumour inducing plasmid
Uv	Ultra violet
Vir	Virulence
X-gal	5-bromo-4-chloro-3-indolyl-β-D- galacto pyranoside
5'	5' terminal phosphate of DNA molecule
3'	3' terminal hydroxyl of DNA molecule

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Chapter1

Introduction

1. Introduction

1.1. Signal transduction system in Bacteria

In bacteria the most common signal transduction system involves phosphoryl transfer and is known as two- component signal transduction. It contains two domains, the receiver and the transmitter domains.

Two-component systems regulate different responses in many different organisms e.g. nutrient acquisition, electron transport systems, plasmid transfer and adaptation to physical or chemical aspects of the environment. It is possible that a single cell may have many two-component systems. In *E. coli*, 178 genes encode products related to regulatory functions, and of those, 62 genes are part of the two-component signal transduction pathways.

In the two-component system, the receiver domain functions as a sensor, usually located in the cytoplasmic membrane, and monitors the environment. The second is a response regulator, which is cytoplasmic and mediates an adaptative response. The sensor component has a kinase function that binds ATP, and when activated, phosphorylates a histidine usually found in the same protein. Furthermore it becomes a substrate for dephosphorylation by the second component.

The second component, the receiver domain, accepts the phosphoryl group from the histidyl-phosphate of the sensor to an aspartate residue. The phosphorylation of the receiver causes a conformational change that regulates the functional state of an output domain to activate specific effector functions such as chemotaxis, flagellar rotation, regulation of transcription virulence, antibiotic resistance or enzymatic catalysis.

Many of the known sensory kinases are transmembrane molecules that combine an external stimulus detection domain and an internal kinase domain into a single molecule. In chemotaxis, however, as discussed later, receptor and kinase function are separated into different molecules.

1.2. Chemotaxis in Bacteria

Chemotaxis, the ability of some bacteria to sense and respond to chemical stimuli in the environment by moving toward attractants (often nutrient sources) and away from repellents (often toxic compounds), is an important survival function for many bacterial species and a frequent feature in bacterial pathogenesis. The process requires the functional activity of some proteins that receive chemical signals and use the information to direct suitable swimming behaviour.

During chemotaxis, for example in enteric bacteria, such as *E. coli*, the chemical environment is sensed through the periplasmic domains of a family of transmembrane chemoreceptors and intracellular signals are produced and transferred to the flagellar motor, through protein-protein interaction, influencing the direction of flagellar rotation and, consequently, cellular movement. The proteins required for chemotaxis in *E.coli* are shown in Fig (1.2.1).

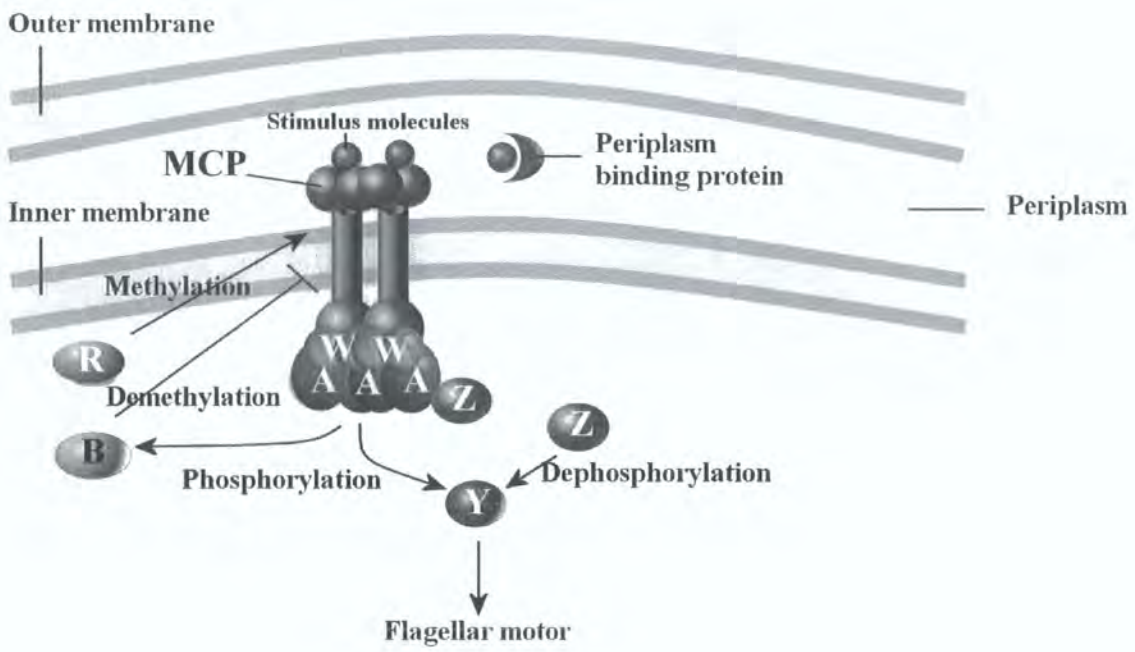

Fig (1.2.1): The classic chemotaxis system in *E.coli*. A: CheA, W: CheW, R: CheR, B: CheB, Y: CheY , Z: CheZ and MCP: Methyl-accepting chemotaxis protein (Figure adapted from: Grebe, T. W. and Stock, J. 1998).

Signal processing and output involve a phosphorelay system. The activity of CheA, a cytoplasmic histidine kinase protein, is affected by its interactions with the chemoreceptors (MCPs). The chemoreceptors bind and sense small molecules or protein ligands and indirectly by conformational changes enhance the activity of the histidine kinase CheA. CheA is inactive when an attractant is bound to the receptor and becomes active when a repellent signal is given. When activated, CheA can autophosphorylate (using ATP) and then transfers the phosphate groups to either of two aspartate autokinases involved in signal output (CheY) and adaptation (CheB). Another protein, CheW couples CheA to chemoreceptor control by physically linking

the proteins in a complex. CheY, the response regulator, catalyses its own acceptance of a phosphate group from CheA on an asparatic acid residue.

The default state of a flagellum is smooth swimming of the bacterial cell in the absence of bound CheY. Only phosphorylated CheY interacts with a flagellum and produces tumbling. CheZ controls the level of phosphorylated CheY by increasing its rate of autodephosphorylation, with the result that tumbles rapidly stop when CheA is turned off (Snezana D., and A. M. Stock, 1998). CheA, CheY, and CheZ usually localize at the cell poles, and clustering is dependent on the presence of MCPs (Sourjik, V., P. Muschler, et al., 2000). CheY and CheZ localization is dependent on CheA, and CheA localization is dependent on CheW (Bourret, R. B. and A. M. Stock, 2002).

Adaptation resets the system by changing the signalling properties of the receptor through reversible methylation. When methylated, the receptor is a more efficient activator of the kinase. Two proteins work together to determine receptor methylation under particular conditions. CheR, a methyltransferase, continuously transfers methyl groups from S-adenosylmethionine molecules to specific sites on the membrane receptor. CheB, a methylesterase, specifically removes methyl groups from the receptor. CheB becomes activated by accepting a phosphate group from CheA and this control largely determines the observed level of methylation at any time (Bren, A. and M. Eisenbach, 2000).

1.2.1. Transmembrane signalling

Most transmembrane signalling is done by chemoreceptors known as methyl-accepting chemotaxis proteins (MCPs). These proteins are components of the

chemotactic response system in Bacteria and Archaea (Grebe, T. W. and J. Stock, 1998). These proteins usually are concentrated and clustered at the cell poles in an evolutionarily diverse range of bacteria and an archea (Gestwicki, J. E., A. C. Lamanna, et al., 2000). MCPs may play a role in the signalling pathway of chemotaxis.

Five closely related MCPs are found in *E.coli*, each between 533 and 553 amino acids in length. These are: Trg, for ribose, galactose and glucose, Tar, for aspartate, Tsr, for serine, Tap, for dipeptides and Aer, which may be a redox detector (Grebe, T. W. and J. Stock, 1998). MCPs are transmembrane proteins with the carboxyl- terminus located in the cytoplasm and the amino-terminus accessible to the external environment (Yost, C. K., P. Rochepeau, et al., 1998). The amino- terminal domain detects attractants and repellents, and carboxyl-terminal domain transfers this information to CheA, allowing the bacterium to swim towards attractants and away from repellents. Serine, aspartate and citrate bind directly to the receptors but maltose, ribose, galactose, glucose and dipeptides bind to the specific periplasmic binding proteins, which then dock with the correct membrane receptors. MCPs also mediate responses to temperature and pH, and serve as receptors for several different repellents. The cytoplasmic domain is very highly conserved between transducers but there is little sequence homology between the periplasmic domains of the different MCPs, therefore they sense different molecules. The cytoplasmic domain contains four or five methylatable glutamate residues, and are therefore called methyl-accepting chemotaxis proteins (Manson, M. D., J. P. Armitage, et al., 1998).

The periplasmic sensing domain is flanked by transmembrane segments, the second of which connect to the cytoplasmic signalling domain through a linker region. The methylation sites responsible for sensory adaptation are located next to the

signalling domain. MCP is known to form stable dimmers, resulting in four transmembrane α-helices, two from each monomer. These are called TM1 and TM2 for one monomer and TM1′ and TM2′ for the second one.

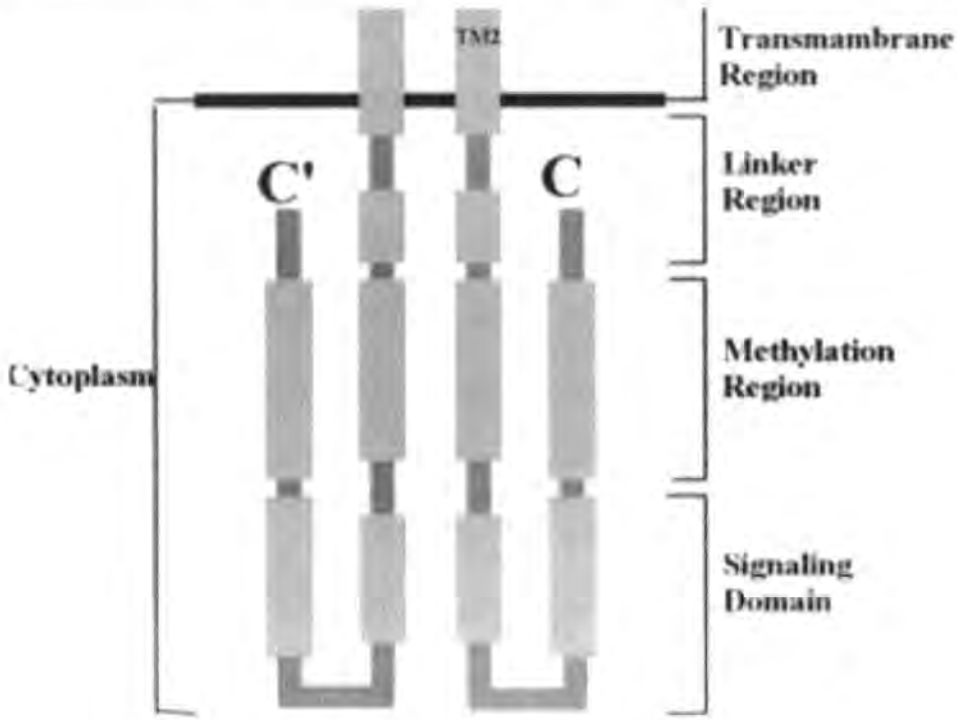

Fig (1.2.2): Schematic model for the cytoplasmic domain of the transmembrane receptor (Figure adapted from: Falke, J. J., et al., 1997).

The receptors are connected using a linker protein, CheW, to a histidine kinase, CheA, generating stable ternary complexes. Active receptors form a supramolecular complex, consisting of about seven receptors, two or four CheW molecules and one CheA dimmer (Bren, A. and M. Eisenbach, 2000).

Methylation and demethylation of homodimeric chemoreceptors such as Tar, Tsr, Trg, Tap and Aer are catalysed by methyltransferases and methylesterases homologous to *E.coli* CheR and CheB (Aravind, L. and C. P. Ponting, 1999). The

pentapeptide sequence Asn-Trp-Glu-Thr-Phe (NWETF), present at the carboxyl terminus of some chemoreceptors (high abundance) gives a docking site for the methyltransferase (Wu, J., et al., 1996) and greatly improves methylation of receptors on which it is present. Receptors naturally lacking the methyltransferase-docking site (low-abundance receptors) are poorly methylated and thus are ineffective, in the absence of high-abundance receptors, in both adaptation and the ability to mediate chemotaxis (Barnakov, A. N., L. A. Barnakova, et al., 1998).

The presence of a docking site on the receptor enhances the rates of demethylation catalysed both by inactivated CheB and by the activated (phosphorylated) enzyme. Phosphorylation of CheB either by free CheA, or by CheA activated in a complex with its receptor, results in increased rates of demethylation for receptors lacking or carrying the docking site (Barnakov, A. N., L. A. Barnakova, et al., 1999).

MCPs are not involved in effector transport, and deletion of any one results in loss of chemotaxis to a few compounds, but no effect on responses to other attractants. The intracellular response of cells to environmental change is mediated by phophorylation and methylation- dependent signalling in a closed system. Transmembrane receptors of extracellular ligands include molecules with histidine kinase or methyl-accepting functions. Homodimeric histidine kinases catalyse transphosphorylation of specific histidine residues and the phosphoryl groups are subsequently transferred to specific aspartyl residues, usually on response regulator domain homologous to *E.coli* CheY.

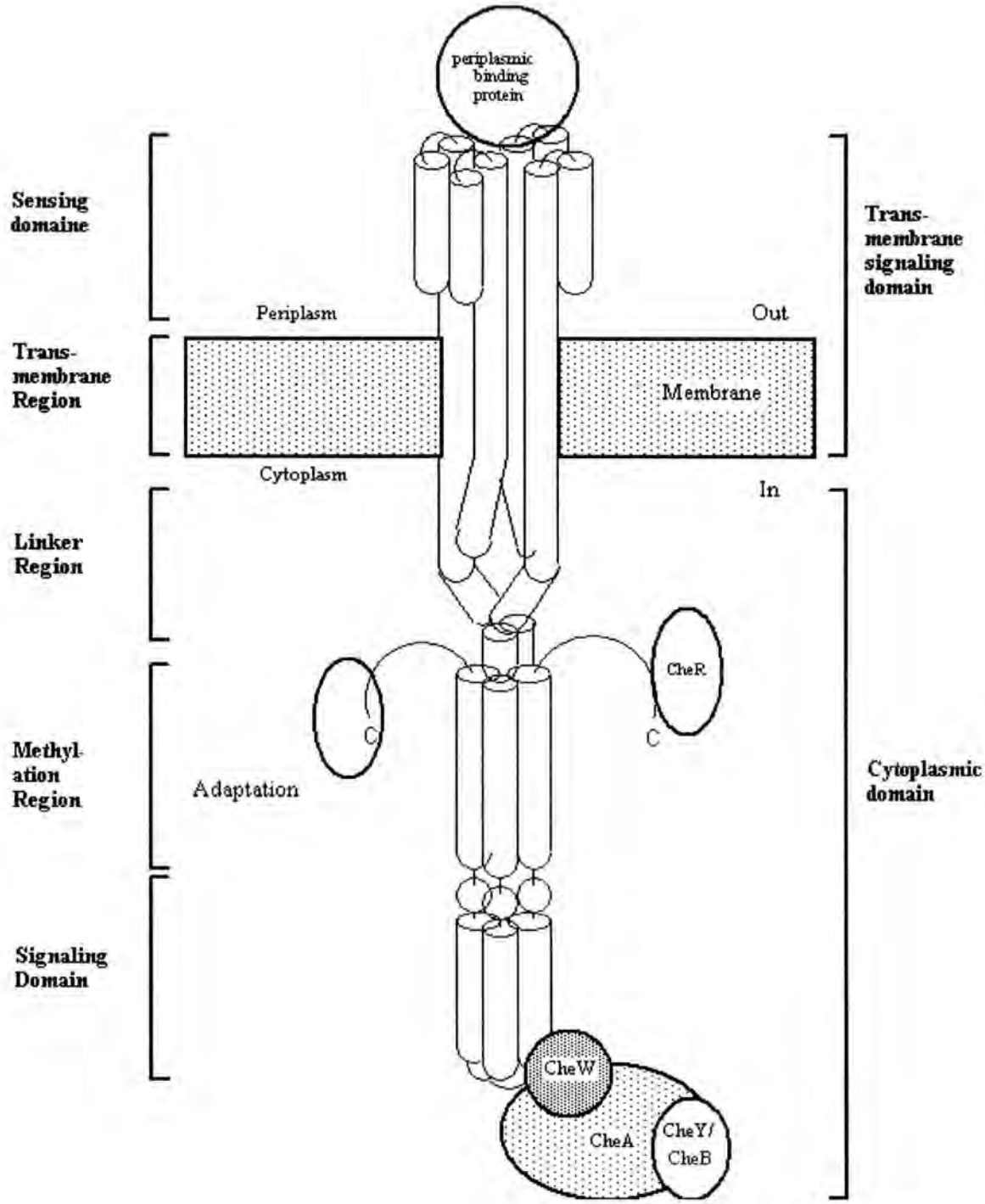

Fig (1.2.3): A typical receptor-kinase signalling complex and protein-protein interaction between receptor and CheA, CheW, CheB and CheR (Figure adapted from: Falke, J. J., et al., 1997).

1.2.2. Cytoplasmic signal transduction

Histidine Kinase CheA: Signals detected by the receptors are used to control the activity of the bound histidine kinase CheA. The isolated kinase is a dimmer of identical 71kDa subunits containing two symmetrical active sites, each of them using Mg^{2+}- ATP to drive phosphorylation of His48 (Surette, M. G., M. Levit, et al., 1996).

Each subunit of CheA can be divided into four functional regions (Parkinson, J. S. and E. C. Kofoid, 1992), at least three of these regions have been shown to be distinct folding domains (Falke, J. J., R. B. Bass, et al., 1997). The N-terminal P1 phosphotransfer domain possesses the reactive His48 residue that serves as the site of autophosphorylation (Morrison, T. B. and J. S. Parkinson, 1994). Autophosphorylation is the most important control point in the chemotaxis signalling pathway. CheA can autophosphorylate itself slowly in the absence of other proteins, but the reaction is accelerated and enhanced several hundred times in the presence of membrane receptors and CheW (Blair, D. F., 1995). The phospho-P1 domain is fully functional as a phosphotransfer substrate for CheY or CheB even when other parts of the CheA are absent (Swanson et al 1993). The P2 response-regulator docking domain, which contains the docking site for CheY and CheB, is C-terminal to the P1 domain (Morrison, T. B. and J. S. Parkinson, 1994). The CheB protein competes with CheY for binding to the P1-P2 region and probably uses the same docking site on the P2 domain (Li et al 1995). Many histidine kinases encode specificity within other domains and lack the P2 domain completely, suggesting that CheA and its homologues have developed this domain for a specific purpose other than phosphotransfer specificity (Parkinson, J. S. and E. C. Kofoid, 1992).

The most highly conserved region of the CheA is its catalytic domain. The CheA catalytic domain folds independently, and the isolated domain can efficiently phosphorylate the isolated P1 domain (Swanson et al 1993). The C-terminal-receptor docking region of CheA is essential for receptor- mediated regulation. Genetic studies suggest that different CheW- and receptor-binding sites exist within this region of CheA (Parkinson, J. S. and E. C. Kofoid, 1992). One possible mechanism of receptor-mediated regulation is suggested by the dimeric structure of CheA, since that enzyme is active only when it is a dimmer (Surette, M. G., M. Levit, et al., 1996).

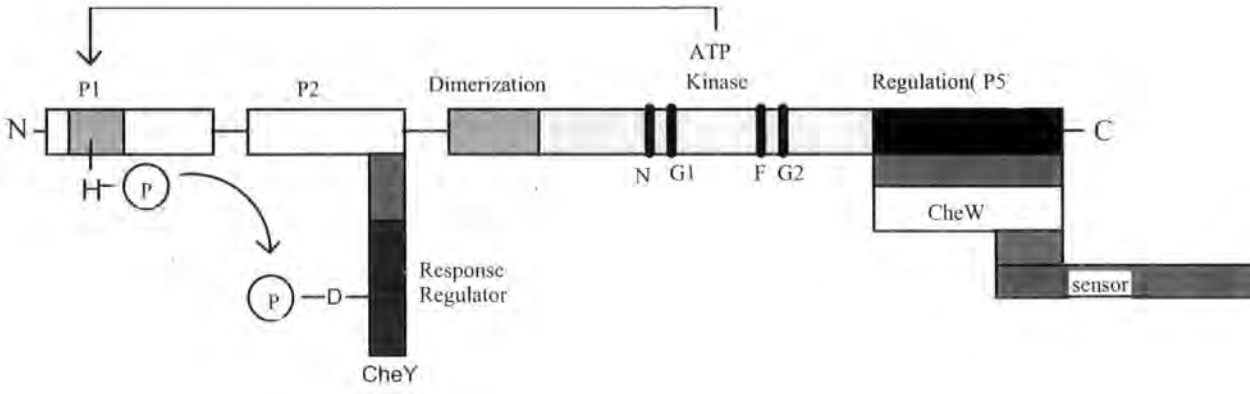

Fig (1.2.4): Domain organisation of the transmitter histidine kinase cheA.

The CheA gene from both *E. coli* and *S. typhimurium* has an alternative translational start site at Met-98 that produces a short variant called CheAs (Stock, J. B. and M. G. Surette, 1996). Bacteria which can carry out chemotaxis express CheA both as the full length and a short form termed CheAs. Both forms have the same C-terminus, but CheAs lacks a sequence of almost 100 residues that contains the P1 domain and cannot be phosphorylated. CheAs is not strictly required for chemotaxis (Sanatinia, H., E. C. Kofoid, et al., 1995) but a 1:1 mole ratio of CheAl to CheAs

provides optimal cellular motility (Wang, H. and P. Matsumura, 1997) and CheAs binds CheY as well as does the full- length CheA protein (Swason et al 1993). Over production of CheAs in wild type cells increased counter clock wise rotation (CCW) (Wang, H. and P. Matsumura, 1996) and this effect is dependent on the presence of CheZ. The CheAs/CheZ complex shows greater dephosphorylating activity on CheY-P in comparison with that observed by the action of free CheZ, alone.

1.2.3. Adaptation pathway

Adaptation, the restoration of the pre-stimulus behaviour in the presence of the stimulus, is a necessary component of chemotactic behaviour. Adaptation resets the system by changing the signalling properties of the receptor through reversible methylation. Two proteins working together to determine receptor methylation under special conditions. CheR is a methyltransferase, which continuously transfers methyl groups from S-adenosylmethionine molecules to specific glutamate sites on the cytoplasmic domain of the receptors during adaptation to positive stimuli. CheB is a methylesterase that removes methyl groups from receptors during adaptation to negative stimuli. This protein also has an amidase activity that catalyzes the conversion of specific glutamine residues of the MCP receptors into glutamate residues (Bren, A., and M. Eisenbach, 2000). The result of this demethylation is the preventation of CheA autophosphorylation and the transient maintenance of a CCW signal (Borkovich, K. A., et al., 1992).

In the chemotaxis system, sensory adaptation is continuous process that enables the cells to make temporal comparisons as they swim about. The adaptation machinery works to cancel recent stimulus responses, so that the organism is ready to

respond to any new changes in chemoeffector concentration. Adaptation is an essential part of chemotaxis because if the receptor can not reset after experiencing a change in receptor occupancy, the receptor would continue to generate a signal and the cell would be unable to respond to future change. Mutation in the adaptation mechanism results in the smooth swimming of the cell.

The relative activities of CheR and CheB determine the methylation level of each MCP species. In the absence of chemical stimuli, about half the sites are methylated. In high attractant or low repellent levels, most sites are methylated, whereas in low attractant or high repellent levels, few sites are methylated.

CheB: The methylesterase CheB is a member of a large and functionally varied family of proteins known as response regulators. These proteins are involved in a wide variety of phosphotransfer-dependent signal transduction pathways found in prokaryotes and eukaryotes (Djordjevic, S., P. N. Goudreau, et al., 1998). Methylesterase CheB (35-kD) functions together with methyltransferase CheR (31-kD) to control and determine the level of methylation of a set of four or five glutamate residues in the cytoplasmic domains of the chemoreceptors (Djordjevic, S., P. N. Goudreau, et al., 1998).

The level of receptor methylation is controlled both globally and locally. Global control includes the change of CheB activity by CheA, CheW, and the receptors. The level of methylation is also controlled locally by the conformation of the receptors themselves. The binding of attractant to a receptor increases the level of methylation of that receptor more than others, presumably by inducing a conformational change that alters the exposure of methylation sites to CheR and CheB.

Regulation of CheB involves many protein-protein interactions. There are three known sites of interaction between CheB and the receptor-CheA-CheW complex: the kinase site at which CheB obtains a phosphate group, the sites at which the enzyme modifies the receptor and the pentapeptide docking site at the carboxyl terminus of the receptor.

CheB has amidase activity and converts the glutamines to glutamates which can subsequently to take part in the methylation/demethylation cycle catalysed by CheR and CheB. CheB, like CheY, is a response-regulator protein with an activity controlled by phosphorylation. CheB has a two-domain architecture, with an N-terminal regulatory domain homologous to CheY and a C-terminal effectors domain with amidase/esterase activity. Structural similarity between the two CheB and CheR suggests an evolutionary and / or functional relationship.

As the autophosphorylating histidine kinase CheA is acting as a phosphoryl donor protein to two response regulators, CheY and CheB. CheY and CheB compete for binding to the P2 domain of CheA and when band, phosphorylation of the N-terminal domain of the intact CheB protein results in enhanced methylesterase activity. The N-terminal domain plays two regulatory roles, functioning to inhibit methylesterase activity when unphosphorylated and to stimulate activity when phosphorylated. The interaction between methylesterase CheB and the chemotaxis receptors is complex and probably contains multiple regions of the CheB molecular surface. There is no phosphatase to increase the rate of CheB dephosphorylation and the protein therefore has developed a more rapid rate of autocatalytic dephosphorylation than CheY-P. Mutants deleted for CheB have over methylated receptors and, as a result, smooth swim constantly.

CheR: Methyltransferase CheR, which utilizes S-adenosyl-methionine as the methyl donor, catalyses S-adenosyl-methionine (AdoMet)- dependent methylation of the γ-carboxyl groups of specific glutamate residues in the chemotaxis receptors. CheR is a two- domain protein with an N-terminal domain that appears to be involved in substrate recognition, and an α/β C-terminal domain that contains sequences typical of AdoMet- dependent methyltransferases.

In the cell, two separate domains of the MCP receptor are involved in the interaction with CheR: a binding domain onto which CheR docks and a domain that is methylated by CheR. Mutants deleted for CheR cannot methylate the receptors and constantly tumble. CheR binds to major chemoreceptors through their C-terminal motif NWETF, which differs from the C-terminal motif in methylation sites (Shiomi, D., H. Okumura, et al., 2000).

1.2.4. Characteristics of other Che proteins:

CheW: The 18 kDa CheW protein couples CheA to the MCP receptor and is required for receptor- mediated activation of histidine kinase activity (Ames, P. and J. S. Parkinson, 1994). In *E.coli*, CheW is an essential protein necessary for MCP-dependent chemotaxis and interaction with the signalling domain of Tsr and the carboxy-terminus of CheA, suggesting a role as a linker protein between the sensor (MCP) and the CheA (Liu, J. D. and J. S. Parkinson, 1991; Bourret, R. B., J. Davagnino, et al., 1993). Neither CheW nor Tsr alone has a significant effect on CheA activity; however all three proteins must be together for activation to occur (Ninfa, A. J. and R. L. Bennett, 1991).

Evidence for the formation of complexes between MCP, CheW and CheA has been identified by immunoelectron microscopy and immunofluorescence light microscopy of *E. coli.* The complex is frequently found in clusters at one or both poles of the cells. In mutant strains where one of MCP, CheW or CheA is absent, the polar clustering of the remaining components is dramatically reduced (Maddock, J.R., and Shapiro, L., 1993).

No catalytic or regulatory activity has been associated with CheW, although Sanders et al. have shown that over-expression of CheW produces a phenotype similar to its deletion (Sanders et al., 1989). In-vitro experiments showed that the kinase activity of CheA was greatly reduced in the presence of high levels of CheW (Ninfa, A. J. and R. L. Bennett, 1991). CheW of *R. sphaeroides,* when cloned into *E. coli,* induced changes in the switching frequency of the flagellar motor of bacterial cell. Over-expressed CheW reduced the switching frequency. Smooth swimming results from a reduction in the level of CheY-P and this suggests that CheA was sequestered by excess CheW in an inactive form (Hamblin, P. A., N. A. Bourne, et al., 1997).

In *E. coli*, deletion of *cheW* results in smooth- swimming and non-chemotactic behaviour (Liu, J. D. and J. S. Parkinson, 1991). In contrast, deletion of *cheW1* in *R. sphaeroides* has no significant effect on chemotactic behaviour when analysed using swarm plate and plug plate chemotaxis assays (Hamblin, P. A., N. A., et al., 1997).

CheY: CheY, a 14kDa polypeptide, is the response regulator protein which serves as a phosphorylation- dependent protein in the bacterial chemotaxis signal transduction pathway. CheY has a special and important place in the chemotaxis pathway because this molecule is the diffusible component that acts as the signal

between the two membrane-localized supramacromoleculear complexes; namely, the chemotaxis receptor clusters that are localised at the two poles of the cell and usually the six to eight flagellar basal bodies that are uniformly distributed (Djordjevic, S. and A. M. Stock, 1998). CheY binds to the P2 domain of CheA and phosphate is transferred from His-48 in a reversible reaction which phosphorylates CheY. Phospho-CheY (CheY-P) diffuses through the cytoplasm and binds to FliM, one of an assembly of proteins called the 'switch' at the base of the flagellar motor. This binding event results in the change of direction of the flagella from the counter clockwise (CCW) to clockwise (CW) rotation.

CheY can also be phosphorylated by small phosphodonors such as acetyl phosphate. The rate of phosphorylation of CheY by small phosphodonors is much lower than that of CheA-mediated phosphorylation (Mayover, T. L., C. J. Halkides, et al., 1999). This CheA-independent phosphorylation indicates that CheY can catalyse its own phosphorylation. Phosphorylation of CheY results in a conformational change that is necessary for activation (Silversmith, R. E. and R. B. Bourret, 1999) and not only reduces the affinity of the CheY to CheA, but also improves its affinity for the protein FliM (McEvoy, M. M., A. Bren, et al., 1999). Even though mutational analysis and NMR data have identified three distinct regions on CheY that interact with its three protein effectors (P2 of CheA, FliM and CheZ), and all are distinct from the phosphorylation site, it can bind to only one protein at a time because the C-terminal portion of CheY is involved in the binding to all these proteins.

Dephosphorylation of CheY-P occurs through autophosphatase activity but the rate of autodephosphorylation is increased by CheZ in enteric species (Eisenbach, M. 1996). CheZ binds CheY in a phosphorylation-dependent process, and the CheY-binding domain is located at the C-terminus of CheZ (Blat, Y. and M. Eisenbach,

1996). The phosphatase activity of CheZ is modulated and occurres only after a chemotaxis response is established.

CheZ: CheZ protein acts to inactivate the tumble signal of the chemosensory pathway by increasing the hydrolysis of phospho-CheY, either directly using a CheZ active site residue to carry out a nucleophilic attack, or indirectly through a conformational change that stimulates the intrinsinc auto-phosphatase activity of the CheY active site (Hess et al 1988). Only phospho-CheY binds to the CheZ protein, and after dephosphorylation CheY is separated from CheZ (Blat& Eisenbach 1994). CheZ can be isolated as a dimmer of 24-kDa subunits. It also forms mixed oligomers with CheAs (but not with the full-length of CheA) that enhance CheY phosphatase activity (Wang, H. and P. Matsumura, 1996, 1997).

CheZ has also been isolated as a high-order oligomer with phospho-CheY but not CheY, and it appears to compete with FliM for CheY-P but not CheY (Blat, Y. and M. Eisenbach, 1996). The inability of CheZ to dephosphorylate FliM-bound CheY-P may be the result of its inability to bind to CheY-P when the latter is bound to FliM (Bren, A., et al., 1996). This protein, like all the other cytoplasmic chemotaxis proteins, can be attached to the receptor supermolecular complex. CheZ homologues have not been found in species outside the γ-subgroup.

CheC, CheD and CheV: These three proteins are absent from *E. coli* and their functions are restricted to some bacteria, such as *Bacillus subtilis*. CheV, has amino acid sequence similarity to CheW and CheY in N-terminal and C-terminal domains, and functions in both adaptation and receptor coupling (Rosario, M. M. L., J. R. Kirby, et al., 1995). CheC and CheD both seem to function in MCP methylation,

but the two proteins appear to act independently (Kirby, J.R., 2001). CheC interacts with flagellar switch components and dissociates when CheY-P binds. Subsequently this protein interacts with the receptor complex to enhance adaptation (Kirby, J. R., C. J. Kristich, et al., 2001).

Homologues of the CheD protein of *Bacillus subtilis* have been found in a large number of bacteria, and it seems to play important role in chemotaxis. CheD catalyzes the amide hydrolysis of the *B. subtilis* chemoreceptor McpA, and also deamidates other *B. subtilis* chemoreceptors such as McpB and McpC. CheD mutant cells do not respond to most chemoattractants and deamidation by CheD is required for *B. subtilis* chemoreceptor to transduce signals to the CheA kinase (Kristich, C. J. and G. W. Ordal, 2002).

1.2.5. Chemotaxis towards phosphotransferase sugars (PTS taxis)

Chemotaxis to sugars transported by the phosphoenolpyruvate phosphotransferase system exhibits a mechanism intermediate between chemotaxis and metabolism-dependent energy taxis. This system is independent from MCPs. It is formed from enzyme I (EI), phosphohistidine carrier protein (HPr) and several membrane-bound sugar specific proteins (enzymeII, EII). During transfer by EII the incoming sugar is phosphorylated by a phosphate group accepted from phosphoenolpyruvate (PEP) and signalled by HPr and EI. The phosphorylation of EI regulates autophosphorylation of CheA (Lux, R., K. Jahreis, et al., 1995). To cause a signal the sugar must be transported but methylation is not necessary and only CheY and CheA in combination with CheW are necessary (Postma, P. W., et al., 1993).

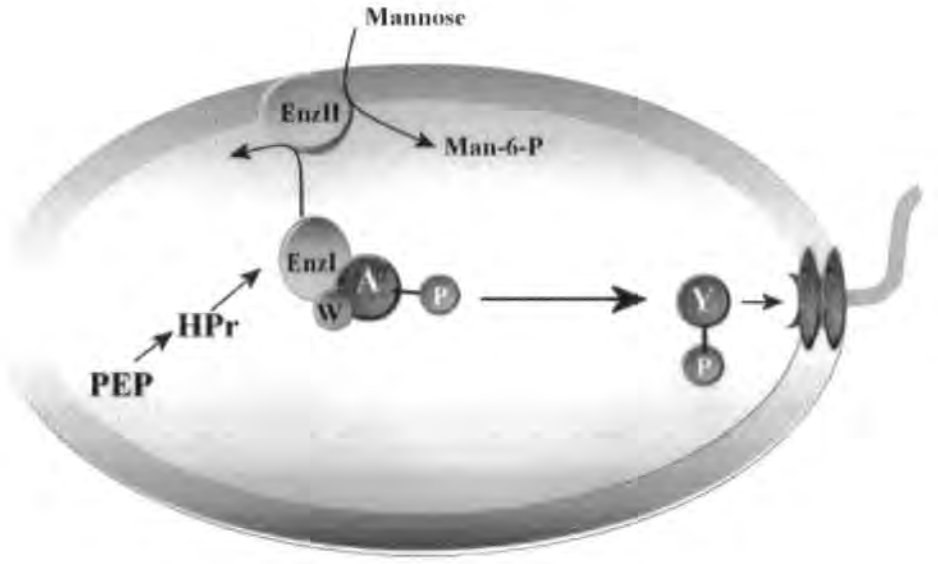

Fig (1.2.5): Diagram showing the signal transduction pathway in *E. coli* for phosphotransferase (PTS) taxis (Figure adopted from: Taylor, B. L., et al., 1997).

1.2.6. Other sensing behaviour in Bacteria (Energy taxis)

In contrast to classical chemotaxis behaviour, in which sensing of stimuli is independent of cellular metabolism, some bacteria monitor their cellular energy levels and in response to a decrease in energy level swim to a new environment that re-energizes the cells. Aerotaxis (taxis to oxygen), phototaxis and taxis to alternative electron acceptors share a common signal transduction pathway, and use some components of classical chemotaxis as well.

1.2.6.1. Aerotaxis

Aerotaxis is a response to changes in respiratory electron flow that results from an increase or decrease in oxygen concentration. In the absence of oxygen, alternative electron acceptors such as fumarate or nitrate support electron flow. In the presence of air the preferred acceptor (oxygen) is used. A transducer senses the change in electron transport and establishes a signal that changes the direction of flagellar rotation and swimming.

The Aer and Tsr proteins in *E. coli* are recognized as transducers for energy taxis. Aer is homologous to *E. coli* chemoreceptors but in other aspects it is unique because it has a PAS domain and flavin-adenine dinucleotide (FAD) cofactor that interacts with a component of the electron transport system.

The *cheA*, *cheW* and *cheY* genes of the chemotaxis operon are essential for aerotaxis, since the aerotaxis transducer (Aer) regulates the CheA histidine kinase. The sensing domain of Aer is a PAS domain that contains the flavin-adenine dinucleotide (FAD) binding site. In addition, the Aer transducer has two cytoplasmic domains anchored to the membrane by one central hydrophobic sequence. All known PAS domains are located in the cytoplasm.

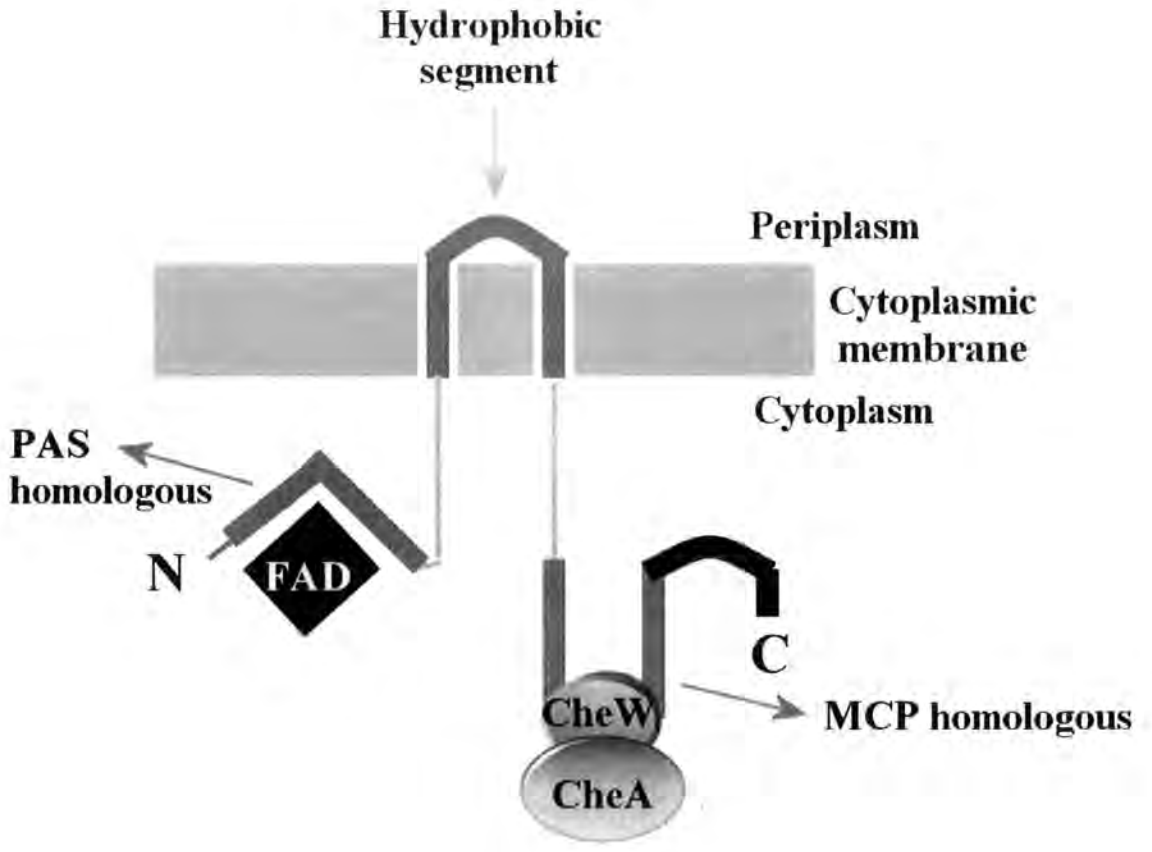

Fig (1.2.6): Suggested model of Aer structure, domain organisation and protein-protein interaction during aerotaxis (PNAS: 2000:97:11:5830-5835).

During signal transduction by Aer, oxidation and reduction of FAD produces the on and off signals for aerotaxis. The PAS domain interacts with a component of the electron transport system. During an increase or decrease in oxygen concentration the redox changes in FAD reflect redox changes in the electron transport system. Subsequently the PAS domain signals the redox status of FAD to the highly conserved signalling domain in the C-terminus. In the presence of CheW, this highly conserved domain regulates the histidine kinase activity of CheA.

The serine receptor Tsr in *E. coli* is also a transducer for aerotaxis. Tsr is a multifunctional protein, in addition to detecting serine and energy, it senses temperature and the repellents leucine, indole and weak acids. The transduction mechanism of Tsr in aerotaxis is unknown. Unlike Aer, Tsr has no known cofactor and some evidence suggests that it has a role in the adaptation pathway, a process that does not occur in Aer-mediated aerotaxis.

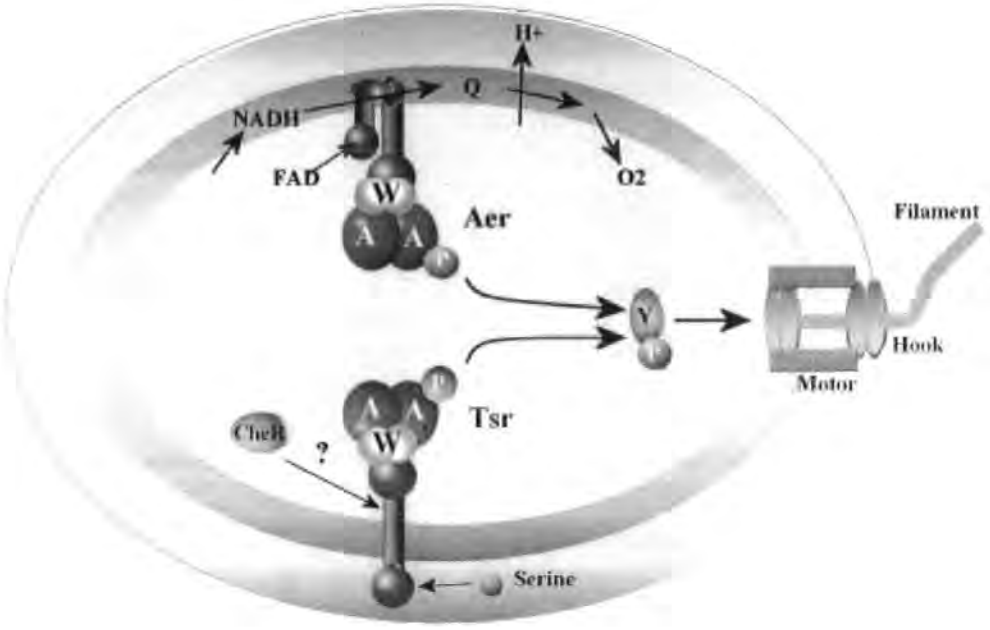

Fig (1.2.7): Scheme showing the signal transduction pathway in *E. coli* for aerotaxis and chemotaxis mediated by Tsr chemoreceptor (Figure adopted from: Taylor, B. L., et al., 1997).

1.2.6.2. Phototaxis

Some bacteria responses to changes in light intensity and colour using the sensory photoreceptors, rhodopsin I and II (SRI and SRII) (Hoff, W. D., K. H. Jung, et al., 1997). Light activated SRI and SRII transmit signals to their specific

transducers, HtrI and HtrII. The Htr proteins contain two transmembrane helices, cytoplasmic methyl-accepting and histidine-kinase- activating domains (Rudolph, J. and D. Oesterhelt, 1995) that are homologous to domains in the chemotaxis transducers of *E. coli*, Tsr and Tar.

There is a specific interaction between SRI and HtrI and between SRII and HtrII. This specificity seems to be encoded in the transmembrane portion of the transducers (Zhang, X. N., et al., 1999) and it has been suggested that Htr transducers physically and functionally interact with their sensory rhodopsins within the membrane (Spudich, J. L., 1998), which results is the control of the flagellar motor switching through a cytoplasmic phosphoregulator.

The mechanism of responding to light in *Halobacterium salinarum* is the best understood system. The two sensory rhodopsins give the bacterial cells the ability to respond to light as an attractant or repellent depending on its colour and the stage of growth of the cells. A change in the oxygen concentration and light wavelength prevent SRII production or produce more of SRI that subsequently induce the production of another two rhodopsins, the transport rhodopsin BR (a proton pump) and HR (a chloride pump). SRI and SRII, in combination with BR and HR functions, transmit signals to HtrI and HtrII through membrane spanning α-helices and the conformation of the signalling domains controls the activity of the CheA and CheY and subsequently control the motor switching function (Armitage, J. P., 1999, Spudich, J. L., 1998).

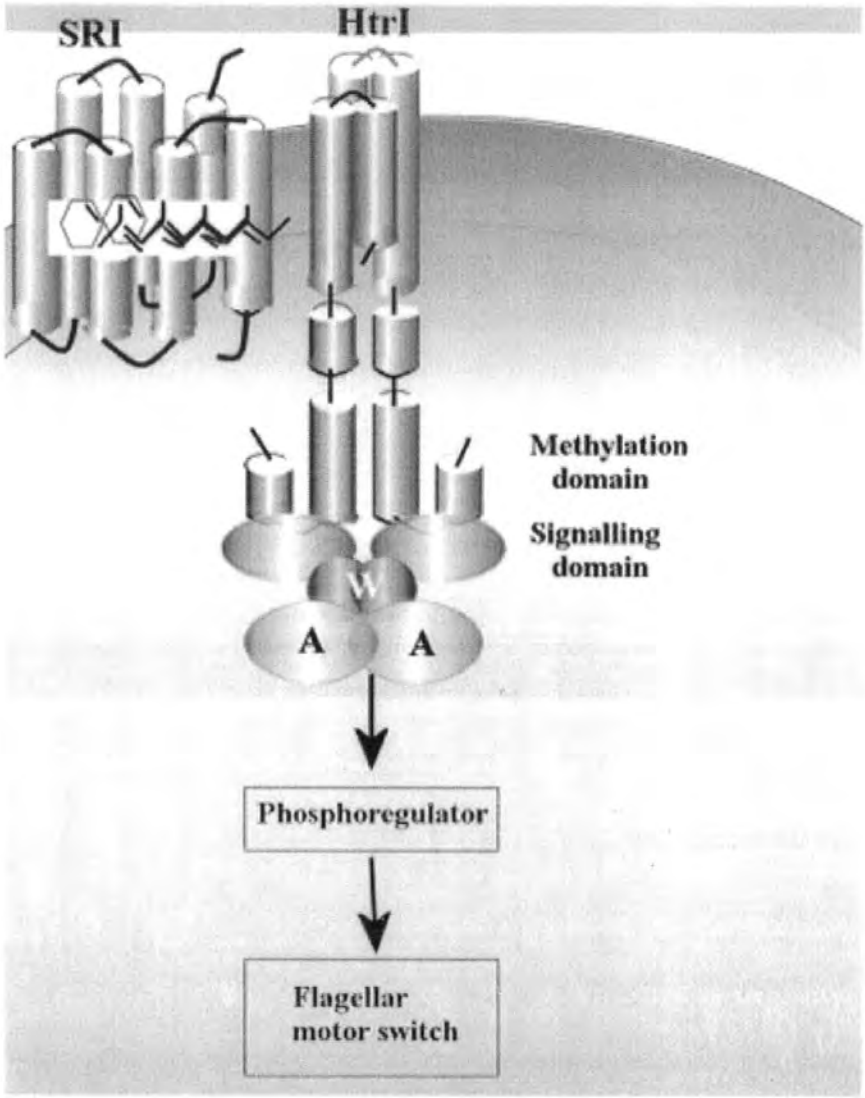

Fig (1.2.8): The schematic figure of phototaxis pathway in *Halobacterium salinarum*, with location of sensors and protein-protein interaction (Figure adapted from: Spudich, J. L., 1998).

1.3. Motility in bacteria

Responses to a variety of environmental information or signals are very well developed in bacteria, and result in suitable movement in response to this information (Macnab, R. M., 1996).

Various styles or types of motility are recognized but most of them are based on the rotation of rigid, extracellular and helical flagellar filaments, driven by a rotary motor fixed in the cell envelope (Berry, R. M. and J. P. Armitage, 1999). Because the filament is normally a left-handed helix, rotation in the counter clockwise (CCW) sense causes the helical wave to travel from proximal to distal and to exert a pushing motion on the cell. Sometimes a tumbling form of motility occurs when the filaments are rotated in the opposite direction, clockwise (CW), when the helical wave travels from distal to proximal. The situation is made possible by structural changes that take place in the filament (Macnab, R. M., 1977).

In *Salmonella*, for example, there are currently 44 known flagellar genes. Twenty-three of these genes encode structural components of the flagellum. Of these components, five (MotA, MotB, FliG, FliM and FliN) are needed for torque generation and three of these five (FliG, FliM and FliN) are also needed for switching. The principal remaining components are the filament, the hook, and the basal body. The basal body has three parts: rod, MS ring and LP ring.

1.3.1. The flagellar structure

With some small differences, the flagella from most bacterial species are built in the same basic ways. The proteins that form most parts of the flagellum have been

identified by electron microscopic studies, genetic and biochemical characterization (Blair, D. F., 1995). The flagellum is formed from a helical filament connected via the hook to the basal body, which is surrounded by several rings of torque-generating particles in the cytoplasmic membrane (Berry, R. M. and J. P. Armitage, 1999). The filament is the propeller, and the basal body and torque- generating particles together are the motor. Within the motor, the basal body is the rotor and rotates relative to the anchored torque- generating particles or stator.

1.3.1.1. The filament

The filament has variable length (5-10μm) but it has a constant diameter of about 20nm through its length (Namba, K., I. Yamashita, et al., 2000). It is built from more than 1000 copies of a single protein, flagellin. The flagellin subunits are synthesized in the cytoplasm, transferred to the outside, and arranged at points on a cylindrical or tubular pattern.

1.3.1.2. The Hook

The filament is connected to the cell by the hook. The structure of hook is very similar to the structure of the flagellum but it is built from a different subunit, called the hook protein (FlgE) (Macnab, R. M., 1996). Three hook-associated proteins (HAP) have been identified in *E. coli*. HAP1 and HAP3 are placed between the hook and filament but HAP2 make a cap at the top of the filament. The hook is flexible and reflecting it is role as a universal causing flagella to come together as a bundle (Berry, R. M. and J. P. Armitage, 1999).

1.3.1.3. Basal body

The hook is connected to a complex structure known as the basal body, which is embedded in the cell wall surface. It is consists two rings (L and P) in a position equivalent to the outer membrane (the L ring is in the Lipopolysaccharide membrane, the P ring is in the peptidoglycan layer), two rings (S and M) in the position of the cytoplasmic membrane and a central rod that link together the rings and the hook.

The basal body complex made up from at least 8 proteins, four of them in the rod (FlgB, FlgC, FlgF, and FlgG), three in the rings (FlgH, FlgI, and FliF forming the L, P, and MS rings respectively) and one whose location is not known (FliE) (Macnab, R. M., 1996).

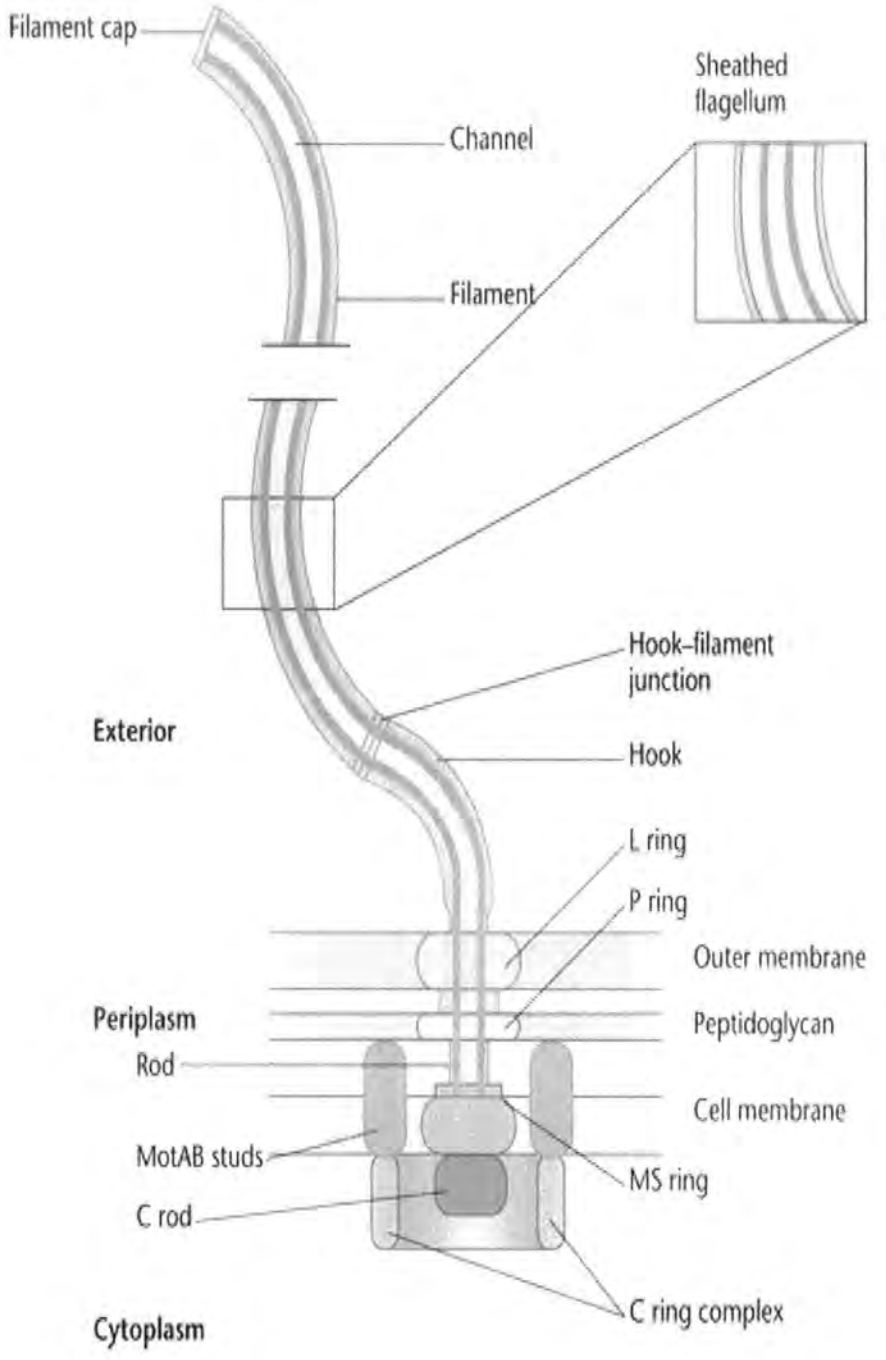

Fig (1.3.1): Schematic structure of the bacterial flagellum (Figure adapted from: Morgan, D. G., and S. Khan, 2001).

1.3.2. Flagellar assembly

Assembly of the flagellum begins with components, such as the rotary motor, (which are closest to the bacterial surface) and ends with the filament (the most distal part of it) (Macnab, R. M., 2000). It is normally starts with the formation of the MS ring, formed from single protein called FliF. At the next step the function of at least four genes (*flgB*, *flgC*, *flgF* and *flgG*) are needed to form the basal-body rod.

The flagellar genes are clustered in three (*E. coli*) or four (*S. typhimurium*) regions on the chromosome. The genes are named *flg*, *flh*, *fli* and *flj* according to the chromosomal region in which they are found. Expression of the flagellar genes is controlled by a regulatory hierarchy that has three levels. Level 1 has two genes (*flhC*, *flhD*) that are required for the expression of level 2 genes, and level 2 genes are required for expression of level 3 genes. Level 1 genes are under control of cAMP (cyclic- AMP) levels and other factors linked to the cell cycle.

Many of the level 2 genes encode components of the basal body and level 3 genes encode components of the filament that are added in the later steps or encode the Che and Mot proteins (Macnab, R. M., 1996).

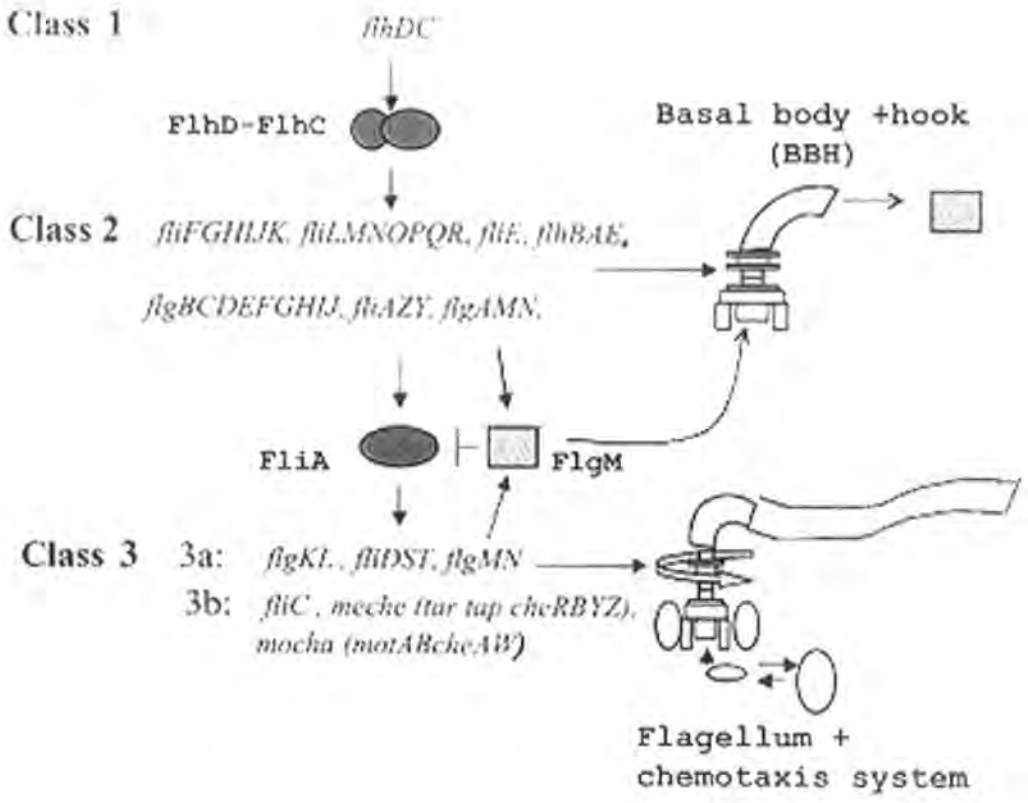

Fig (1.3.2): The genetically defined sequence of flagellar assembly in *E. coli* (Figure adopted from: Kalir, S. et.al. 2001).

1.3.3. Mot proteins

Two proteins, MotA and MotB, are integrated into the cell membrane and are necessary for motor rotation. Electron microscopic studies suggested that they form a ring of proteins surrounding the MS ring of the basal body (Khan, S., M. Dapice, et al., 1988).

1.3.3.1. Motor function and torque generation

As mentioned above, some studies indicate that MotA and MotB interact with each other via their periplasmic domains and both of them form the complete stator (Garza, A. G., L. W. Harrishaller, et al., 1995). Each flagellar motor contains several MotA/MotB complexes that surround the MS-ring. The Fli complex is attached to the face of the M-ring protein on the base of the flagellum, and contains of three proteins, FliG, FliM and FliN. Genetic studies have shown that only FliG, MotA and MotB are directly involved in torque generation.

The mechanism of flagellar rotation involves electrostatic interactions between the rotor and stator (Zhou, J. D., S. A. Lloyd, et al., 1998). Regarding to motor function, studies on *motA* mutants have shown that MotA, possibly in association with MotB, acts as a proton translocating protein (Zhou, J. D. and D. F. Blair, 1997), which forms a proton channel anchored to the cell wall by MotB. Proton flow through the inside of this channel produces the torque or rotation (Bren, A. and M. Eisenbach, 2000).

MotB contains a conserved aspartic acid, Asp32, that is essential for rotation. According to a current hypothesis the protonation of Asp32 in MotB produces a conformational change that affects a cytoplasmic domain of MotA (containing a residue known to interact with the rotor), which works on the rotor to drive rotation (Kojima, S. and D. F. Blair, 2001).

This model provides potential mechanistic models of switching from one direction of rotation to the other, but the mechanism of signal propagation within the switch subsequent to CheY~P- FliM binding is not yet fully known.

Phosphorylated CheY (CheY~P) diffuses through the cytoplasm and binds to switch. FliM appears to be the major target of CheY~P interaction. It appears that CheY~P binding to FliM is essential but insufficient for the generation of CW rotation.

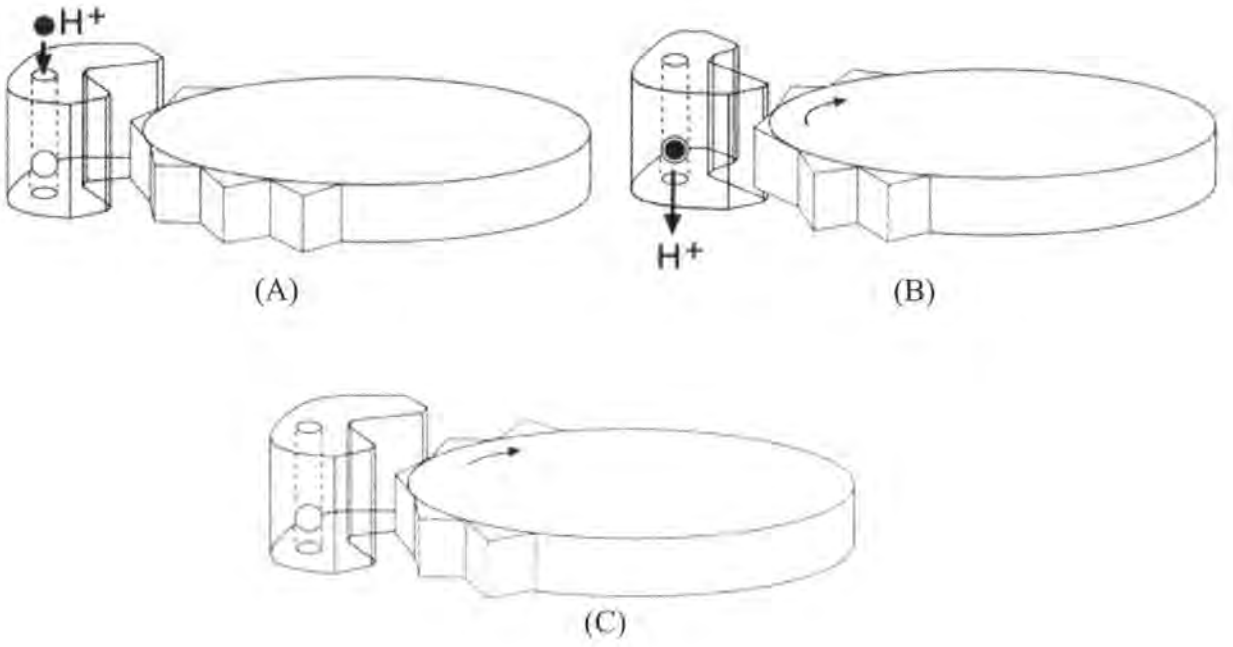

Fig (1.3.3): Suggested model of stator conformational changes in the generation of torque by the flagellar motor. Asp32: Asparatic acid 32, H+: Proton (Figure adapted from: Kojima, S., and D. F. Blair, 2001).

1.3.4. The switch complex

Three genes were identified in *E.coli*, named *fliG*, *fliM* and *fliN*, that are expressed in early steps of the flagellar biosynthesis. The resulting products are the

proteins FliG, FliM and FliN which are located in cytoplasm, and together comprise a gearbox, termed the "switch complex", because certain mutations in these proteins affect the switching between CW and CCW rotation. This structure is located on the cytoplasmic face of the MS-ring, and is the element of the supramolecular complex onto which CheY~P docks, and which determines the direction of flagellar rotation.

All three genes have diverse roles in producing four distinct mutant phenotypes: (a) non-flagellate (*fla*) (occurring at three loci *flg*, *flh* and *fli*) where the flagellar structure is incomplete, (b) paralysed (Mot-), where the flagellum is completed but does not rotate, (c) smooth-swimming[che-(ccw)] and (d) tumble[che-(cw)] where the flagellum is made and rotates but does not reverse with suitable frequency, therefore chemotaxis does not occurr (De Rosier, D. J., 1998). Thus, these proteins are important for the mechanism of rotation as well as for controlling its direction. Analysis of mutants suggests that FliM is the most important, central component of the switch and is the target for binding of the switch regulator, CheY~P. The primary function of FliN is flagellum- specific export rather than torque generation. The characteristics of FliG mutants suggest that it could be involved in torque generation.

1.4. The α- subgroup of *Proteobacteria*

1.4.1. The family of *Rhizobiaceae*

The members of this family, *Rhizobium (Sinorhizobium), Agrobacterium*, *Bradyrhizobium* and *Pyhllobacterium*, usually have one polar or subpolar flagellum and 2-6 peritrichous flagella. All species except *Agrobacterium*, in association with plants and during the symbiosis procedure, incite cortical hypertrophies on plants. Nodules are produced on roots of *Leguminous* species and on leaves of certain plants in the family of Rubiaceae by strain of *Phyllobacterium.*

The nitrogen-fixing group usually subdivided into two groups: The fast growing genus e.g. *Rhizobium* and the slow growing genus the same of *Bradyrhizobium*. Two further genera have recently been described, *Azorhizobium* and *Sinorhizobium*. Some of the *Rhizobiaceae* affect plant development and cause plant disease. Examples are *Agrobacterium tumefaciens* and *A. rhizogenes* that produce crown gall and hairy root on plants.

1.4.2. Genus *Agrobacterium tumefaciens* (descriptive information)

This bacterium is bacilliform, 0.6- 1.0 μm by 1.5- 3.0μm, gram negative and motile by flagella circumthecally arranged, near one end rather than ringing the middle portion of the cell. Colonies are usually convex, circular, smooth and non-pigmented. Members of this genus invade the crown, roots and stems of the great variety of dicotyledonous and some monocotyledonous plants, via wounds, causing

the transformation of the plant cells into autonomously proliferating tumour cells. The induced plant diseases are commonly known as crown gall, hairy root and cane gall.

Tumour induction by *Agrobacterium* is correlated with the presence of a large tumour- inducing plasmid (Ti- plasmid). *Agrobacterium* species are soil inhabitants. The type species is *Agrobacterium tumefaciens*.

1.5. Sensing behaviours in α–subgroup of *Proteobacteria*

1.5.1. Chemotaxis in α- subgroup of *Proteobacteria*

The genetic organisation of the chemosensory genes of several members of this subgroup has been characterised and found to be very similar (Greck, M., J. Platzer, et al., 1995, and Ward, M. J., A. W. Bell, et al., 1995). The organisation is very different from that seen in *E. coli* (Armitage, J. P. and R. Schmitt, 1997). In all members of this subgroup at least two copies of the *cheY* genes have been found, but no *cheZ* genes.

1.5.1.1. Chemotaxis in *Sinorhizobium meliloti* (formerly named *Rhizobium meliloti*):

The chemotaxis genes are part of a large operon containing three novel open reading frames (*orf1*, *orf2* and *orf9*) and the six familiar che genes, *cheA*, *cheW*, *cheR*, *cheB*, *cheY1* and *cheY2*. *Sinorhizobium meliloti* has chemotaxis behaviour different from *E. coli*. In this bacteria *cheY1* and *cheYII* have different role. *cheYI* alone does not mediate chemotaxis in the wild type but it is necessary for full tactic response.

Furthermore both CheYII and CheYI require phosphorylation by CheA for activity. CheYII is the main response regulator directing chemokinesis. In comparison CheYI has a small role in chemokinesis, but interferes with smooth swimming (Sourjik, V. and R. Schmitt, 1996).

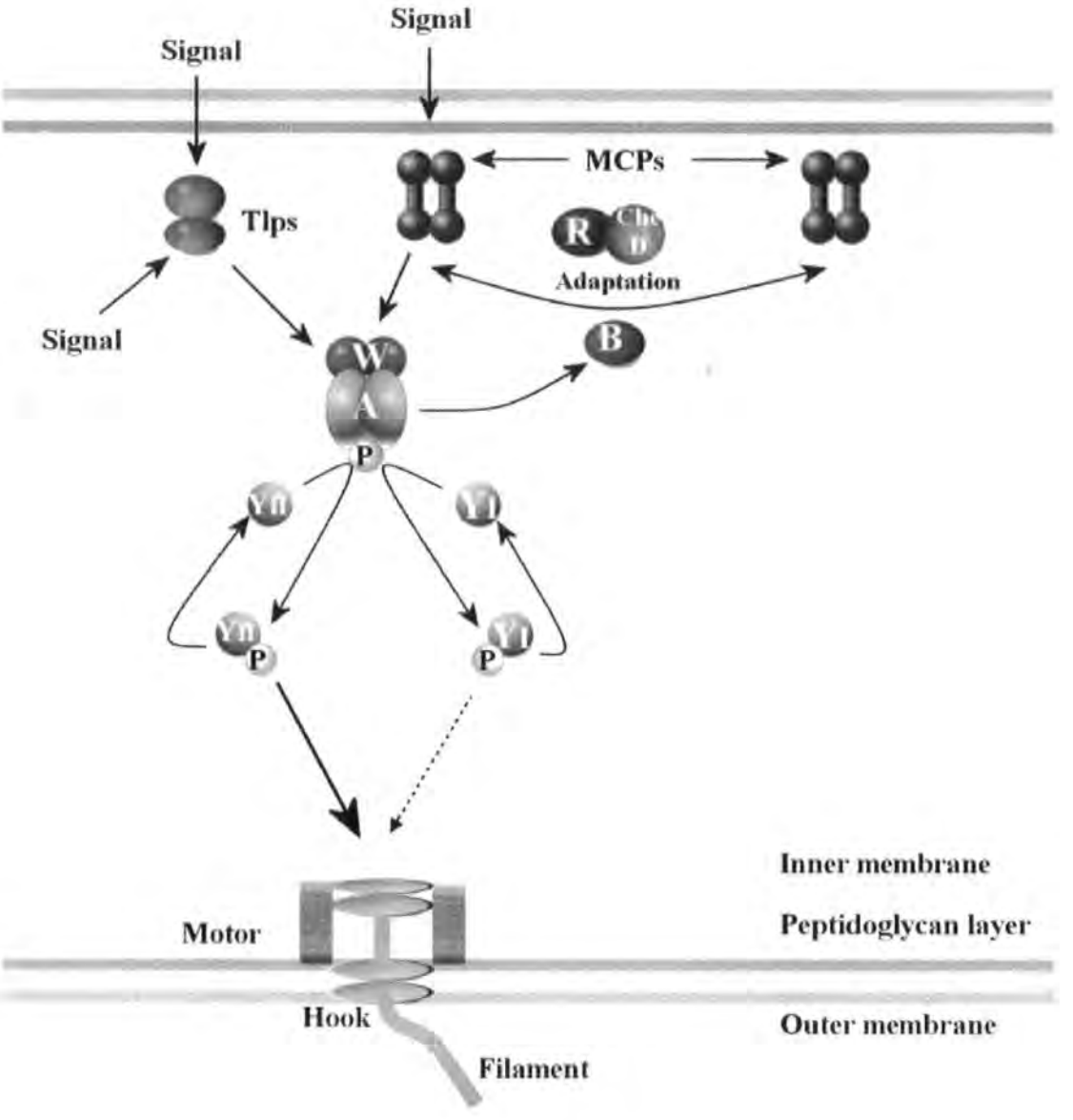

Fig (1.5.1): Accepted model for the sensory pathway of *Sinorhizobium meliloti* (Microbiology: 1997:143:3671-82).

1.5.1.2. Chemotaxis in *Rhodobacter sphaeroides*

The chemotaxis operon of *R. sphaeroides* contains *orf1*, *cheY1*, *cheA*, *cheW*, *cheR*, *cheY2*, *orf2* and *orf3*. In this bacterium a second operon has been identified with a second functional set of the chemotaxis genes *cheA*, *cheW*, *cheR* and a third copy of the cheY genes (Hamblin, P. A., B. A. Maguire, et al., 1997). Further investigation showed that *R. sphaeroides* has multiple copies of chemotaxis genes (two *cheA*, two *cheR*, one *cheB*, three *cheW* and five *cheY*) (Shah, D. S. H., S. L. Porter, et al., 2000).

Rhodobacter sphaeroides has more complex chemotactic behaviour than *E. coli* because it has multiple copies of chemotaxis genes. Tethered cell analysis suggested that CheY4, CheY5 are the motor- binding response regulators. CheA2 mediates an attractant response via CheY4 but CheA1 and CheY5 appear to mediate a repellent response. CheY3 facilitates signal termination, and CheY1 and CheY2 can substitute. CheW3 (but not CheW2) restored swarming to a CheW mutant of *E. coli* (Shah, D. S. H., S. L. Porter, et al., 2000). In addition CheW2 and CheA2 are required for the normal localisation of McpG and for normal chemotactic responses (Martin, A. C., G. H. Wadhams, et al., 2001), although the roles of these genes are different in aerobic or anaerobic conditions. In this bacterium, *cheR2* and *cheB1* were essential for normal chemotaxis. *cheR2* and *cheB1*, but not *cheR1*, were able to complement the equivalent *E. coli* mutants, but none of these proteins were required for correct polar localisation of the McpG in *R. sphaeroides* (Martin, A. C., G. H. Wadhams, et al., 2001).

Analysis of the DNA sequence directly upstream of the chemotaxis operon of *R. sphaeroides* identified a single gene that has strong similarity to the methyl-accepting chemotaxis protein (Ward, M. J., D. M. Harrison, et al., 1995). Using

antibody raised against the highly conserved domain of *E. coli* Tsr at least one MCP-like protein was detected in *R. sphaeroides*. Analysis using western blotting and immunogold electron microscopy showed that expression of these proteins is environmentally regulated and that receptors are targeted to two different cellular locations, the poles and the cytoplasm of the cell (Harrison, D. M., J. Skidmore, et al., 1999).

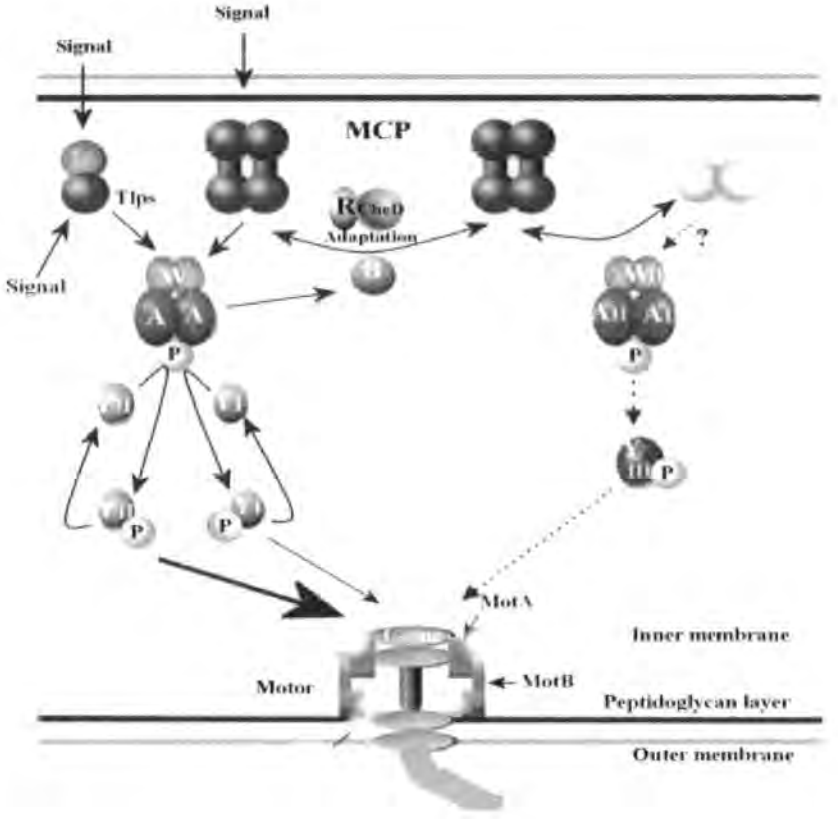

Fig (1.5.2): Multiple chemosensory pathway in *Rhodobacter sphaeroides*.

1.5.1.3. Chemotaxis in other members of α-subgroup of *Proteobacteria*

Sequencing of the genomic DNA of *Azospirillum brasilense* indicated that a region of five open reading frames translated in one direction as encoded homologues of *cheA*, *cheW*, *cheY*, *cheB* and *cheR* genes of the chemotaxis operon of *E. coli* (Hauwaerts, D., G. Alexandre, et al., 2002). 4Kb DNA region of *Rhizobium leguminosarum bv. Viciae* was also sequenced, and found to encode a chemoreceptor (Brito, B., J. M. Palacios, et al., 1996). Another investigation showed that *R. leguminosarum* contains at least five MCP- encoding genes (McpB to McpF) and some of these play a role in early steps in the plant- microbe interaction (Yost, C. K., P. Rochepeau, et al., 1998).

Phylogenetic and genomic analysis has shown three groups of chemotaxis operons in the α-subgroup of Proteobacteria. Group I is exemplified by the operons of *S. meliloti*, *A. tumefaciens*, *R. sphaeroides* and *Caulobacter crescentus*. Group II is exemplified by the chemotaxis operon from *Rhodospirillum centenum* and the best example of group III is the major chemotaxis operon of *R. sphaeroides*. Some species have more than one operon, belonging to different groups. In *S. meliloti* and *C. crescentus* the group I operons are the major ones. The major chemotaxis operon in *R. sphaeroides* belongs to group III.

The mechanism of sensing an environmental change seems to vary across the α- subgroup. *Caulobacter crescentus*, has membrane- spanning MCP related to those in *E. coli*, at the other, *R. sphaeroides*, primarily senses its metabolic state using both cytoplasmic and membrane- bound sensors to signal changes to the flagellar motor, with *S. meliloti* taking an intermediate position.

1.5.2. Energy sensing in α-subgroup of Proteobacteria

In *Rhodobacter sphaeroides* energy taxis is a very common behaviour and electron transport-sensing mediates aerotaxis, photo responses, electron acceptor taxis and taxis toward metabolized substrates. *R. sphaeroides* contains a homologue of the Aer protein identified in *E. coli*, the change in electron transport rate signalled through CheAII and the second chemosensory phosphor-relay system was induced. This signal, in combination with others, produces a balance response at the flagellar motor (Armitage, J. P. and R. Schmitt, 1997). In *Sinorhizobium meliloti* all natural amino acids induce chemotaxis, and it seems that energy-dependent signals are important role in this bacteria. *S. meliloti* cells respond to changes in oxygen concentration by changing their swimming speed. *Azospirillium brasilense* is the first bacterial species in which redox taxis was reported. The major response in *A. brasilense* is aerotaxis and attracts the bacteria to an oxygen concentration that supports a maximum energy level in bacterial cells.

A variety of taxis play major roles in establishing symbiotic, pathogenesis and associative relationships between plants and Rhizobacteria. If the strains of this group are motile then they are able to travel distances in soil between the plant roots. Several line of evidence suggest that taxis in plant-associated bacteria is towards metabolites (Armitage, J. P. and R. Schmitt, 1997).

In symbiotic strains eg. *Sinorhizobium meliloti* different metabolizable substrates cause chemotaxis and in some cases chemoreceptors are cytoplasmic proteins that respond to intracellular signals. The carbon sources that support the fastest growth are also the best chemoattractants, a behaviour typical of energy-

dependent taxis. In *Azospirillium* species, aerotaxis and taxis toward some substrates (malate, succinate) are the most important responses.

1.6. Motility in α-subgroup of *Proteobacteria*

Members of α-subgroup of proteobacteria, such as *Agrobacterium*, *Rhizobium (Sinorhizobium)*, *Azospirillium*, *Caulobacter* and *Rhodobacter* have a motility system which differs from entric bacteria. Cells of *S. meliloti*, unlike Entrobacteria, swim by unidirectional rotation of their rigid flagella (Gotz, R. and R. Schemitt, 1987) and swimming cells respond to material stimuli by changing their flagellar rotary speed (Sourjik, V. and R. Schmitt, 1996).

Unlike the Entrobacteria, in this group two different CheY response regulators act in concern with new MotC and MotD motor proteins to control flagellar rotary speed (Greck, M., J. Platzer, et al., 1995). The organisation of the *S. meliloti* chemotaxis (*che*), flagellar (*fla*, *flg*, *flh* and *fli*) and motility (*mot*) genes is very different from that in Enterobacteria, since all known 41 genes are clustered in one chromosomal region (Sourjik, V., W. Sterr, et al., 1998). Furthermore, in *S. meliloti* two related members of the luxR family, VisN and VisR (for vital of swimming) have been identified (Sourjik, V., W. Sterr, et al., 1998). VisN and VisR act as the master controls of a gene cascade that encodes flagellar, motor and chemotaxis proteins (Sourjik, V., P. Muschler, et al., 2000).

1.7. Virulence in *A. tumefaciens*

Tumor formation by this bacteria is induced through transfer of approximately 15 genes of bacterial DNA (Zhu, J., et al., 2000, Zupan, J., T. R. Muth, et al., 2000, Zupan, J. and P. Zambryski, 1997). The transferred DNA (T-DNA), which is on a 200Kb tumor- inducing plasmid (Ti- plasmid), is translocated to the plant cell, where it is integrated into the plant genome (Ziemienowicz, A., B. Tinland, et al., 2000). This transfer process requires the products of 20 known vir genes located on a non-transferred portion of the Ti-plasmid as well as a smaller number of chromosomally-encoded proteins (Winans, S. C., 1992).

Most transferred genes can be categorized into two distinct groups: The first group, when expressed, induce the production of opines, a group of carbon compounds that are produced from plant metabolism and used by *A. tumefaciens* as a nutrient source (Dessaux, Y., A. Petit, et al., 1993). The second group mediates the over production of the phytohormones, auxin and cytokinin, which cause neoplastic growth and crown gall tumor formation in some parts of the plants (Kalogeraki, V. S. and S. C. Winans, 1998).

To start the process of transfer, virulence genes (from *vir* region) must be induced in response to chemical signals from the plant wound site, which include low pH, phenolic compounds, and monosaccarides of the plant cell wall (Kemner, J. M., X. Y. Liang, et al., 1997). The signals are recognized by three proteins, the VirA-VirG, two component transduction system and ChvE, a periplasmic sugar binding protein (Peng, W. T., Y. W. Lee, et al., 1998).

1.7.1. Attachment of *Agrobacterium* to the plant cell

Bacterial attachment to the host cell is necessary for virulence, and non-attaching bacterial mutants normally cannot incite disease (Matthysse, A. G., 1987). *Agrobacterium* attaches to the plant cell in two steps. The first step is mediated by a cell associated, acetylated, acidic capsular polysaccharide; the second step involves the elaboration of cellulose fibrils by the bacterium. A region of the chromosome of *A. tumefaciens* containing genes (*att* genes) required for bacterial attachment to host cell has been identified.

The *att* region includes two segments of DNA, these are *attA1- attE,* which has homology with ABC transport system, and *attR*, which has homology to cetyl transferases (Reuhs, B. L., J. S. Kim, et al., 1997). The genes located in the *att* region have been organized into nine operons which contain 26 genes. Non-attaching mutants have reduced ability to colonize roots, suggesting that att genes are involved in the colonization of roots (Matthysse, A. G., 2001).

The chromosomal virulence genes *chvA*, *chvB* and *pscA* are involved in the synthesis, processing and export of cyclic β-1, 2 glucan. *Agrobacterium* strains with mutation in chvA or chvB show attachment deficiencies (Douglas, 1985). Attachment occurs at the cell wall surface of wounded plant tissue and two plant cell wall proteins mediate bacterial attachment, a vitronectin- like protein (Wagner, V. T., and A. G. Matthysee, 1992) and a rhicadhesin- binding protein (Swart, S., et al., 1994).

1.7.2. Ti- Plasmid: Structure and Function

In past Ti- plasmids were generally classified by the type of opines that could be catabolised by genes on the plasmid. However, this nomenclature is becoming less satisfactory because all known Ti- plasmids catalyse the catabolism of more than one opines (Zhu, J., P. M. Oger, et al., 2000). The most common type of Ti- plasmid is the octopine-type. This Ti- plasmid contains 155 open-reading frames. All Ti- plasmids have simillar functions and generally contain five region:

1. The T- region that is transferred to the plant cell.
2. The *vir* region, which directs the process of transfer of the T- DNA
3. The *rep* region, required for replication of the Ti- plasmid
4. The *tra* and *trb* loci that is required for the conjugal transfer of the Ti-plasmid
5. Genes that direct uptake and catabolism of opines.

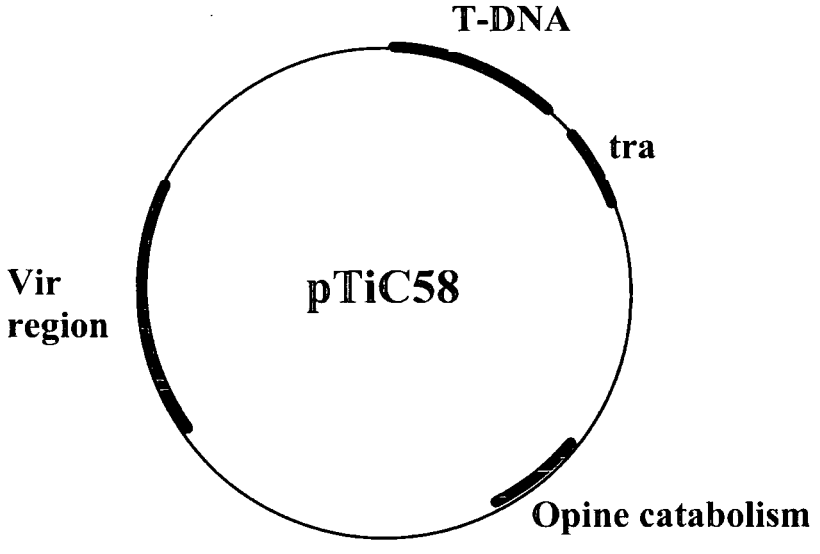

Fig (1.7.1): Map of *A. tumefaciens* pTiC58 plasmid.

1.7.2.1. T- DNA structure

Typically T-DNA contains two regions, T_L- DNA and T_R- DNA, that join together to make T- region. Each region is flanked by cis- acting, 25bp direct repeats called border sequences (Zambryski, P., 1988). The left border of the T_L- DNA is responsible for T-DNA transfer, but in comparison the right border is essential and acts in a polar fashion (Miranda, A., G. Janssen, et al., 1992). T_L- DNA and T_R- DNA encodes several proteins. One group of these genes directs the production of plant growth hormons, auxin and cytokinin, responsible for the gall formation of the transformed plant cell (Binns, A. N., and P. Castantino, 1998). The second group of genes induce the production of various type of opines by host cells.

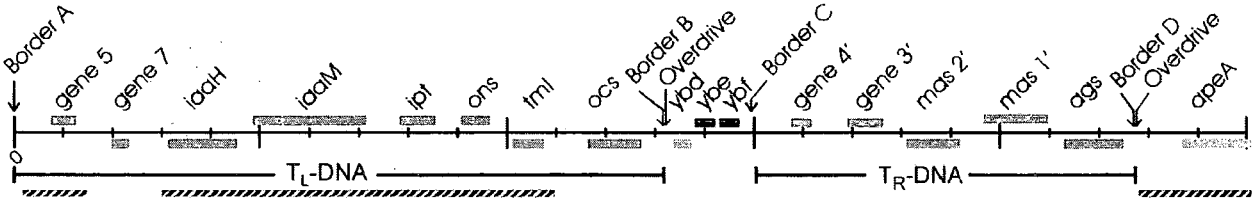

Fig. (1.7.2): Genetic map of T-DNA region of the octopine-type Ti plasmid (Journal of Bacteriology: 2000: 182(14): 3885-3895).

1.7.2.2. Vir region

Proteins responsible for T-DNA processing and transfer are encoded by the *vir* region, which is separate from T-DNA region. Twenty genes have been identified, which are expressed in six operons, *virA*, *B*, *C*, *D*, *E* and *G*. The *vir* regulon is induced

in response to a variety of chemical signals that are released from plant wounds. The signals are perceived by the VirA-VirG system. VirA is a transmembrane sensory histidine kinase which phosphorylates the response regulator VirG. Phospho-VirG binds to binding sites in vir region and activates transcription of the other genes in this region (Jin, S. G., R. K. Prusti, et al., 1990).

The proteins required for cutting the left and right border sequences are encoded by *virD1* and *virD2* (Yanofsky, M. F., S. G. Porter, et al., 1986). VirC1 and VirC2 are not required for T- region processing but are necessary for efficient T-strand transfer into plant host cell. The *virB* operon contains 11 genes. All of them except *virB1* are essential for tumorigenesis (Berger, B. R. and P. J. Christie, 1994) All proteins of this operon are localised to the inner or outer membrane of the bacterial cell (Thorstenson, Y. R., G. A. Kuldau, et al., 1993).

VirE2 and VirD2 contain nuclear localization sites (NLS) and mediate transport of the T-DNA from cytoplasm to the nucleoplasm inside the plant host cell (Zupan, J., T. R. Muth, et al., 2000). The *virB* and *virD* operons together make a complete set of conjugation proteins. Some members of the vir regulon are not essential for tumorigenesis in all hosts and may be required only in specific hosts or may play other roles. These include *virF*, *H*, *J*, *K*, *L*, *M*, *P*, and *virR* (Kalogeraki, V. S. and S. C. Winans, 1998).

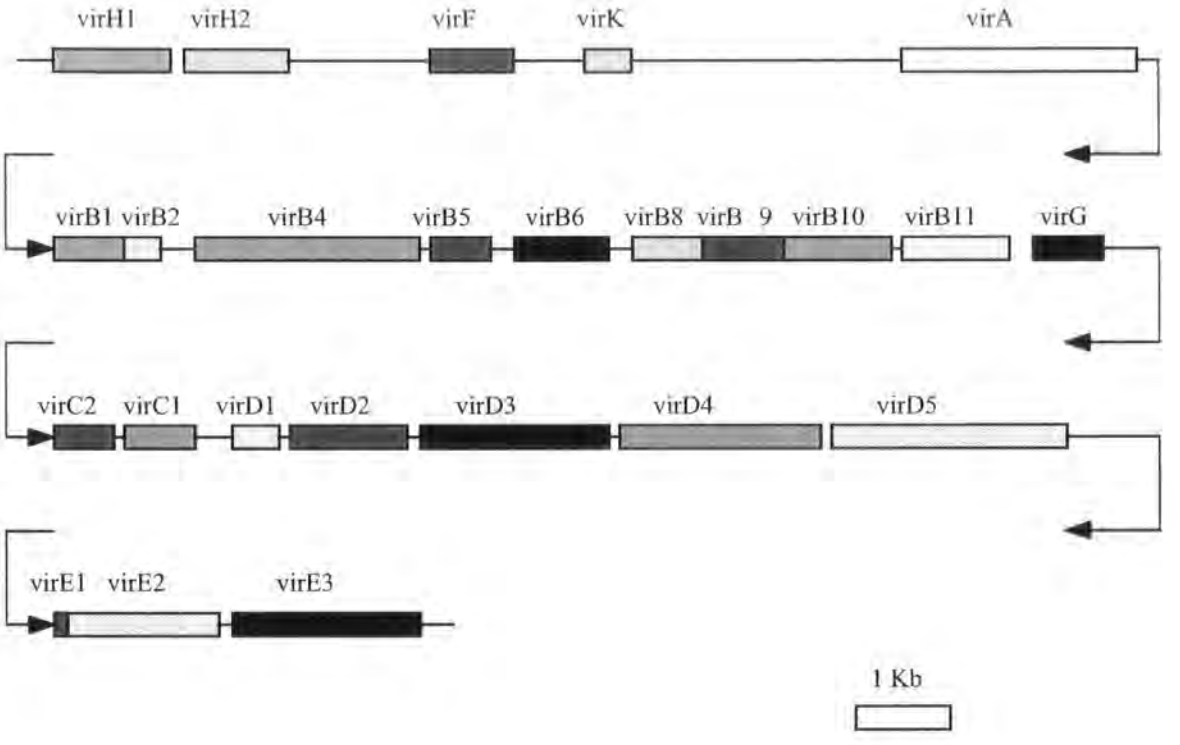

Fig (1.7.3): Map of *A. tumefaciens vir* region (Science: 2001:299:2317-2323).

1.7.3. T- DNA transfer process

The process initiates when bacterial cells perceive certain phenolic and sugar compounds from wounded plant cells (Winans, S. C., 1992). These compounds act as a inducers of the *vir* genes. Phenolic compounds such as acetosyringone and related compounds are recognized by *virA*. Autophosphorylation of VirA protein and transphosphorylation of VirG protein results in the activation of *vir* genes. The activation of *virD1* and *virD2* results the cleavage of Ti- plasmid in the border sequences, and production of a single stranded (ss) copy of the T-DNA (T-strand) with a single molecule of the Vir protein. VirD2 covalently binds at the 5′ end, coated

with the ssDNA binding protein, VirE2, and produces the T- complex (Zupan, J., T. R. Muth, et al., 2000).

T-DNA borders act as sites of initiation and termination for T- strand production and nicking enzymes (VirD1, VirD2), which produce ss nicks in T- DNA borders. In *Agrobacterium* VirD1 firstly recognises and binds to the T-DNA border, to enhance the binding of VirD2, working as a relaxase (Pansegrau, W. and E. Lanka, 1996).

During transfer of VirD2:T-strand and before transfer into the plant cell, the nucleic acid is coated by ssDNA-binding protein, VirE2, (Zupan, J. and P. Zambryski, 1997). Some investigators have proposed that VirE2 and VirD2:T-strand are exported independently from the bacterium and that the formation of the T-complex is completed in the plant cell cytoplasm (Lee, L. Y., S. B. Gelvin, et al., 1999).

Structure and function of T- transporter: The T-complex transporter is assembled from 11 proteins encoded by the *virB* operon and *virD4*. This transporter mediates transfer of the T- complex to plant cell, conjugal transfer of plasmids into the bacterium and also transfer of some proteins, such as VirE2. The assembly of T-transporter is started by the hydrolysis of the peptidoglycan layer by VirB1 (Baron, C., M. Llosa, et al., 1997). VirB6, VirB7, VirB8, VirB9 and VirB10 are the constituents of the T- DNA transport pore (Kumar, R. B., Y. H. Xie, et al., 2000), and through it VirB2 and VirB5 migrate to the cell surface to form the T- Pilus. Energy to make the assembly and mediate translocation is supplied by the three ATPases (VirB4, VirB11 and VirD4) (Christie, P. J., 1997).

The VirB apparatus delivers the T-complex to the cytoplasm of the plant cell. The carboxyl- terminus of VirD2 contains a nuclear localization signal (NLS) that mediates import of T- strand through the nuclear pore. The VirE2 protein also has a

role in nuclear import. This protein has a nuclear localization site that mediates transport of the T- DNA from the cytoplasm to the nucleoplasm (Citovsky, V., J. Zupan, et al., 1992). For import of short ssDNA, VirD2 is sufficient, but import of long ssDNA requires VirE2. Both proteins, VirE2 and VirD2, are required for efficient import of the T-DNA complex into plant nuclei (Ziemienowicz, A., T. Merkle, et al., 2001).

In the final step, the ssDNA of the T-complex is integrated into plant chromosome. After nuclear import, the T-strand made ds-DNA with the concomitant displacement of VirE2. Most models propose illegitimate recombination for T- strand integration (De Buck, S., A. Jacobs, et al., 1999). VirD2 initiates integration by ligating the 5′ end to an exposed 3′- OH in plant chromosome. VirE2 interacts with nuclear factors (VIP2) that mediate interaction with chromatin and facilitate integration of the T-strand.

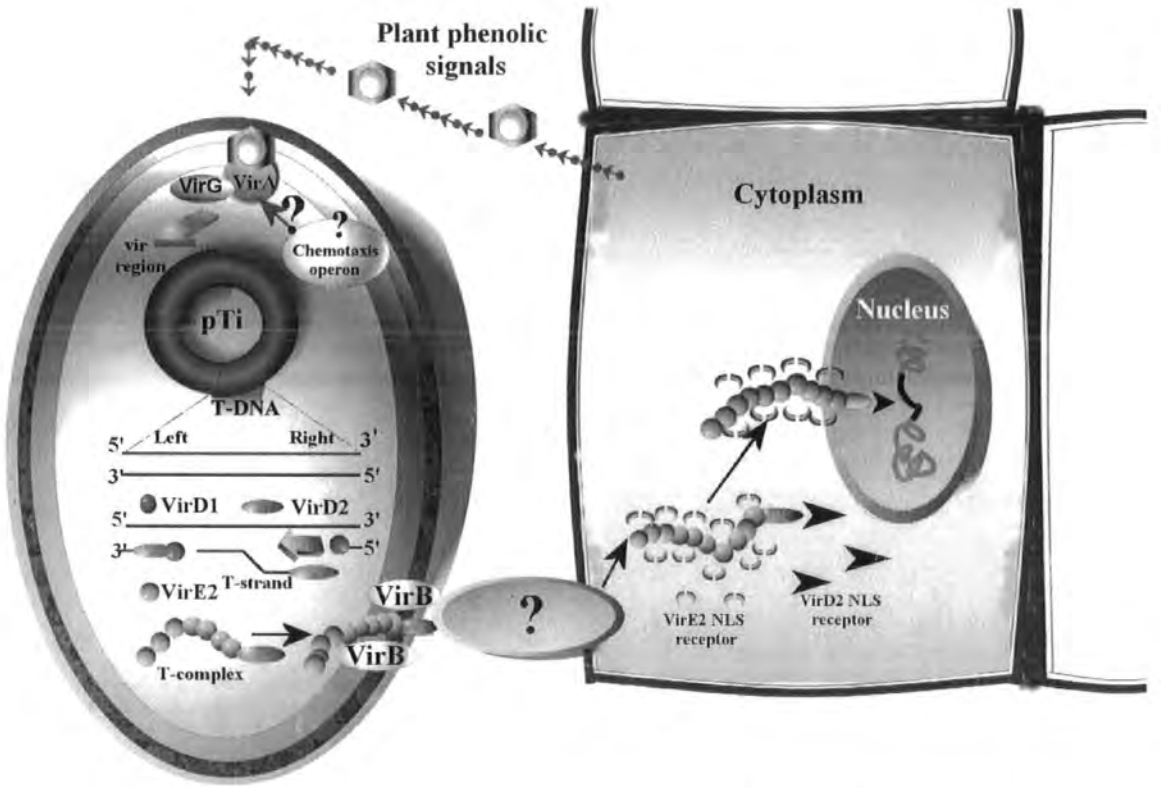

Fig (1.7.4): The basic steps in the transformation of plant cells by *A. tumefaciens* (Figure adopted from: Zupan, J., et al., 2000).

1.8. Chemotaxis and motility in *Agrobacterium*

1.8.1. VirA and *vir* genes activation

The *vir* genes are induced in response to chemical signals produced at the plant wound site. These signals include low pH, phenolic compounds, and monosaccharide components of the plant cell wall (Cangelosi, G. A., R. G. Ankenbauer, et al., 1990, Shimoda, N., A. Toyodayamamoto, et al., 1993, Stachel, S. E., E. Messens, et al., 1985). The signals are perceived by the chromosomally encoded sugar- binding protein, ChvE, and the Ti-plasmid- encoded proteins, VirA and VirG. VirA/VirG are members of the highly conserved class of two-component sensory transduction proteins. VirA is a transmembrane sensory histidine kinase which phosphorylates the response protein VirG (Jin, S. G., R. K. Prusti, et al., 1990, Winans, S. C., R. A. Kerstetter, et al., 1989). Phospho-VirG binds to binding sites designated *vir* boxes, located upstream of each *vir* promotor and co-ordinately activates transcription of these promoters (Han., D.C., and S. C. Winans, 1994, Jin, S. G., R. K. Prusti, et al., 1990).

The most important and key molecule that permits *Agrobacterium* to sense environmental conditions suitable for T-DNA transfer is the VirA protein, which is anchored to the cytoplasmic memberane by two transmembrane domains (TM1 and TM2). This protein contains four other domains: an amino- terminal periplasmic domain and three cytoplasmic domains. The cytoplasmic domain include a linker, a kinase, and a carboxyl-terminal region termed the receiver because it contains a region that is homologous to the phosphorylatable receiver domain of VirG. The

periplasmic domain is required for detection of monosaccharides and has homology with the chemoreceptor Trg (Chang, C. H., and S. C. Winans, 1992).

Genetic analysis showed that the Trg- homologous region of *virA* is not essential for the enhancement of *vir* gene expression by sugars (Toyoda-Yamamoto, A., N. Shimoda, et al., 2000). The kinase domain is critical for tumorigenesis (Jin, S. G., T. Roitsch, et al., 1990). There are differences in *vir* gene induction by a variety of different phenolic compounds which is determined by the *virA* gene of *Agrobacterium*. The *virA* locus determines which phenolic compounds can function as *vir* inducers, suggesting that VirA directly senses the phenolic compounds and responds to them for *vir* activation (Lee, Y. W., S. G. Jin, et al., 1996). The site of interaction of the phenolic compounds with VirA is in the linker domain of VirA (Chang, C. H., and S. C. Winans, 1992). Mutation in the TM2 region adjacent to the cytoplasmic linker abolished induction of *vir* genes. In the linker domain, sites essential for *vir* induction by phenolic compounds were distributed over the entire region (Toyoda-Yamamoto, A., N. Shimoda, et al., 2000).

virA and *virG* are the Ti-plasmid loci required for chemotaxis towards acetosyringone, suggesting a multifunctional role for the VirA/G system: at low *vir* inducer concentration, it induces chemotaxis, at high concentration, it effects *vir*-induction (Shaw, C. H., G. J. Loake, et al., 1991). For chemotaxis, *vir* induction does not appear to be required, the constitutive level of *virA/G* expression being sufficient to effect chemotaxis. Under peak chemotaxis condition, *vir* induction is unlikely to occur (Shaw, C. H., A. M. Ashby, et al., 1988).

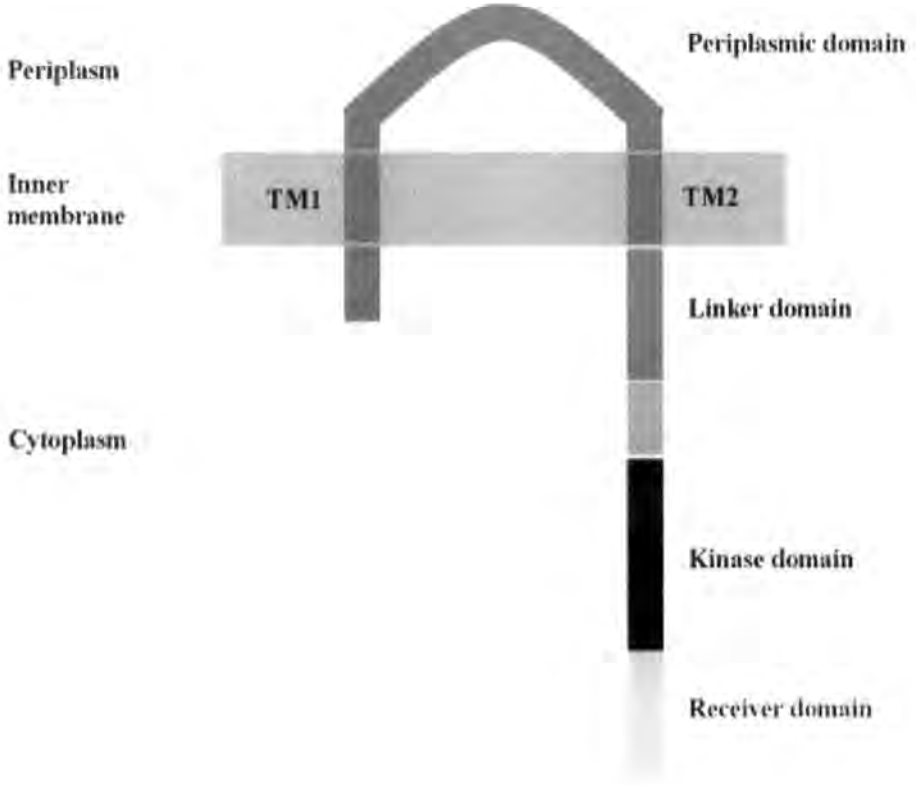

Fig (1.8.1). Functional domains of the sensor molecule, VirA. (Figure adapted from Lee, W., et al., 1996).

Monosaccharides, which act as inducers of *vir* genes, are derived from components of the plant cell wall. They are bound by the periplasmic sugar-binding protein, ChvE, which then interacts with the periplasmic region of the membrane-bound VirA molecule of the VirA/VirG sensor–regulator pair to activate transcription of the *vir* regulon (Huang, M. L., et al, 1990, Shimoda, N. A., et al, 1990). Most of the periplasmic domain of VirA is required for the interaction with, or response to, ChvE (Doty, S. L., M. C. Yu, et al., 1996). All *Agrobacterium* strains containing a defective *chvE* are defective in *vir* gene induction (Huang, M. L., et al, 1990). ChvE also

mediates chemotaxis toward various sugars, presumably by interacting in the sugar-bound form with an unidentified membrane- bound receptor (Cangelosi, G. A., R. G. Ankenbauer, et al., 1990). Specific inducing sugars can broaden the specificity of the phenolic compounds which VirA senses. This broadened specificity results from the increased level of ChvE through induction by arabinose via the regulatory protein GbpR. (Peng, W. T., Y. W. Lee, et al., 1998).

A chemotaxis operon has been identified in *A. tumefaciens*. The operon begins with *orf1*, a protein product showing strong sequence identity to Mcps protein, followed by *orf2*, *cheY1*, *cheA*, *cheR*, *cheB*, *cheY2*, *orf9* (which shows the strong similarity with *cheD* of Bacillus subtilis) and *orf10*. Complete deletion of this operon results in severely-impaired chemotaxis. *cheW* is not found in the che operon (Wright, E.L., et al., 1998) but elsewhere in *A. tumefaciens* genome (Wood, D. W., J. C. Setubal, et al., 2001). *orf2* has sequence identity with *cheZ*.

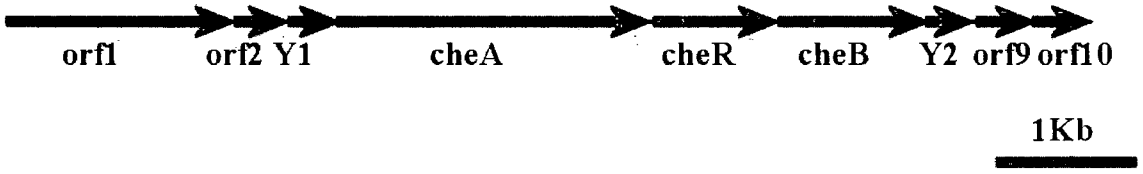

Fig (1.8.2): Map of *A. tumefaciens* chemotaxis operon.

1.8.2. Motility in *Agrobacterium*

For motility, *A. tumefaciens* produces flagella that are circumthecally arranged, near one end rather than ringing the middle portion of the bacilliform cell, and they are not peritrichously situated (Chesnokova, O., J. B. Coutinho, et al., 1997). The

flagella rotate unidirectionally, clockwise, driving the bacterium at 60 μm/s (Shaw, C.H., 1991). This type of flagellar arrangement might play a role in facilitating bacterial chemotaxis and expedite the infection process because cells are frequently attached to plant cells in a polar fashion (Smith, V.A., et al., 1978).

Motility by *A. tumefaciens* plays a critical role in the early infection process, but probably it does not influence virulence. Direct inoculation of plants showed that non- motile mutants were equally as virulent as the parent strains (Bradley, D. E., C. J. Douglas, et al., 1984). Another group reported chemotaxis by *A. tumefaciens* towards plant phenolics such as acetosyringone, and observed that non- motile mutants were non- chemotactic and incapable of colonising the roots of young potato seedlings (Shaw, C. H., G. J. Loake, et al., 1991). These observations would suggest that motility, and presumably flagella, may be a necessary component for ecological fitness in natural environment (Chesnokova, O., J. B. Coutinho, et al., 1997). The size and weight of the tumors induced by mutant strains (with a deletion of *flaABC* of motility gene region) were consistantly smaller than those induced by the wild type (Chesnokova, O., J. B. Coutinho, et al., 1997).

Genes concerned with motility have been identified (Deakin, W.J., et al., 1997., 1999., Chesnokova, O., J. B. Coutinho, et al., 1997). These genes are clustered in *Agrobacterium*. The cluster contains genes concerned with filament and basal body structure, assembly and export, motion and switching (Deakin, W.J., et al., 1999). The *Agrobacterium* cluster shows considerable similarity with that from another member of the α- subgroup of Bacteria, *Sinorhizobium meliloti* (Sourjik, V., W. Sterr, et al., 1998).

1.9. Aims of this work

This project aims to describe how *Agrobacterium tumefaciens* processes sensory information and to determine which cytoplasmic components of the chemotaxis operon have a major role in signal transduction in the chemosensory pathway. Another aim is to find possible MCP genes in *A. tumefaciens* and to investigate the role of this protein(s) in the acquisition of sensory information from chemoattractants.

Chapter 2

Materials and Methods

2. Materials & Methods

2.1. Materials

All inorganic chemicals were of Analar quality and purchased from BDH Chemical Ltd., Poole, Dorset, U.K., unless otherwise specified.

All organic chemicals and enzymes were from Sigma Chemicals plc., Poole, Dorset, U.K., unless otherwise specified.

Lab M nutrient broth (no.2) and Lab M nutrient Agar were from Amersham Ltd., Bury, U.K..

Nylon hybridisation transfer membranes and radiochemicals were from Amersham Biosciences U.K. Limited, Amersham Place, Little Chalfont, Bucks HP7 9NA, U.K.

Bacteriological Agar (no.1) and yeast extract were from Oxiod Ltd, Basingstoke, Hants, U.K..

Trypticase peptone was from BBL, Cockeysville, U.S.A..

Restriction endonucleases, T4 DNA ligase, Klenow fragment enzyme, Taq polymerase, corresponding buffers, X-gal, IPTG and λ-DNA were from NBL fermentas, Cramlington, Northumberland, U.K., Boehringer Mannheim Ltd, Lewes, U.K., or Helena Biosciences, Tyne & Wear, U.K..

Agarose was from BRL, Gaithersburg, U.S.A..

Metaphor agarose was from FMC Bioproducts, Rockland, U.S.A..

Ficoll 400 and Sephadex G-50 were from Pharmacia fine chemicals, Uppsala, Sweden.

Fuji RX-100 x-ray film was from Fuji Photo film Co, Ltd., Japan. Polaroid film was from Polaroid Ltd., St. Albans, Hertfordshire, U.K..

Filter paper (3MM) and laboratory sealing film were from Whatman International Ltd., Maidstone, U.K..

Nitrocellulose discs (25mm, 0.22μm pore size) were from Schleicher & Schuell, Postfach 4, D-3354, Dassel, Germany.

Oligonucleotides for use in PCR analysis were from MWG-Biotech, Germany and PE-Applied Biosystems U.K., Cheshire, U.K..

Nitrocellulose discs (13mm, 0.8μm pore size) for use in chemotaxis assay were from Scientific Laboratory Supplies Ltd, Wilford Industrial Estate, Nottingham, U.K..

IsotonII for use in Coulter Counter Multisizer II was from Coulter Electronics Limited, Northwell Drive, Luton, U.K.

2.2. Bacterial strains

2.2.1. *E.coli* Strains

Strain	Characterization	Source
DH5α	supE44 ΔlacU169 (f80 lacZΔM15) hsdR17 RecA1 endA1 gyrA96 thi-1 relA1	Woodcock et al.(1989)

2.2.2. *Agrobacterium tumefaciens* strains

Strain	Characterization	Source
C58C1	Wild-type chemotaxis, Ti-, Rif+	VanLarebeke et al. (1974
C1-delY1	Chemotaxis mutant-created by in-frame deletion of *cheY1*, Rif+	This work
C1-delY2	Chemotaxis mutant-created by in-frame deletion of *cheY2*, Rif+	This work
C1-delB	Chemotaxis mutant-created by in-frame deletion of *cheB*, Rif+	This work
C1-delR	Chemotaxis mutant-created by in-frame deletion of *cheR*, Rif+	This work

2.3. Plasmid

2.3.1. Plasmid vectors

Plasmid	Characterization	Source
pBluescriptSK+	2.96 Kb Cloning vector, Amp®	Lab stock
pJQ200uc1	Suicide vector, Gm®	Quandt and Hynes (1993)
pJQ200mp18	Suicide vector, Gm®	Quandt and Hynes (1993)
pK18mobSacB	Allelic exchange vector, Kan®	Schafer et al. (1991)

2.3.2. Recombinant plasmids containing cloned chemotaxis genes

Plasmid	Characterization	Source
pDUB1911	C58C1 cosmid library clone containing *BamH*I fragment from chromosomal DNA in the vector pLAFR3, Tc®	Lab stock

2.3.3. Recombinant SK+ plasmids containing subcloned *A. tumefaciens* chemotaxis genes

Plasmid	Characterization	Source
pELW6	pBluescript SK+ containing 1.7Kb *EcoR*I fragment from pDUB1911	Wright, E. L., 1999
pELW1	pBluescript SK+ containing 4KbKb *Hind*III fragment from pDUB1911	Wright, E. L., 1999
pELW2	pBluescript SK+ containing 4KbKb *Hind*III fragment from pDUB1911	Wright, E. L., 1999
pELW61	1309 bp *Sac*II fragment isolated from pELW6 and subcloned into pBluescript sk+	This work
pELW62	363 bp *Sac*II fragment isolated from pELW6 and subcloned into pBluescript sk+	This work
pELW63	1190 bp *EcoR*V fragment isolated from pELW6 and subcloned into pBluescript sk+	This work
pELW64	1373 bp *XbaI/Hind*III fragment isolated from pELW6 and subcloned into pBluescript sk+	This work
pELW65	547 bp *Hinc*II fragment isolated from pELW6 and subcloned into pBluescript sk+	This work
SK+/cheR	1827bp *Pst*I fragment from pELW1 and subcloned into pBleuscriptsk+	This work
SK+/cheB	1935bp *Rsa*I fragment from pELW1 and subcloned into pBluescriptsk+	This work

SK+/cheY1	773bp *Pvu*II/*Hinc*II fragment from pELW2 and subcloned into pBluescript sk+	This work
SK+/cheY2	957bp *Bgl*II/ *Mlu*I fragment from pELW1 and subcloned into pBluescript sk+	This work
SK/delY1	~3.4Kb PCR product resulting of deletion of *cheY1* gene from SK/*cheY1*	This work
SK/delY2	~3.8Kb PCR product resulting of deletion of *cheY2* gene from SK/*cheY2*	This work
SK/delR	~3.8Kb DNA product resulting of deletion of *cheR* gene from SK/*cheR*	This work
SK/delB	~3.7Kb PCR product resulting of deletion of *cheB* gene from SK/*cheB*	This work

2.3.4. Recombinant pK18mobsacB plasmids containing subcloned *A. tumefaciens* chemotaxis genes

Plasmid	Characterization	Source
pK18/delY1	490bp *EcoR*I/*Hind*III fragment from SK/delY1 ligated into pK18*mobsacB* vector	This work
pK18/delY2	990bp *Hind*III/*Xba*I fragment from SK/delY2 ligated into pK18*mobsacB* vector	This work
pK18/delR	900bp *EcoR*I/*Xba*I fragment from SK/delR ligated into pK18*mobsacB* vector	This work
pK18/delB	750bp *Hind*III/*Xba*I fragment from SK/delB ligated into pK18*mobsacB* vector	This work

2.3.5. Plasmid used in mutant construction

Plasmid	Characterization	Source
pRK2013	Helper plasmid for conjugation into *A. tumefaciens* , Km®	Figursky and Helinski (1979)
pDUB2033	Neomycin resistance cassette, Amp®, Neo®	Lab stock

2.4. Bacterial growth, media, condition and procedure

Lab M nutrient broth no.2 (LM-broth):

25g made up to 1 litre with distilled water gives final concentration of 5g/l beef extract, 10g/l balanced peptone no.1, 5g/l NaCl pH: 7.5 ±0.2.

For swarm plates bacteriological agar was added at a concentration of 0.2-0.35%.

Lab M nutrient agar (LM-agar):

28g made up to 1 litre with distilled water, gives final concentration of 5g/l peptone, 3g/l beef extract, 8g/l NaCl, 12-15g/l Agar no.2, pH: 7.3±0.2.

MinA+Glucose:

20ml 5X MinA salts, 2ml 10% Glucose, 0.1ml 1M $MgSO_4$ made up to 100ml with distilled water (5X MinA salts contains 52.5g K_2HPO_4, 22.5g $KHPO_4$, 1.0g $(NH_4)_2SO_4$ and 2.5g Sodium citrate, $2H_2O$ per 1000ml).

If required, bacteriological agar was added to final concentration of 1-1.2% for solid plates.

Chemotaxis media:

1ml 0.1M EDTA and 10ml 1M K_2HPO_4 (pH: 7.0) made up to 1 litre with distilled water.

Antibiotics were added to sterile media to give a final concentration of:

For *A. tumefaciens*: Erythromycin 100μg/ml, Kanamycin 50μg/ml, Neomycin 100μg/ml, Rifampicin 100μg/ml and Tetracycline 15μg/ml.

For *E. coli*: Ampicillin 50μg/ml, Gentamycin 10μg/ml, Kanamycin 50μg/ml, Neomycin 100μg/ml and Tetracycline 15μg/ml.

For selecting against the inactivation of the β-galactosidase gene, by insertion of DNA fragments into the multiple cloning sites of pBluescriptSK+ and other plasmids, 50μl of 20mg/ml X-gal (in dimethyl formamide) were spread over the surface of agar plates using sterile glass spreader.

All cultures were sterilized by autoclaving at 20PSI for 20 minutes, unless otherwise specified.

Liquid cultures were inoculated with a flamed loopful or with 5μl of overnight culture using sterile tip.

Solutions and bacterial suspensions were spread onto agar plates using a glass spreader which had been sterilized by flame and then cooled with 70% ethanol.

Bacterial colonies were inoculated into the centre of swarm plates using sterile needle (stab inoculation), or 3-5 μl of bacterial suspension was placed on agar plates as a spot with sterile tip.

Liquid bacterial cultures were incubated on an orbital shaker at about 200rpm at 37°C for *E. coli* and 28-30°C for *A. tumefaciens* strains.

Short-term (up to 2 months) stocks of cultures were kept at 4°C. For long-term storage, stocks were made in liquid media, glycerol was added to final concentration of 15-20% and after quick freezing in liquid nitrogen, kept at -80°C.

All glassware, plasticware and other equipment was sterilized by autoclaving at 15-20PSI for 15-20 minutes. Otherwise solutions were filter-sterilized through a 0.22μm nitrocellulose filter into sterile container.

2.5. Isolation of DNA

2.5.1. Alkaline lysis plasmid minipreps

A single colony of bacteria was grown for overnight in 5 ml sterile LM broth media containing the appropriate antibiotic selection. The cells were harvested by centrifugation in a microfuge at full speed (14000 rpm) for 1.5 minutes. The supernatant was discarded and the pellet was resuspended in 200 µl of ice-cooled solution I:

Solution I: 50mM glucose, 25mM Tris.Cl (pH 8.0), 10mM EDTA (pH 8.0)

200µl of solution II was added and the contents mixed by gentle inversion.

Solution II: (prepared fresh) 1.0 ml of 0.4 M NaOH and 1.0 ml of 2% SDS

Then 200µl of ice- cooled solution III was added and shaked gently to mix.

Solution III: 1.15 ml glacial acetic acid, 2.85 ml distilled water, 6 ml 5M potassium acetate, pH: 4.8

The tube was then centrifuged in a microfuge at 14000 rpm for 5 min. The supernatant was transferred to a clean tube and centrifuged for 1 minute. The supernatant was transferred to new eppendorf tube, 2 volumes of absolute ethanol (100%) were added at -20°C, and the solution was left on ice for at least 15 minute to enhance the precipitation of the DNA. The tubes were centrifuged at full speed for 15 minutes at 4°C. Then the ethanol was poured off and 1 ml of 70% ethanol (-20°C) was added and the sample was centrifuged at room temperature for 5 minutes. The ethanol was removed from the tube, and the pellet was left at 37°C to dry for a few minutes. The final DNA pellet was resuspended in 50µl of T.E. Buffer (containing RNAase to final concentration of 20µg/ml).

T.E. Buffer: Tris.Cl (pH 7.5) 1.0mM, EDTA (pH 8.0) 0.01 mM

(When necessary plasmid DNA was isolated and purified with Qiagen Qiaprep spin columns, according to the manufacturer's instruction).

2.5.2. Isolation of chromosomal DNA

A single colony of bacteria was grown to logarithmic phase in 5 ml LB medium containing appropriate antibiotic. Cells were collected in 1.5 ml eppendorff tubes and pellets were left at -20°C for 30 minutes. The pellet was resuspended in 200µl of TE buffer and incubated with 8µl lysozyme (10µg/ ml) at 37°C for 30 min. Then the cells were lysed by addition of 40µl of 4M sodium perchlorate, 24µl of 10% SDS and 8µl of proteinase K (20µg/ ml stock) and after mixing by inverting, incubated at 45°C. After 2 hr the DNA was precipitated by adding 2 volumes ethanol (100%, -20°C), incubated at -20°C for 30 minutes and centrifuged for 5 minutes (13000rpm, 4°C). The ethanol was discarded, the pellet was washed with 1ml 70% ethanol, dried for a few seconds and resuspended in 500µl TE buffer.

The DNA was mixed with an equal volume of Phenol: chloroform: isoamyl alcohol (25:24:1), inverted at room temperature to mix completely then the solution was centrifuged for 3 minutes or until the three phases were separated completely. The aqueous layer transferred to a clean tube and this procedure was repeated three times; each time the aqueous layer was transferred to a clean tube followed by extraction once with chloroform:isoamyl alcohol (24:1). The DNA was precipitated by adding 2 volumes of ethanol (100%) and 0.1 volume 3M sodium acetate (pH: 4.8), and then incubated at -20°C overnight. It was subsequently centrifuged for 5 minutes, washed with 1ml 70% ethanol and left at 37°C until dried completely, and then

resuspended in 50µl TE buffer (with RNAase (10mg/ml stock)) to a final concentration of 20µg/ml.

2.5.3. Preparation and purification of salmon sperm DNA

100mg of highly polymerized DNA from salmon sperm (Sigma) is dissolved in 10 ml sterile distilled water in a sterile 50ml polypropylene centrifuge tube. 1.28ml of 0.25M EDTA and 4.8ml of 1M sodium hydroxide was added and the solution has boiled in a water bath for 20min. Then tube was cooled in ice, and 1.1ml of 2M Tris-Cl (pH: 7.5), 4.8ml of 1M hydrochloric acid were added and well mixed. This was followed by addition of 10ml phenol/chloroform/isoamyl alcohol (25:24:1), and the tube was shaken well (to mix completely to make one phase), then vortexed and centrifuged at full speed for 10 min. The aqueous layer was collected and transferred to new tube, and 2ml 3M sodium acetate (pH: 5.6) and 40ml 100% ethanol (-20°C) was added. The solution was left overnight at -20°C, or for 1hr at -80°C.

The mixture was subsequently centrifuged (13000rpm, 4°C, 15min), the ethanol was discarded and pellet was washed 2-3 times with 70% ethanol. The pellet was dried, redissolved in 15-20ml of TE buffer and stored at -20°C.

2.6. DNA manipulation

2.6.1. Phenol:Chloroform extraction of DNA

The DNA was usually suspended in 500µl TE buffer. Then to the suspension an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed

by inverting until the suspension become one phase, and then the phase were separated by centrifugation. It was repeated three times and each time the aqueous layer was transferred to the clean tube. The DNA was then extracted once with chloroform:isoamyl alcohol (24:1), precipitated by addition of 2 volumes of ethanol (100%) and 0.1 volume 3M sodium acetate (pH: 4.8).

After overnight incubation at -20°C the sample was centrifuged (13000rpm, 5min, 4°C), the ethanol was discarded and the pellet was washed with 1ml 70% ethanol. Then the ethanol was removed, the DNA dried and resuspended in 50µl TE buffer containing RNAse (10mg/ml) to a final concentration of 20µg/ ml. The sample was stored at -20°C.

2.6.2. Spectrophotometric quantification of DNA solution

A dilution of 1:50 of the DNA samples (dissolved in 50µl of TE buffer) was made in sterile distilled water and the absorbance was read at 230nm, 260nm and 280nm with a Pu8740 UV/vis scanning spectrophotometer (Philips), using sterile distilled water as blank. As an A260nm of 1.0 is equivalent to a concentration of 2.5 µg /µl of double stranded DNA and according with it sample DNA concentration could be calculated.

2.6.3. Restriction endonuclease digestion of DNA

The following procedure was used for a typical reaction containing 1 µg of DNA. Plasmid DNA was digested in a total volume of 50µl, with 0.1 volume of 10X restriction endonuclease buffer, 1 µg of DNA, 1-3 units of restriction endonuclease

enzyme and sterile distilled water was added to the final volume. The components were mixed well and the reaction was incubated at the recommended temperature (usually 37°C) overnight.

If more than one enzyme was to be used in the same reaction and the recommended buffers were different, the Y+/tango buffer (MBI Fermentas) was used according to the manufacturer's instruction. The reaction was stopped by addition of 1µl of EDTA (0.5M, pH 8.0) or incubation at 65°C for 20 minutes.

2.6.4. Agarose gel electrophoresis

Gel electrophoresis of DNA was usually carried out using a minigel (77 × 55 mm, 60 ml vol.). Minigels were run in Pharmacia gel apparatus GNA-100 electrophoresis tanks. The concentration of agarose could be varied depending on the size of DNA. Usually a 0.7- 1.5% agarose gel was used. The required amounts of agarose were mixed and dissolved in 1×TAE buffer by heating in a microwave oven.

1×TAE Buffer: - [0.04 M Tris-Acetate, 0.001 M EDTA (pH 8.0)]

The solution was cooled to 60° C, and the agarose poured into the gel mould with a suitable well comb in place. When the gel had set, it was put in a tank and covered with 1×TAE buffer.

The samples and size marker were mixed with 6×loading buffer (bromophenol blue 0.25%, xylene cyanol 0.25%, glycerol 30%), and 20-30µl of the mixture was slowly pippeted into the slots of the submerged gel using a disposable micropipette. Electrophoresis was carried out at 5-10 V / Cm for the required amount of time. The gel was then stained with ethidium bromide (10mg/ml stock solution) to final concentration of 0.5µg/ml for 30-60 minutes. Excess ethidium bromide was removed

by washing the gel in distilled water for 10-15 minutes. DNA fragments within the gel were visualised on a transilluminator and photographed with a Polaroid RP4 camera, using a red filter onto Polaroid 667 film, or recorded using Gel Doc 2000 (Bio-Rad). The size markers used were λ- DNA digested with *Hind*III or *Hind*III/*Eco*RI.

2.6.5. Isolation of DNA fragments from agarose gel using silica fines

The required DNA bands were cut out from an agarose gel using a sterile razor blade and placed into an eppendorf tube. 800 µl of sodium iodide solution [90.8g sodium iodide and 1.5g Na_2SO_3 were dissolved in 100ml of distilled water, filter sterilised and saturated with 0.5g Na_2SO_3 (stored at 4°C in dark bottle to protect it from the light) was added and the tube incubated in a water bath at 70°C for about 5 minutes or until the agarose has completely melted. The tube was mixed by inversion and allowed to cool to room temperature for 5 minutes. A suspension of silica fines (50% slurry in water) was vortexed and 5µl was added to DNA solution. The solution was mixed for 1 minute and left for 10 minutes at room temperature with inverting continuously.

The tube was centrifuged for 30 seconds, the supernatant discarded and the pellet washed with 1ml 70% ethanol. The solution was centrifuged for 30 seconds, the 70% ethanol was removed and pellet was dried using tissue or by placing it at 37°C with lied open. The silica fines were resuspended in 30µl TE buffer and incubated at 37°C for 10 minutes with occasional shaking. The samples were centrifuged for 30 second and the supernatant containing the DNA was transferred to new sterile tube. The silica fines were then resuspended in 20 µl TE buffer and incubated as before.

Then, the tube was centrifuged for 30 seconds and supernatant containing the DNA added to other 30 µl of sample to give a total volume of 50µl of DNA solution.

Preparation of the silica fines:

2.5ml of silica was resuspended in distilled water to give a total volume of 5ml. The suspension was stirred for 1 hour and left to settle for another one hour. The suspension was centrifuged at 7000rpm for 10 minute, the supernatant was discarded and the pellet was resuspended in 1.5 ml of distilled water plus 1.5 ml of nitric acid. The suspension was then heated to 98°C and allowed to cool to room temperature. The silica fines were then repeatedly washed with sterile distilled water until the pH was greater than 5.5. Silica fines were stored at 4°C as a 50% slurry in sterile distilled water.

(When necessary the DNA fragments were isolated from the gel using QIAEX II Agarose gel extraction Kits according manufacturer's instruction).

2.6.6. Ligation of DNA fragments

T4 DNA ligase was used to ligate DNA fragments with compatible cohesive or blunt termini. In a microcentrifuge tube, 10-15µl of vector DNA and foreign DNA to be inserted, were digested with restriction enzymes, when necessary dephosphorylated using alkaline phosphatase, mixed at a ratio (insert: vector) of 3:1 (cohesive termini) or 1:1 (blunt end) with maximum 300-400ng DNA. 0.1 volume of 10× ligase buffer, 2µl polyethylene glycol (to increase the rate of ligation of blunt ended DNA) and sterile distilled water up to 20µl were added, then 2u (for sticky

ended) and 4u (for blunt ended) T4 DNA ligase was added. The tube was incubated overnight at 4°C (sticky ended) or 24-48hr at 16°C (blunt ended). 3-10µl of the ligation mix was then used immediately by adding to competent cells, or the T4 DNA ligase was inactivated by heating at 65°C for 10 minutes.

2.6.7. Dephosphorylation of DNA fragment

To reduce self-ligation of vector restricted with enzymes giving blunt ends, the vector was usually dephosphorylated before mixing with DNA fragment. 5µl of 10× reaction buffer (0.1M Tris-Cl pH: 7.5, 0.1M $MgCl_2$) was added to 10-40µl of DNA solution, followed by sterile distilled water to a total volume of 49µl and 1u/µl alkaline phosphatase. The reaction mixture was incubated at 37°C for 30 min or 3hr at room temperature and then the reaction was stopped by heating at 85°C for 15 min.

2.6.8. Filling- in Recessed 3'- Termini

To the 30µl of DNA (0.1- 4µg) digested with enzyme the following components were added:

- 10× reaction buffer 3.5µl
- 4dNTPs 1µl (0.05mM final concentration)
- Klenow fragment 0.5µl

The reaction mixture was incubated at 37°C for 10 minutes and reaction was stopped by heating at 75°C for 10min.

2.6.9. Polymerase chain reaction (PCR)

All polymerase chain reactions were performed in a Perkin-Elmer thermal cycler. Reaction was carried out in a total volume of 25µl and the following components were added in order:

1.	dH_2O to make a final volume of	25µl	
2.	10× reaction buffer	2.5µl	
3.	dNTPs	2.0µl	(each at 1mM)
4.	5' primer	1.0µl	(10pmol/µl)
5.	3' primer	1.0µl	(10pmol/µl)
6.	$MgSO_4$	3.0µl	(2.5mM)
7.	Template DNA	Y* µl	
8.	Taq polymerase**	0.3µl	(5u/µl)

*Y= The amount of template DNA was varied, it is usually 100ng for plasmid DNA and 500- 1000ng for genomic DNA.

**= Taq polymerase usually was added after the initial denaturation step if it was longer than 3 minutes.

Primer design:

Primers were generally designed to contain an equal number of G+C and A+T, usually 10 of each to make sure the Tm of both primers are the same. Furthermore, care was taken to ensure the primers would not self anneal or hybridise to each other.

When required, the appropriate restriction enzyme target sequences was usually added to 5'-end of each primers followed by 2-5 extra bases.

PCR protocol:

The temperatures and times used in PCR were as follow:

Starting		Denaturation	94°C	2-5 min.
15-20 cycle:	{	Denaturation	94°C	45 sec.
	{	Annealing*	55- 65°C	60 sec.
	{	Extension**	72°C	5 min.
Final extension			72°C	10 min.

* The annealing temperature varied depending on the Tm of each pair of primers and was established empirically.

The following formula was used to determine the Tm of primers:

$$Tm = [(No.\ G+C) \times 4 + (No.\ A+T) \times 2] - 5°C$$

** The extension time depended on the length of PCR product, usually 1min for 1Kb when the Taq polymerase was used.

- Before starting the PCR 25µl of mineral oil was added to the top of the mixture.

2.7. Transformation of *E. coli*

2.7.1. Preparation of competent cell (Hanahan methods)

5 ml of LM broth was inoculated with *E. coli (DH5α)* and incubated overnight at 37°C. A dilution of 1:100 was made in fresh LM broth and the cells grown for 2.5-3 hour or until to OD600 of 0.3-0.35 or OD550 of 0.45- 0.55. The culture was aseptically transferred to a sterile and ice- cold propylene tube, chilled for 5 minutes on ice, then cells were centrifuged at 4000rpm for 7 minutes at 4°C. The supernatants were discarded and the cell pellets resuspended in 40% of the original culture volume of solution A.

solution A :
- 30mM potassium acetate,
- 100mM rubidium chloride,
- 10mM calcium chloride
- 50mM manganese chloride and 15% glycerol.

The solution was adjusted to pH 5.8 with 0.2M acetic acid, filter sterilised and stored at 4°C. The tubes were incubated on ice for 5 minutes and cells were recovered by centrifugation as before. The supernatants were poured off and the pellets resuspended in 4% of the original volume of solution B:

Solution B:
- 10mM MOPS
- 75mM $CaCl_2$
- 10mM $RbCl_2$
- 15% glycerol

The solution was adjusted to pH 6.5 with KOH, filter sterilised and stored at 4°C. The tubes were left on ice for 15 minutes, then suitable volumes (100-200µl)

were added to pre- chilled eppendorf tubes, frozen in liquid nitrogen and stored at -80°C. These cells can be used for transformation for at least 3 months.

2.7.2. Transformation procedure

Competent cells were removed from the -80°C freezer, thawed and then placed on ice for 10 minutes. DNA was added up to 5% volume of the competent cells with pre-cooled sterile tips, and the tubes stored on ice for at least 15 minutes. The cells were heat shocked in a water bath at 42°C for 90 seconds then rapidly transferred to an ice bath. Cells were chilled for 2-5 minutes and then 800µl of LM broth were added. The cultures were warmed to 37°C in a water bath, and then the tubes were transferred to a shaking incubator for 1 hour at 37°C.

Finally an appropriate volume of transformed competent cells, usually 10% and 90%, were spread onto selective agar plates with a sterile glass spreader. The medium was incubated at a suitable temperature until bacterial colonies appeared.

2.7.3. PCR product cloning

Some polymerases such as Taq-polymerase have specific extension activities. This characteristic will decrease ligation and cloning efficiencies of the PCR product. Therefore, unless it is known which base is extended onto the end of the completed PCR molecules, blunt-ended cloning should be used. With the one-tube PCR cloning method, polishing and ligation is completed in one step. The components were as follows:

- 10µl of the purified PCR product
- 2µl 10× T4-DNA ligase buffer
- 2µl T4-DNA ligase (2u/µl)
- 5µl T4-DNA polymerase (1u/µl)
- 0.5µl dNTPs (2.5mM)

The reaction mixture was incubated for 1-2 days at 16°C and then was used in transformation procedure directly.

2.8. DNA Hybridisation procedure

2.8.1. Radio labelling of DNA fragment

30- 50 ng of the DNA fragment (28µl total volume) was boiled for 5 min and immediately held on ice for 2min. The components for radio labelling were as follows:

- 10µl OLB* buffer
- 2µl BSA (10mg/ml)
- 5µl P^{32}-dCTP (50 µCi)
- 2µl klenow enzyme (2u/µl)
- Sterile distilled water (up to 50µl)

The mixture was incubated at room temperature overnight and the reaction was stopped by adding 5µl of 10%SDS.

* Preparation of OLB buffer:

Solution O: (1.25M Tris-Cl, 0.125M $MgCl_2$), pH: 8.0

Solution A: To 1ml of solution O added the following components:

- 18µl 2- mercaptoethanol,
- 5µl dATP, 5µl dGTP, 5µl dTTP [each as a 0.1M stock in (3mM Tris-Cl, 0.2mM EDTA, pH: 7.0)] store at -20°C

Solution B: 2M HEPES, titrated to pH: 6.6 with 4M NaOH. Store at -20°C

Solution C: Hexadeoxyribonucleotides in TE buffer at 90 OD units/ml. Store at –20°C

Mix solution A: B: C in a ratio of 100: 250: 150 to make OLB buffer, store at -20°C

2.8.2. Purification of probe using Sephadex G-50 chromatography

A 10% suspension of Sephadex G-50 was prepared in sterile distilled water. The solution was vortexed completely, washed with sterile distilled water several times to remove unsettled Sephadex particles, and stored at room temperature under distilled water. To make a column, the bottom of the 10ml plastic pipette was blocked by sterile cotton wool and Sephadex solution was added to make a column of about 10cm. Immediately afterwards, the column buffer was added to fill the column.

Column buffer: - (0.15M NaCl, 10mM EDTA, 0.1% SDS, 50mM Tris-Cl, pH: 7.5)

Prior to loading the labelling reaction onto the column, 200µl of column buffer was added and the labelling mixture was pipetted onto the column. The purified labelled

DNA was collected at the bottom of the column from unincorporated sample and stored at -20°C until required.

2.8.3. Preparation of Southern blots

The DNA samples were digested with suitable restriction enzymes, electrophoresed in a 0.7-1% agarose gel, and after staining with ethidium bromide the gel was photographed with the ruler side beside it to estimate the correct size of the fragments after hybridisation. Gels known to have DNA fragments greater than 10kb were soaked in 0.25M HCl for 10-15 minutes or until the colour of loading buffer changed to yellow, and washed twice with distilled water. After that, the gel was soaked in denaturation buffer with shaking for 30 min and rinsed twice with distilled water.

Denaturation buffer: - 1.5M HCl, 0.5M NaOH

The gel was then soaked in neutralization buffer for 30 min with shaking. After washing the gel twice with distilled water the blot was set up.

Neutralization buffer: - 1.5M NaCl, 0.5M Tris-Cl pH: 7.2, 0.001M EDTA

A reservoir of 20×SSC was set up, a platform was placed over this reservoir and three pieces of Whatman 3mm paper (pre soaked in 20× SSC) put on the platform with its ends dipped into the reservoir.

The gel was placed on the 3mm paper (wells uppermost) and a piece of Hybond-N nylon membrane was placed on top of it (the same size of the gel and pre-soaked in 20×SSC). Any air bubbles between gel and nylon were removed carefully then 3 sheets of Whatman paper were placed on top (the same size of the gel and pre soaked in 20×SSC). Finally several paper towel (10 cm high) were placed on top,

stock covered with a glass plate and a 500g weight placed on top of it. The blot was left for at least 16hr.

20×SSC: - 3M NaCl, 0.3M Na Citrate pH: 7.0

After transfer, all the sheets above the nylon membrane were removed carefully, the position of the wells marked on the membrane, and the membrane was washed in 2×SSC. The nylon membrane was air dried for up to 1hr and then wrapped in cling film. The membrane was exposed to UV light for 2 minutes (each side) to fix the DNA fragments to the membrane.

2.8.4. Hybridization of Radio-labelled probes to Southern blots

Hybridization reactions were carried out using Techne hybridization tubes in a Techne Hybridizer HB-1 oven. The blots were put inside the hybridization tube and 200µl of pre-hybridization solution was added per cm^2 of filter (usually 25-30ml per blots).

Pre- hybridization solution: - 5×SSC,

- 5× Denhardt's solution (1% ficoll, 1% poly vinyl pyrrolidone, 1% BSA fractionV)
- 0.5% SDS
- 0.1% pyrophosphate
- 100µg/ml denatured Salmon Sperm DNA

The tubes were incubated at 65°C for 2hr with rotation in oven. At the end of this period 100- 200µl of the labelled probes (denatured by boiling for 5 min and immediately put in an ice bath for 2 minutes) were added to the tube. The tube was incubated in oven at 65°C for at least 12hr. The pre-hybridization solution was poured

off and the blots were washed within the tube twice with 2× SSC, 0.1% SDS for 10 min at room temperature followed by one wash in 0.1× SSC, 0.1% SDS for 15 min at 65°C or until ≤20 cpm was obtained by Geiger counter. The filter was wrapped in cling film.

2.8.5. Detection of hybridization blot

The labelled blots were placed onto Whatman paper and put inside the film holder with the intensifying screen on top of the filter. In the dark room a sheet of X-ray film was either pre-flashed once to increase its sensitivity, or without pre-flash, placed between the intensifying screen and the blot. Exposure was carried out at -80°C for varying amount of time depending on the probes and the counts obtained. The exposed films were developed using the automatic X-ray film processor.

2.8.6. Stripping blots for reuse

Blots were stripped by adding a boiling solution of 0.1% SDS and were then allowed to cool to room temperature. Stripping was usually allowed to proceed for at least 12hr, after that the filter was wrapped in cling film and exposed again to check that probes were removed completely.

2.9. DNA Sequencing

Sequencing of the DNA samples were done by DNA sequencing service. DNA sequencing was carried out with an Applied Biosystems 373A or 377A DNA

sequencer, using double stranded DNA templates and dye terminator chemistries. Usually, the universal 21m13 forward and reverse primers were used. The DNA Strider programme was used to determine the Restriction endonuclease and ORF maps of DNA fragments. Nucleotide sequence searches were carried out using Blastn programme. Amino acid sequences were analysed by BlastX for homology with protein database programmes.

2.10. Mutagenesis

2.10.1. Gene replacement mutagenesis

Gene replacement mutagenesis was carried out according to the Quandt & Hynes method (Quandt & Hynes, 1993). Three suicide vectors were used in this method, pJQ200uc1, pJQ200mp18 and pJQ200SK. These vectors contain the following elements: The RP4 origin of transfer (which allows the mobilization of the constructs into most Gram-negative bacteria), *sacB* gene of *Bacillus subtilis* as a source of positive selection for loss of the vector, and the *lacZ*α gene for easy identification of cloned fragments. The only difference between these vectors is in the restriction enzymes available in their multiple cloning sites (MCSs), thus allowing the simplified cloning of DNA fragments.

The pJQ200uc1 was used in this work to carry out gene replacement experiments leading to the insertional inactivation of chemotaxis genes. Genes to be mutated were ligated into pJQ200 vector. The 1.2kb EcoRI fragment of pDUB2033, containing a neomycin resistance cassette, was then ligated into the gene and positive constructs obtained by selection on plates containing gentamycin and neomycin.

2.10.2. In-frame deletion method

In-frame deletion mutagenesis was carried out according to the Schafer method (Schafer, A., et.al., 1994). According to this method by deleting single gene, all other genes are left correctly in-frame, hence allowing phenotypic studies of the effects of mutating a single gene. The basic vector used in this work was the allelic exchange vector pK18mobsacB. The vector constructed from mobilizable pK18 and contains The *Km®* gene, a β-galactosidase gene for identification of correct transformants, genetically modified *sacB* gene, which confers sucrose-sensitivity to bacterial hosts, multiple cloning sites (MCS) and a functional mob site for conjugation into Gram-negative bacteria. The gene to be deleted plus 200-500bp of the upstream and downstream flanking sequence was cloned into pBluescriptSK+. The coding region of gene was deleted using a protocol based on the PCR reaction. PCR primers (sense and antisense) were designed to amplify the regions of the flanking sequences of the gene and also the vector backbone. Nucleotide sequencing of primers contains only the first 9bp or the last 3bp of the gene, but also had an additional restriction enzyme site at their 5' termini plus 2-5 extra bases.

After PCR, the DNA products were electrophoresed, isolated and digested with suitable enzyme then religated and transformed into *E. coli (DH5α)*. Sequencing of the fragment usually carried out to confirm that the PCR reaction and religation had taken place correctly. The constructs were isolated from bacterial strains and digested with suitable enzymes to excise the fragment containing the specific gene deletion plus flanking sequence and cloned into pK18mobsacB vector. The final construct was mobilised into *A. tumefaciens (C58C1)* by tri-parental mating.

pK18*mobsacB* is only able to survive in *A. tumefaciens* by integration into the genome via homologous recombination. The first transconjugants were selected on LM-agar plates containing rifampicin and kanamycin. The genotype of resulting colonies was confirmed by PCR and Southern blotting. To select double cross-over events a single colony, previously identified, was grown for 24 hours in LB medium containing rifampicin only. Serial dilutions of the culture were plated onto MinA-agar plates containing rifampicin and 10-15% sucrose, and incubated at 28-30°C for 48 hours. The resulting colonies were then picked from plates and replica streaked onto LM-agar containing rifampicin only or rifampicin plus kanamycin.

Recombinants were sensitive to kanamycin, indicating the excision of the plasmid by second cross-over events. This second cross-over either restored the wild type situation or led to a mutant with the deletion left in the chromosome. The mutants were checked once again by PCR analysis and Southern blotting.

2.11. Conjugation of plasmids into *Agrobacterium*

The final plasmid clones resulting from the mutagenesis process maintained in *E.coli* were mobilised into *Agrobacterium* by the pRK2013-based tri-parental mating system (Ditta et al., 1980). Cultures of donor, recipient and helper strains were grown to mid log phase in selective media. Then the cell cultures were sedimented by centrifugation (4000rpm, 5min) and resuspended in the LB broth to the same volume as before. For all matings, 300µl of recipient cells, 200µl of donor cells and 150µl of helper plasmid were pipetted into 1.5ml Eppendurf tube. The mixture was centrifuged for 30-60 seconds and resuspended in 100µl of LB broth by pippeting. The mating

mixture was dropped onto a sterile nitrocelulose filter (0.22μm pore size), which was placed on non-selective LM agar plate.

The plates were incubated at 28-30°C for overnight to allow to mating occur. After the mating period, the discs were transferred to a universal bottle containing 10ml of 10mM $MgSO_4$ and vortexed to wash the cells. Serial dilutions then were made from the bacterial suspension and plated onto selective medium. Selection plates were incubated at 28°C for 2 to 3 days.

2.12. Swarming behaviour assay

Swarm plates were prepared as follows. To LabM nutrient broth, bacteriological agar was added at a concentration of 0.2-0.22%. Usually 3μl sample of cells grown in LB medium were placed on swarm plates and incubated at 28-30°C for up to three days. The swarm- ring size was measured after 10, 24, 48 and 56hr.

2.13. Chemotaxis assay

2.13.1. Preparation of attractant

Stock solutions of attractant were prepared from 10^{-2} to 10^{-4} M using ethyl acetate: methanol (1:1) as a solvent. Solutions (10^{-5} to 10^{-8} M) were prepared for assay by dilution of the stocks with chemotaxis media. The control solution consisted of chemotaxis media with an equal concentration of solvent but no attractant.

Chemotaxis media: - (0.1 mM EDTA, 0.01 M KH_2PO_4 (pH:7.0).

2.13.2. Blind-well chamber assay

Exponential phase cultures of each *Agrobacterium tumefaciens* mutants were grown for 24 hr in MinA+G, then were washed twice with chemotaxis medium. The pellet was resuspended in chemotaxis media to a cell density of about OD= 0.1 at 600nm. The lower chamber was filled with 200µl of cell suspension, sealed with 13mm diameter filter (Sartorius, 8µm pore size), and 400µl of attractant was added into upper chamber. The chamber was inverted for 3hr at 28°C. After that, 200µl was removed from upper chamber into 20ml IsotonII and the number of bacteria count using coulter counter.

The method selected for quantitatively measuring chemotaxis has been modified from Adler (1969) and it is more quickly than which measures, by viable plate counts, the number of bacteria moving into a capillary tube under a chemotactic stimulus. The capillary assay is slow and need over night incubation of the plated bacteria (Armitage, J. P., et al., 1977).

The chemotaxis assay presented uses a Coulter counter to measure the percentage of a suspension of motile bacteria passing through a membrane filter in a determined time, into a cell free buffer, containing a chemotactic stimulus.

Chemotactic chamber: In blindwell chemotactic chambers used in this experiment, the lower wells had a capacity of 200µl, and the upper wells had a capacity of 400µl; a cellulose nitrate membrane filter (13mm diam., 8µm pore size) was clamped between the two chambers.

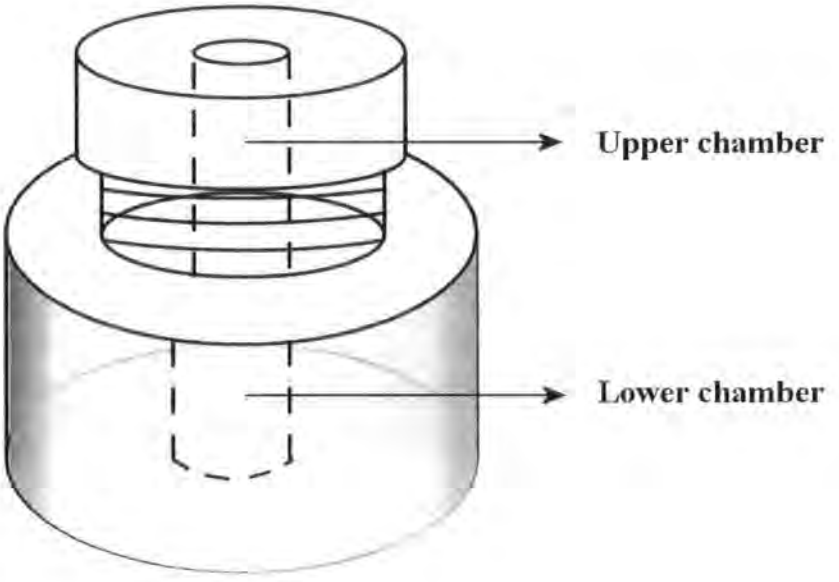

Fig. (2.12.1): The schematic diagram showing blind well chamber

Chemotaxis media: A chemotactic media, which consisted of 0.01M-potassium phosphate buffer pH: 7.0, (containing 0.1mM EDTA) was used. This medium provides conditions necessary for optimum motility of bacteria. The EDTA was added as a chelating agent because distilled water can contain traces of heavy metal ions, that inhibit motility. A buffer system was used to keep the pH at the optimum.

Effect of growth condition and stage of bacterial growth: The MinA media plus 20% glucose (final concentration of 0.2%) was used as a growth medium. Media was inoculated by bacteria and allowed to grow to stationary phase (overnight) at

28°C with rotary shaking. Some bacteria e.g. *E. coli* without shaking the bacteria were poorly motile. Bacteria in the early exponential phase are the best sample for chemotaxis.

Requirement of wash media: Bacterial cells should be washed to make a suspension of bacteria free of the growth medium to remove any unwanted attractants or repellents, and to reduce the high ionic strength of the growth medium which could be inhibit the motility.

Chemotaxis measurement: chemotaxis was measured by adding 200µl of bacterial suspension in upper chamber, after 2hr incubation at 28°C, to 20ml IsotonII and counting using coulter counter according to manufacturer's instruction.

IsotonII: 7.9g NaCl, 1.9g Na_2HPO_4, 0.4g EDTA, 0.4g KCl, 0.2g NaH_2PO_4, 0.3g NaF, per 1000ml of water and filter sterilized

Mechanism of measuring by coulter counter: The Coulter method of sizing and counting of particles is based on measurable changes in electrical impedance produced by non-conductive particles suspended in electrolytes. A small aperture between electrodes is the sensing zone through which suspended particles pass. In the sensing zone, each particle displaces its own volume of electrolytes. The volume displaced is measured as a voltage pulse, the height of each pulse being proportional to the volume of the particle. The quantity of suspension drawn through the aperture is precisely controlled to allow the system to count and size particles in an exact volume. With this method several thousand particles per second are individually counted and sized.

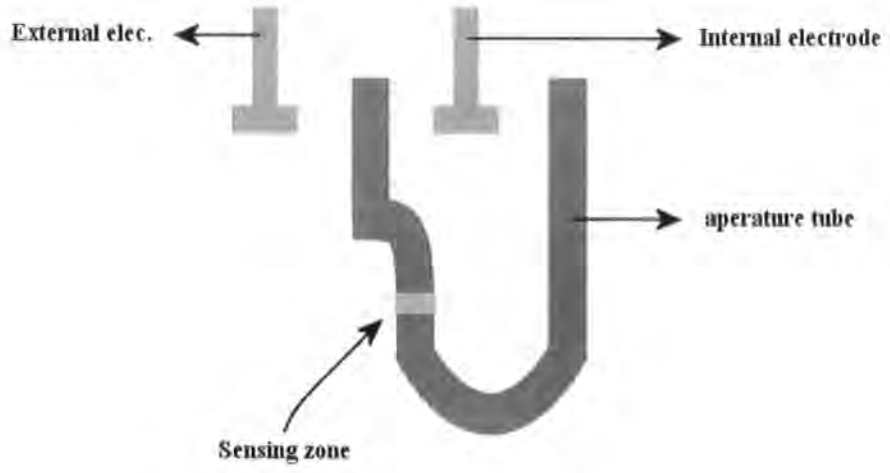

Fig. (2.12.2): A schematic diagram showing the sensing zone of coulter counter.

An aperture size of 30μm was used and the Coulter counter was set up to measure sizes between 0.6μm and 8μm. The system was set up to count 100μl of sample during 30 seconds. Before start the counting of the samples, the background of IsotonII alone was determined and when the count was less than 500 then the counting of samples were started. For each sample reading was done two times and each sample counted at least two times.

The number of particles per ml was calculated as follows:

n: number of particles counted in 100μl/30second

N= 200×n = number of particles per 20ml of Isoton plus 200μl sample

N= number of particle in 200μl of sample

x= 5×N = number of particle per 1ml of sample

X= [x1* + x2**]: 2 – x3***

*x1: number of particle counted/ml at the first reading

**x2: number of particle counted/ml at the second reading

***x3: number of particle counted/ml of IsotoneII alone

X= Net number of particle counted/ml of sample

Presentation of results: As discussed Coulter counter readings were converted to numbers of cells per ml. Control values (parallel assays using chemotaxis medium as attractant) were subtracted from each number/concentration to give a correct number of cells attracted by the acetosyringone. The number of cells attracted were presented graphically and also presented in the form of the chemotaxis index (CI).

Chemotaxis index: The CI is a measure of the proportion of cells in the bacterial population attracted towards the attractant. The CI corrects for differing initial cell density, and arises from the observation that for a given strain, the proportion of cells attracted to a particular attractant concentration is constant (Loak, et.al, 1992).

$$CI = \frac{\text{[Cells in upper chamber] - [cells in control assay upper chamber]}}{\text{[Cells initially introduced to lower chamber]}} \times 100\%$$

Chapter3

Identification of the MCP-encoding gene in *A. tumefaciens*

3.1. Background

As part of the signal transduction pathway involved in chemotaxis in *E. coli*, methyl groups are transferred to MCPs from S-adenosyl methionine. A number of observations provide evidence to suggest that a similar system operates in *A. tumefaciens*: (1) antisera raised to transducer Trg of *E. coli* cross reacts with proteins of approximately 60 – 65 kDa in *A. tumefaciens* (Morgan D. G., et al., 1993). (2) The periplasmic domain of VirA contains a sequence conserved with the MCPs, and which ChvE interacts (Cangelosi, G. A., et al., 1990). (3) *A. tumefaciens* met-auxotrophs do not display chemotaxis unless supplemented with methionine. Partial chemotaxis can be restored by supplementation with substrates for S-adenosyl methionine synthase (Shaw, C. H., 1996). (4) The *orf1* of chemotaxis cluster of *A. tumefaciens* shows the greatest homology with known MCPs of *C. crescentus* (McpA), *R. sphaeroides* (TlpA) and *E. coli* Tar and like Tlp appears to lack a membrane-spanning domain (Wright, E. L., et al, 1998). (5) Oligonucleotide probes corresponding to the conserved signalling domain of *E. coli* MCPs hybridise to a number of DNA fragments in *A. tumefaciens* (Shaw, C. H., 1996).

The results of a Southern blot using a 0.7 kb MCP fragment from plasmid MCP3.2.7P of *Rhizobium leguminosarum* digested with *Hind*III/*Pst*I as a heterologous probe showed that 1.7 kb *Eco*RI fragment of a cosmid library of pDUB1911 from *A. tumefaciens* chromosomal DNA, contains a MCP-encoding sequence. This fragment was subcloned into pBluescriptSK+, forming the plasmid pELW6 (Wright, E.L., et al., 1998).

3.2. Sequencing of the MCP gene

pELW6 was sequenced fully in both directions. The plasmid was digested with suitable restriction enzymes, the resulting fragments were subcloned in cloning vector, pBluescriptSK+, transformed into *E. coli (DH5α)*, and sequenced as discussed previously.

Table (3.2.1)

Plasmid	Relevant genotype
pELW61	1309 bp *Sac*II fragment isolated from pELW6 and subcloned into pBluescriptSK+
pELW62	363 bp *Sac*II fragment isolated from pELW6 and subcloned into pBluescriptSK+
pELW63	1190 bp *EcoR*V fragment isolated from pELW6 and subcloned into pBluescriptSK+
pELW64	1373 bp *Xba*I – *Hind*III fragment isolated from pELW6 and subcloned into pBluescriptSK+
pELW65	547 bp *Hinc*II fragment isolated from pELW6 and subcloned into pBluescriptSK+

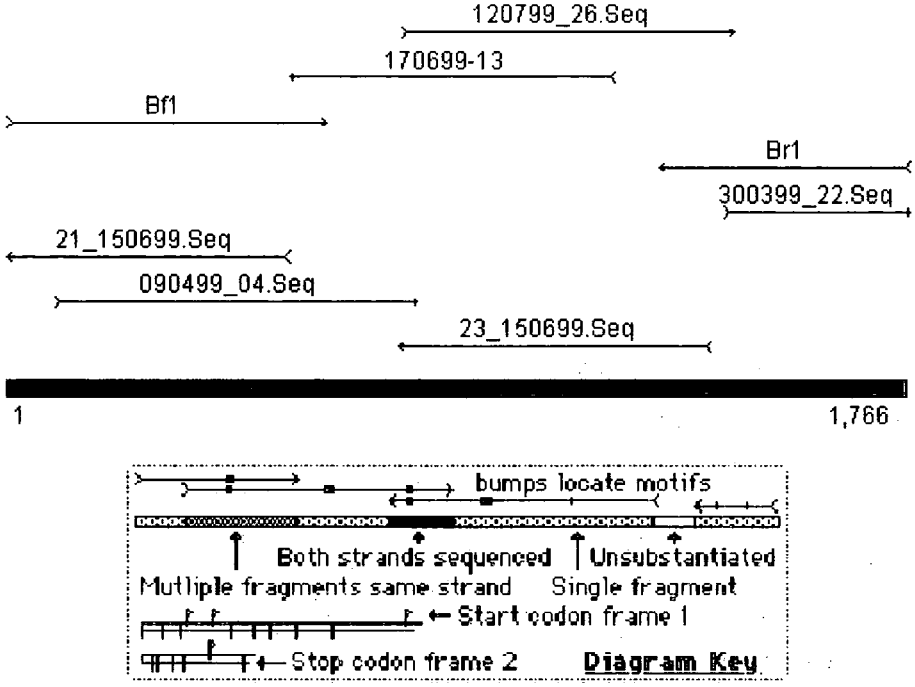

Fig (3.2.1): Diagram showing sequencing of pELW6 using sequencer program

Fig. (3.2.2): Nucleotide sequencing of pELW6 (1765 bp)

pELW6 was analysed by Blastn for homology with *Agrobacterium tumefaciens* strain C58 complete genomic sequence.

```
ref|NC_003062.1| Agrobacterium tumefaciens strain C58 circular chromosome, complete
            sequence
          Length = 2841581

 Score = 3420 bits (1725), Expect = 0.0
 Identities = 1759/1765 (99%), Gaps = 4/1765 (0%)
 Strand = Plus / Plus


Query: 1      gaattcggcaatatcgcccgcgctctggtgattttccgcgagaatgcgattgagaagctg 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 516354 gaattcggcaatatcgcccgcgctctggtgattttccgcgagaatgcgattgagaagctg 516413


Query: 61     gcgatcgagggcaaaagtgcgcaggaacggctccgcggccgagtccgagcgacaccgcaa 120
              |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct: 516414 gcgatcgagggcaaaagtgcgcaggaacg-ctccgcggccgagtccgagcgacaccgcaa 516472


Query: 121    cgatgccgagaagcaggaactggacggccagatcgagtttgcagtgggcgagatcgcctc 180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 516473 cgatgccgagaagcaggaactggacggccagatcgagtttgcagtgggcgagatcgcctc 516532


Query: 181    cggcctcggcaggctttcgcgtggcgatctgagccgcacgatcgagacgccctttgcagg 240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 516533 cggcctcggcaggctttcgcgtggcgatctgagccgcacgatcgagacgccctttgcagg 516592


Query: 241    ccgcctcgaccggctgcgcacggatttcaacgaatccttgctcaaccttcgcgatgcgct 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 516593 ccgcctcgaccggctgcgcacggatttcaacgaatccttgctcaaccttcgcgatgcgct 516652


Query: 301    ggggcagatccgtgagcgcacgctcatcattcagaatagtggcatcgaaatcgaacagtc 360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 516653 ggggcagatccgtgagcgcacgctcatcattcagaatagtggcatcgaaatcgaacagtc 516712


Query: 361    ttcggtcgatctgtcgaaacgcacggaaaatcaggccgcctcgctggaggagaccgccgc 420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 516713 ttcggtcgatctgtcgaaacgcacggaaaatcaggccgcctcgctggaggagaccgccgc 516772


Query: 421    tgccgtggaggagatcaccgctaccgtgagatcgtcggccgagcgggcacgggaggcaaa 480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 516773 tgccgtggaggagatcaccgctaccgtgagatcgtcggccgagcgggcacgggaggcaaa 516832


Query: 481    tgaggccgtacgcgtcaccaagcagagcgccgacagttccggttcggttgtcagcaatgc 540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 516833 tgaggccgtacgcgtcaccaagcagagcgccgacagttccggttcggttgtcagcaatgc 516892


Query: 541    cgtcgacgccatgagccgtatcgaaggcgcctcgcgcaagatcgagcagatcatcgaggt 600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 516893 cgtcgacgccatgagccgtatcgaaggcgcctcgcgcaagatcgagcagatcatcgaggt 516952


Query: 601    catcgacgacatcgccttccagaccaatcttctcgcttctcaatgccggcattgaagcgg 660
              |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct: 516953 catcgacgacatcgccttccagaccaatcttctcgct-ctcaatgccggcattgaagcgg 517011


Query: 661    cgcgcgcgggtgaggcgggcaagggttttgccgtcgtggcgcaagaagtgcgcgaacttg 720
              |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct: 517012 cgcgcgcgggtgaggcgggcaagggttttgccgtcgtggcgcaggaagtgcgcgaacttg 517071


Query: 721    cccagcgctcttgccgacgcaacccgtgaaatcaagcagctcatcaaccagtcgacccat 780
```

```
                ||||||||||| ||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct: 517072 cccagcgctct-gccgacgcagcccgtgaaatcaagcagctcatcaaccagtcgacccat 517130

Query: 781    gaggtcagctccggttcgaagctggtgcaggaggcgggcaccgttctctccgcccatcag 840
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct: 517131 gaggtcagctccggttcgaagctggtgcaggaggcgggcaccgttctctccgcc-atcag 517189

Query: 841    ccggcagatcgtgactgtcagccagcatgtcgaaaccatcgcgacggcgacgcaggacca 900
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 517190 ccggcagatcgtgactgtcagccagcatgtcgaaaccatcgcgacggcgacgcaggacca 517249

Query: 901    gtcctcagcccttcacaacgtcaacggctccgttaaccagatggaccagatgacgcagca 960
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Query: 1021   ggcgctgctcgatctggtacagcggttccagatggagcaggggtctgctgccggttcggg 1080
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Query: 1081   tcgattgaaccgggcggcctgatctctccatttttgaaagtcaatgcagctcccggatcg 1140
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Query: 1141   caaggtccgggagtttttattcggccgctgcccgcgccgtaccggatatctggttgatat 1200
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Query: 1201   aggcacgcaacgccgcaggcttaaccggtttgtgctgcacggagatcccgtatttctccg 1260
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Query: 1261   catcgctgcgcacttccgggctgcgatccgccgttaccagcagggcagggatcgtcttgc 1320
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 517610 catcgctgcgcacttccgggctgcgatccgccgttaccagcagggcagggatcgtcttgc 517669

Query: 1321   cgtaaaatgtcctgatgaggcggatggcgctgataccgtcaccatcatctagatgatagt 1380
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 517670 cgtaaaatgtcctgatgaggcggatggcgctgataccgtcaccatcatctagatgatagt 517729

Query: 1381   cggcgatgatgacatccggggccgcggccagagtgaggaagggttcttccagcgccgcca 1440
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Sbjct: 517730 cggcgatgatgacatccggggccgcggccagagtgaggaagggttcttccagcgccgcca 517789

Query: 1441   cggaacctgcgggcaagacatcgcaaccccatcccgtcaaaagcagcgtcatgccctcga 1500
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Sbjct: 517790 cggaacctgcgggcaagacatcgcaaccccatcccgtcaaaagcagcgtcatgccctcga 517849

Query: 1501   ggattttcggctcgttgtcgatgcacagcacccgaatgccatgaagacggtcgcttgctg 1560
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 517850 ggattttcggctcgttgtcgatgcacagcacccgaatgccatgaagacggtcgcttgctg 517909

Query: 1561   cgggactggttgtcccgccctccgtcttggcaggagcaagacgatccgcctcacgcggca 1620
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 517910 cgggactggttgtcccgccctccgtcttggcaggagcaagacgatccgcctcacgcggca 517969

Query: 1621   gatggatgcggaaggtcgtgcctttgccgggggtggaaatgagctgcaccggatggtgca 1680
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Query: 1681   gcatgcgtgatagccgatcgacgatggaaaggccgagcccaaggccagacgccgtttttg 1740
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Sbjct: 518030 gcatgcgtgatagccgatcgacgatggaaaggccgagcccaaggccagacgccgtttttg 518089

Query: 1741   ccccttcgtccagccgggcgaattc 1765
              |||||||||||||||||||||||||
Sbjct: 518090 ccccttcgtccagccgggcgaattc 518114
```

Fig. (3.2.3): Comparison of nucleotide sequences of pELW6 with *Agrobacterium tumefaciens* strain C58 complete genomic sequence.

The subclones were sequenced and the deduced amino acid sequences were analysed by BlastX for homology with the protein database. One open reading frame, that when translated, showed a strong identity to Mcp gene. The highest alignment scores occurred with the *McpA* gene recently identified from *A. tumefaciens* (Goodner, B. et al., 2001), the Mcp gene of *Rhizobium sp.* (Frieberg, C., et al., 1997), McpG of *R. leguminosarum* (Yost, C. K., et al., 1998) and McpA of *Caulobacter crescentus* (Alley, M. R., et al., 1992).

```
R.leguminosarum      MKNIMSFGVVARLALGFGVLLLLMVGLTIYSTEEVALINDKLGAMNDVNSVKQRFAINYR 60
C.crescentus         MKRIRLVDLPLIIKIGFAPAFALLM-LAVMAGGAILVQKSQSAALKQVVERDMRQNLEIQ 59
pELW6                ------------------------------------------------------------
A.tumefaciens_C58_   MALGNCFMHNRLFKSVAGKVVALTIGLITLSVAAVGFSTYIRLKDNIITTALRDTHGAMR 60

R.leguminosarum      GSVHDRAIAIRDVTLVTSDDE-RKTAEALI-----GKLAASYAENEKRMADMVASPAGAT 114
C.crescentus         RISKRISNINGELFVVMTHKAGNIDVDKND-----ARMAAVLVETDAVKKDLLALKSKLP 114
pELW6                ------------------------------------------------------------
A.tumefaciens_C58_   GMAILYEMKVGGVALEMVDGDLKSVGRASIGTMRDNDLVDRTAAGNGGIATVFEAKAGEF 120

R.leguminosarum      EQEKTILSEIADIQAKANPLVAQIIALQEKGDGEAARKILLEQARPAFVAWLGAINKFID 174
C.crescentus         AEEQPKIAELIKSLEECRSAIDTVSGMISVDFNMAAG--FIAPFEEQYVKMTGQLDQVVA 172
pELW6                ------------------------------------------------------------
A.tumefaciens_C58_   IRLTTNLKNEKGERAAGTKLATDHPAFEKVSKGEAYFGTATLFGTSYMTGYMPVTNKTGA 180

R.leguminosarum      YQEALNKSI-GGEVRSSASGFKPFALTALGIAAVLSLVAAAVTARTIVGPLAKLQLSLKA 233
C.crescentus         AANQRIESE-SAKRQAQATAAMSVTIIMSLLTLGAVGALAFLTVMTTRKSINDIAAATDK 231
pELW6                ------------------------------------------------------------
A.tumefaciens_C58_   TVGILFVGVPMDFYNAQIYSLRDMMVVCGALAMLGVGLLAYFVIKRTLQPLSKLTDAVKS 240

R.leguminosarum      MADGNLDGDRRLEARGDEIGKLARAVAGLRDAISAKAEREADAEAKRAVSERHRLEQDAD 293
C.crescentus         LSKGDNSIDLEKMTRGDELGGIVTALKVFRDNQVHLEQLRADQEKSAALTADERRSKEAA 291
pELW6                ----------------EFGNIARALVIFRENAIEKLAIEGKSAQERSAAESERHRNDAE 43
A.tumefaciens_C58_   LSDGDLETPIPYATNTNEFGNIARALVIFRENAIEKLAIEGKSAQERSAAESERHRNDAE 300
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                                    *:* :. *:  :*:        ...   . : :  .*  ::*

R.leguminosarum      ERRTLAEQTDKAVGQLGDALQALADGDLTQQIGTPFIPSLEKLRADFNSAVEKLRAAMQK 353
C.crescentus         AA-AAAQEASLVVSNLAEGLEKLASGDLTFRVTADFPGDYRKLKDDFNAAMGSLQETMKV 350
pELW6                KQ-ELDGQIEFAVGEIASGLGRLSRGDLSRTIETPFAGRLDRLRTDFNESLLNLRDALGQ 102
A.tumefaciens_C58_   KQ-ELDGQIEFAVGEIASGLGRLSRGDLSRTIETPFAGRLDRLRTDFNESLLNLRDALGQ 359
                           : . .*.::...*  *: ***:  : : *     :*: *** :: .*: ::

R.leguminosarum      VAQNASAIAAGAQEIRSASDDLAKRTEQQAASVEETAAALEEITTTVADSSNKAQEAGQL 413
C.crescentus         IAASTDGLSTGADEIAHASDDLSRRTEQQAASLEETAAALDELTATVRRTAAGARQASDV 410
pELW6                IRERTLIIQNSGIEIEQSSVDLSKRTENQAASLEETAAAVEEITATVRSSAERAREANEA 162
A.tumefaciens_C58_   IRERTLIIQNSGIEIEQSSVDLSKRTENQAASLEETAAAVEEITATVRSSAERAREANEA 419
                     :   :  :  .. **  :* **::***:****:******::*:*:**  ::  *::*.:

R.leguminosarum      VRKTKDSAERSGSVVRDAVDAMGKIESSATEIGSIIGVIDEIAFQTNLLALNAGVEAARA 473
C.crescentus         VSTTRGEATHSGQVVHQAVSAMGEIEKSSGQISQIIGVIDEIAFQTNLLALNAGVEAARA 470
pELW6                VRVTKQSADSSGSVVSNAVDAMSRIEGASRKIEQIIEVIDDIAFQTNLLALNAGIEAARA 222
A.tumefaciens_C58_   VRVTKQSADSSGSVVSNAVDAMSRIEGASRKIEQIIEVIDDIAFQTNLLALNAGIEAARA 479
                     *  *: .*  **.** :**.**..** :: :* .** ***:**************:*****

R.leguminosarum      GEAGKGFAVVAQEVRELAQRSAKAAKEIKELINASNGHVKSGVALVGETGKALKEIAEQV 533
C.crescentus         GEAGRGFAVVAQEVRALAQRSAEAAKEIKALISSSTQQVSQGVSLVGQTGEALQRIVTKV 530
pELW6                GEAGKGFAVVAQEVRELAQRSADAAREIKQLINQSTHEVSSGSKLVQEAGTVLSAISRQI 282
A.tumefaciens_C58_   GEAGKGFAVVAQEVRELAQRSADAAREIKQLINQSTHEVSSGSKLVQEAGTVLSAISRQI 539
                     ****:********** ******.**:*** **. *. .*..*  ** ::* .*. *  ::

R.leguminosarum      QQVDGNVGAIVGASQEQATGLKEINTAVNRMDQGTQQNAAMVEEATAASHNLAKEADALF 593
C.crescentus         GEIDALVTEIAASAAEQATGLNEVNTAVNQMDQVTQQNAAMVEQSTAATHSLKGETAELV 590
pELW6                VTVSQHVETIATATQDQSSALHNVNGSVNQMDQMTQQNAALAEQSSAASRVLSGEVEALL 342
A.tumefaciens_C58_   VTVSQHVETIATATQDQSSALHNVNGSVNQMDQMTQQNAALAEQSSAASRVLSGEVEALL 599
                       :.  *  *. :: :*::.*:::* :**:*** ******:.*:::**:: *  *.  *.

R.leguminosarum      QLLGQFNIGGAVAPKRASQPAAAASHAQPAPSPARQMIAKVG---KSFQTNGNAALAGD- 649
C.crescentus         RLMARFQVG-SGSSSYARPAVADAGHHAPARNPVAEQQARLNTFARPGRSSGSAALAQAP 649
pELW6                DLVQRFQMEQGSAAGSGRLNRAASLHFKSMQLPDRKVREFLFGRCPRRTGYLVDIGTQRR 402
A.tumefaciens_C58_   DLVQRFQMEQGSAAGSGRLNRAA---------------------------------------- 622
                      *: :*::  . :.  .    *

R.leguminosarum      ----WEEF---------------------------------------------------- 653
C.crescentus         ASDGWEEF---------------------------------------------------- 657
pELW6                RLNRFVLHGDPVFLRIAAHFRAAIRRYQQGRDRLAVKCPDEADGADTVTIIMIVGDDDIR 462
A.tumefaciens_C58_   ------------------------------------------------------------

R.leguminosarum      ------------------------------------------------------------
C.crescentus         ------------------------------------------------------------
pELW6                GRGQSEEGFFQRRHGTCGQDIATPSRQKQRHALEDFRLVVDAQHPNAMKTVACCGTGCPA 522
A.tumefaciens_C58_   ------------------------------------------------------------

R.leguminosarum      ------------------------------------------------------------
C.crescentus         ------------------------------------------------------------
pELW6                LRLGRSKTIRLTRQMDAEGRAFAGGGNELHRMVQHAPIDDGKAEPKARRRFCPFVQPGEF 582
A.tumefaciens_C58_   ------------------------------------------------------------
```

Fig. (3.2.4): Alignment of the pELW6 with McpA from *Caulobacter crescentus* McpA from *A. tumefaciens* (C58) and *R. leguminosarum*.

Analysis of the translated protein sequence strongly predicted a C-terminal cytoplasmic domain with homology to the methylation domains and signalling domain of the Mcp genes identified in other bacteria. As discussed earlier Mcp receptors are membrane proteins, about 550 amino acids in length. pELW6 contains part of an *Agrobacterium* Mcp gene.

Chapter4

Mutagenesis

4. Mutagenesis

In-frame deletion method

In this method a single gene was deleted leaving all other genes in frame. The basic allelic exchange vector used in this method is pK18*mobsacB*. This vector combines the useful properties of the pK plasmids (e.g., multiple cloning site, *lacZα* fragment, sequencing with M13 primers), and also has the Broad-Host-Range transfer (bhr) machinery of plasmid RP4 and a modified *sacB* gene from *Bacillus subtillis*. This construct can be transferred by RP4- mediated conjugation into *Agrobacterium*. The *sacB* gene confers sucrose sensitivity to *Agrobacterium* and transformants containing pK18*mobsacB* were not able to grow on media containing 10% sucrose.

Generally, gene that should be deleted with 200- 500bp DNA fragment ligate into pBluescript SK+, then two primers (usually 20-30bp length) containing only 9bp of the starting sequence of the gene, and 3bp of the end of the gene were designed and gene was deleted using PCR.The PCR product containing upstream and downstream flanking sequences of the gene were then ligated into pK18*mobsacB* vector and the final construct introduced into *Agrobacterium tumefaciens* using Tri-parental mating method.

pK18*mobsacB* is unable to replicate in *A. tumefaciens*, and transconjugants only arise after integration of the plasmid into the chromosome by homologous recombination. Intermediate strains containing constructs in pK18*mobsacB* were selected on media containing Rifampicin and Kanamycin. To enhance the double cross-over events, cells from first step were incubated overnight in media containing Rifampicin only; subsequently serial dilutions of culture were plated onto MinA

media plus 10% sucrose. After 3-4 days several colonies from each dilutions were selected and then sensitivity to Kanamycin checked. Colonies sensitive to Kanamycin indicated the excision of the vector during second crossing-over event. This second crossing-over event restored the wild type genotype, or led to a mutant with the deletion fragment left in the chromosome. The resulting mutant was then checked by PCR and Southern blot using the deleted fragment as a probe.

4.1. Mutagenesis of *cheY1* gene

pELW2, containing *cheY1,* was digested with *Pvu*II/ *Hinc*II, and after electrophoresis the 773bp fragment containing *cheY1*, a 264bp region upstream and 142bp region downstream of *cheY1* was isolated from the gel. This fragment was ligated into pBluescriptSK+ digested with *EcoR*V and transformed into *E. coli* (DH5α) to produce SK+/ cheY1. This new construct was checked by digestion with appropriate restriction enzymes. To delete the *cheY1* gene two primers were designed as below:

cheY1a (forward): 5'- GGACT CCATGG TTT CTT CAC TTT TGC ATC- 3'

cheY1b (reverse): 5'- GGACT CCATGG TGA TAC GGG ACA TTT CAC- 3'

Primer cheY11 contained upstream flanking sequence, the first 9bp of the *cheY1* gene, plus five extra bases (GGACT) and *Nco*I site (CCATGG) at the 5' end to use in religation of the PCR product. Primer cheY12 contained downstream flanking sequence, the last 3bp of the cheY1 gene, plus an *Nco*I site and five extra bases at the 5' end.

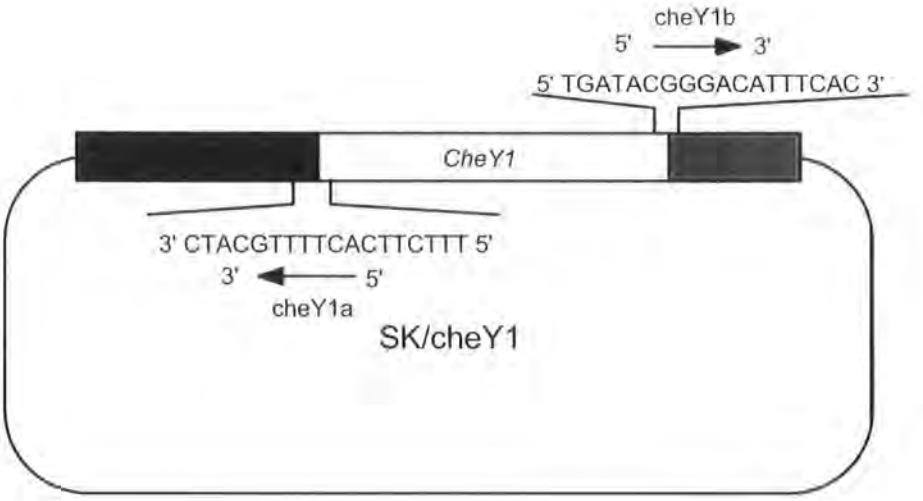


Fig. (4.1.1) Diagram of Sk/cheY1 plasmid showing sequence traces across deletion junctions and position of cheY1a and cheY1b primers.

Amplification by polymerase chain reaction was carried out using SK+/ cheY1 as DNA template. After amplification, 5µl of the PCR reaction was loaded onto an agarose gel, and separated by electrophoresis. The expected fragment of approximately 3.4 Kb (corresponding to the vector plasmid, and the upstream and downstream sequences flanking cheY1) was isolated from the gel (see fig. 4.1.2).

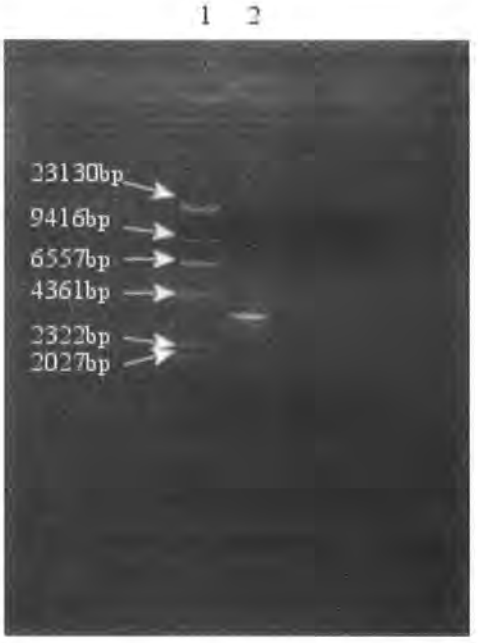


Fig (4.1.2): 0.8% agarose gel electrophoresis showing the expected fragment for *cheY1* in-frame deletion. Lane1: λDNA/HindIII lane2: in-frame deleted PCR product (SK/delY1).

The isolated PCR product was digested with *Nco*I, religated together using T4 DNA ligase and cloned into *E. coli*. This procedure gave no transformants. The PCR product was therefore re-isolated and treated in a one-step polishing and ligation reaction using DNA polymerase and T4 DNA ligase (see methods section).

The reaction was incubated at 16°C for 2 days, then transformed into *E. coli* to produce transformants containing the plasmid (SK+/ delY1). The plasmid was isolated from transformed cells and checked by digestion with *EcoR*I/ *Hind*III. The deletion produced in this plasmid was checked by DNA sequencing.

The new construct (SK+/delY1) was excised by digestion with *EcoR*I/ *Hind*III, separated by electrophoresis and the 490bp fragment containing the upstream and

downstream flanking sequences of *cheY1* was isolated from the gel. This fragment was ligated into pK18*mobsacB* previously restricted with *EcoRI*/ *Hind*III and the product was transformed into *E. coli* to generate the final construct (pK18/delY1). This new construct was checked by digestion with appropriate restriction enzymes and the result was confirmed by sequencing the plasmid DNA as well. The *E. coli* strain containing plasmid pK18/ delY1 has subsequently been used as a donor cell in a tri- parental mating.

Tri- parental mating method: Cultures of donor, recipient and helper strains were grown to mid log phase in selective media. Then the cell cultures were sedimented by centrifugation (4000RPM, 5min) and resuspended in the LB broth to the same volume as before. For all matings, 300µl of recipient cell, 200µl of donor cell and 150µl of cell containing helper plasmid were pipetted into 1.5ml Eppendorf tube. The mixture was centrifuged for 30-60 seconds and resuspended in 100µl of LB broth by pippeting. The mating mixture was dropped onto sterile nitrocellulose filters (0.22µm pore size), which were placed on non-selective LM agar plate.

. The mating mixture was incubated at 28- 30°C over night then serial dilutions were made and plated onto selective media (containing Rifampicin and Kanamycin). After 3 days incubation several colonies were picked and restreaked on the same media to produce the stable resistant intermediate colonies. Chromosomal DNA of some intermediate strains were isolated and checked by PCR to confirm the result.

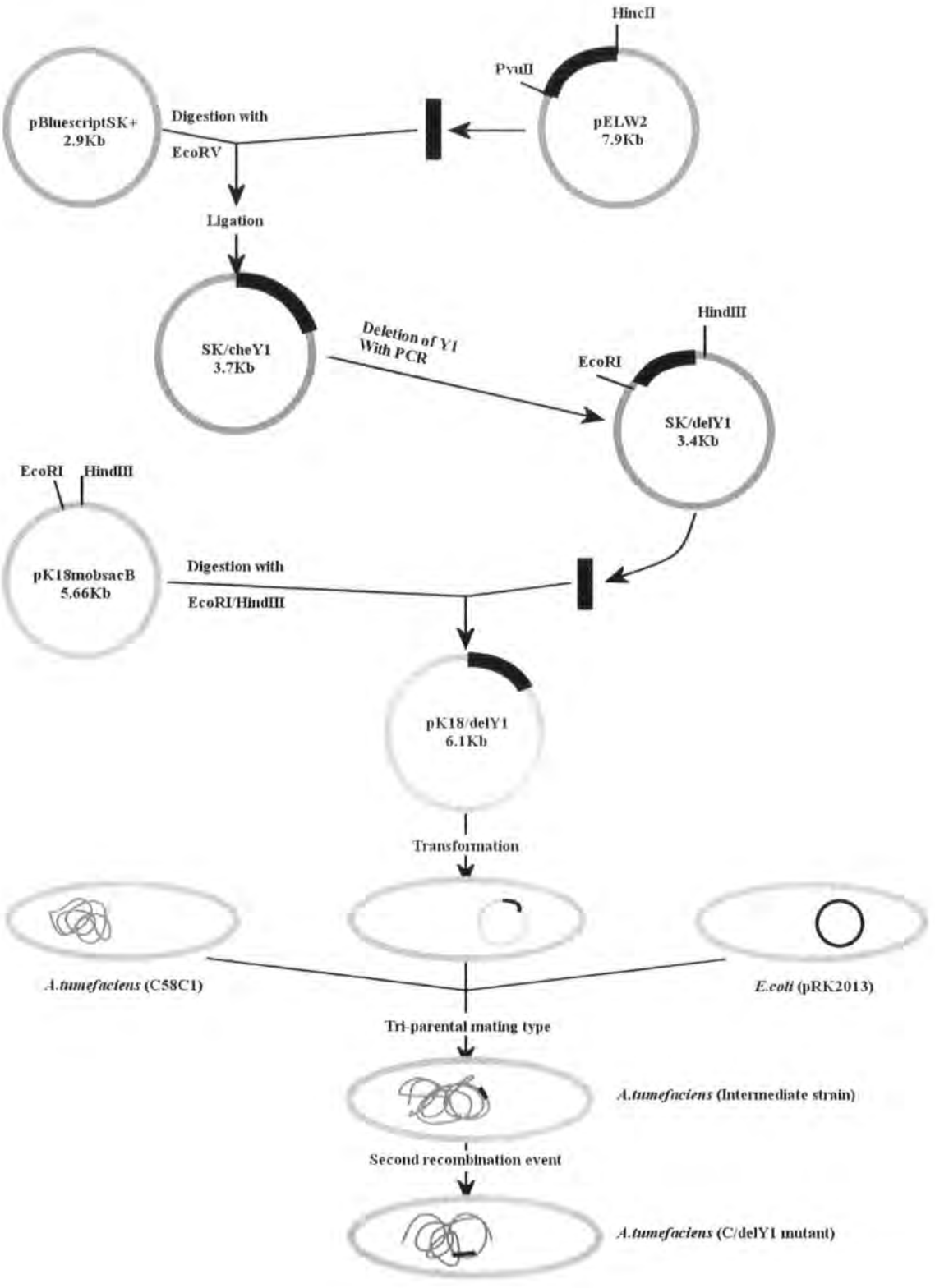


Fig (4.1.3): Diagram showing schematic steps for construction of *cheY1* in-frame deletion mutant of *A. tumefaciens*.

Polymerase chain reaction for mutant verification: Two primers were designed for use in PCR:

cheY11: 5'- GCT GGA TCT CAA CGA AGC AT-3'

cheY12: 5'- ACG GTC CCC GTC ATT CAA TT- 3'

After amplification the PCR reaction was separated by agarose gel electrophoresis. The predicted sizes of the PCR products from the wild type *cheY1* gene and the deleted *cheY1* gene were 773bp and 408bp, respectively. As shown in fig 4.1.2 some strains produced the expected fragments of wild type and intermediates.(wild type fragment about 773bp, intermediate strain fragments, 773bp plus 408bp deleted fragment).

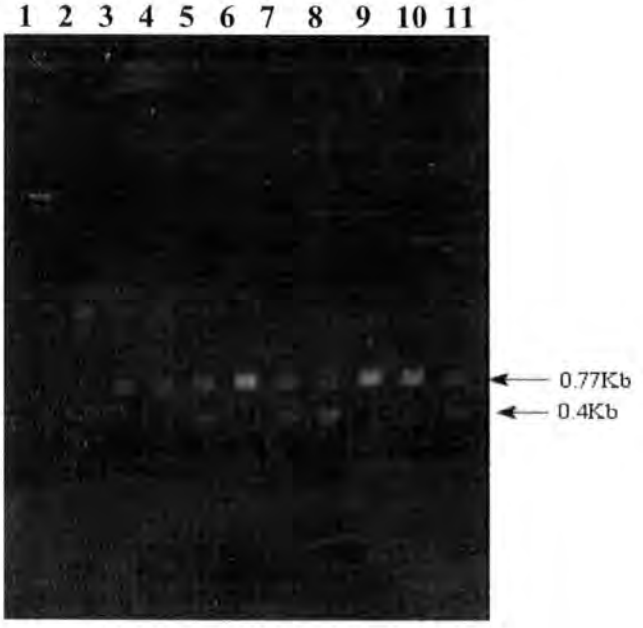


Fig. (4.1.4): 1% agarose gel electrophoresis of PCR product of intermediate strains of *A. tumefaciens* lane1: λDNA/*EcoR*I:*Hind*III, lanes 5, 7, 8 and 11 correct the intermediate strains.

Second recombination event:

Four intermediate strains that had show the expected fragments in PCR, indicating that they contained both wild-type and deleted *cheY1* genes, were grown overnight in liquid media containing rifampicin only. Serial dilutions were made and plated on MinA media plus 10-15% sucrose. After 3 days incubation at 28°C the number of colonies grown were as below:

Concentration	Number of colonies
Neat	> 200
10^{-1}	> 50
10^{-2}	~ 20
10^{-3}	1

Several colonies were picked up and restreaked on LA media containing Rifampicin then on media containing Rifampicin and kanamycin to check the sensitivity to kanamycin. Several kanamycin- sensitive colonies were grown in LB+ rifampicin media, and chromosomal DNA was isolated to check by PCR and Southern blot whether the desired deletion was present.

PCR was performed as described previously, and after the reaction, 5µl of PCR product was analysed by electrophoresis on 1% agarose gel (see fig. 4.1.5).

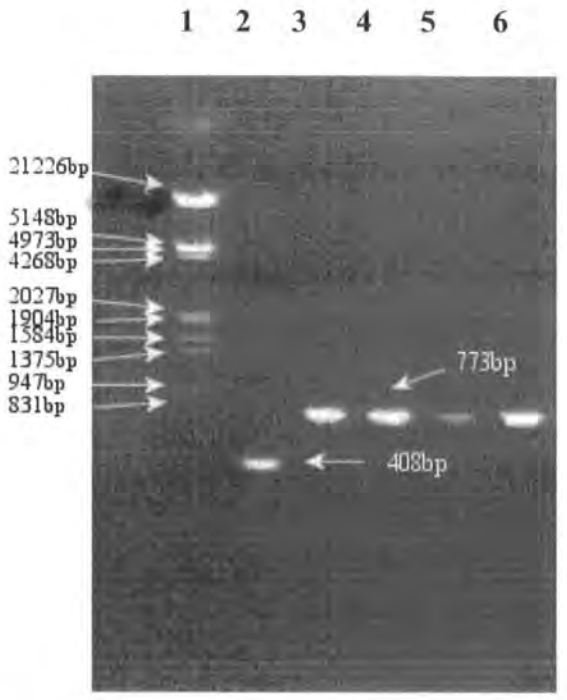


Fig (4.1.5): 1% agarose gel electrophoresis showing the PCR products from genomic DNA of *cheY1* in-frame deletion mutant strains produced using cheY1a and cheY1b primers. Lane1: λ/*EcoR*I:*Hind*III, lanes 2, 3, 4, 5, 6 possible mutant strains.

As shown in fig (4.1.5) only lane 2 (cheY1-12) showed the correct PCR product corresponding to a deleted fragment (408bp). This strain was selected as a mutant and was checked by Southern blotting.

Southern blot analysis of *cheY1* mutant:

The chromosomal DNA of two intermediate strains, one possible mutant, cheY1-12, and wild type strain (C58C1) were digested with *EcoR*V and *Hind*III. The Southern blot performed as described before. The *cheY1*-deleted fragment containing upstream and downstream flanking sequence of the *cheY1* was used as probe.

The following diagram shows the expected fragments for each strain:

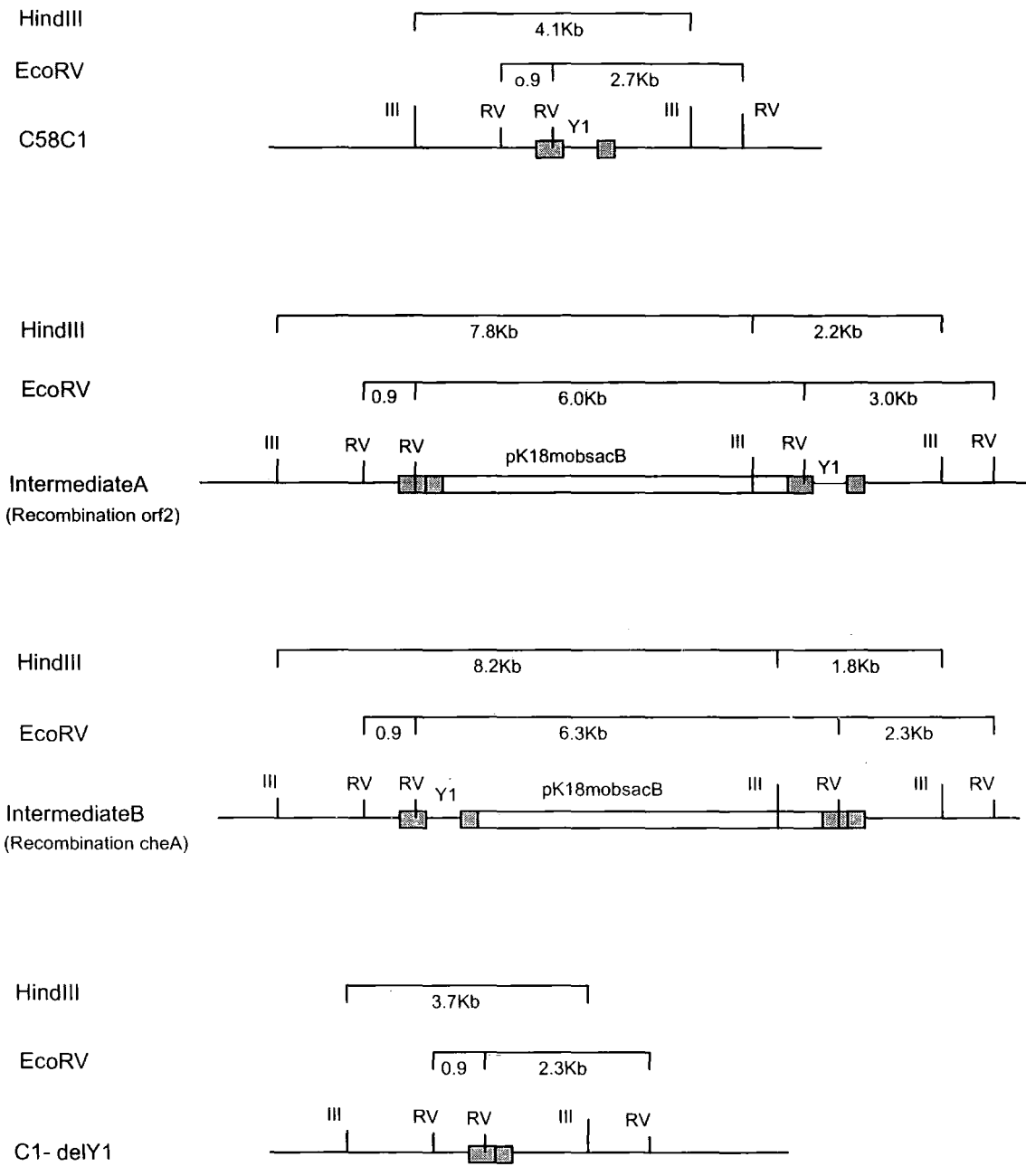


Fig. (4.1.6): Diagram showing the position and expected size of the *EcoR*V and *Hind*III fragments for each *A. tumefaciens* wild type, two possible intermediates and the *cheY1* in-Frame deletion mutant strains.

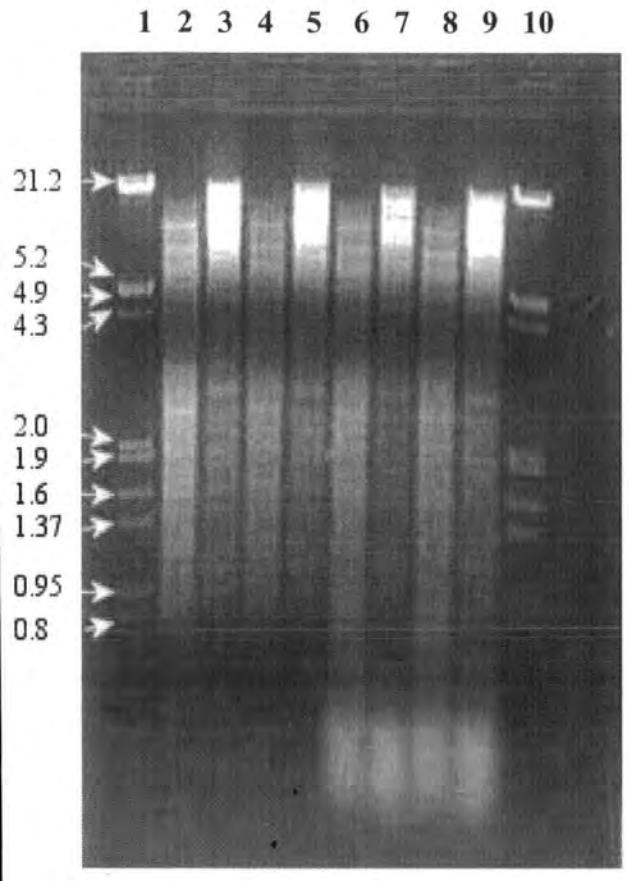


Fig (4.1.7)

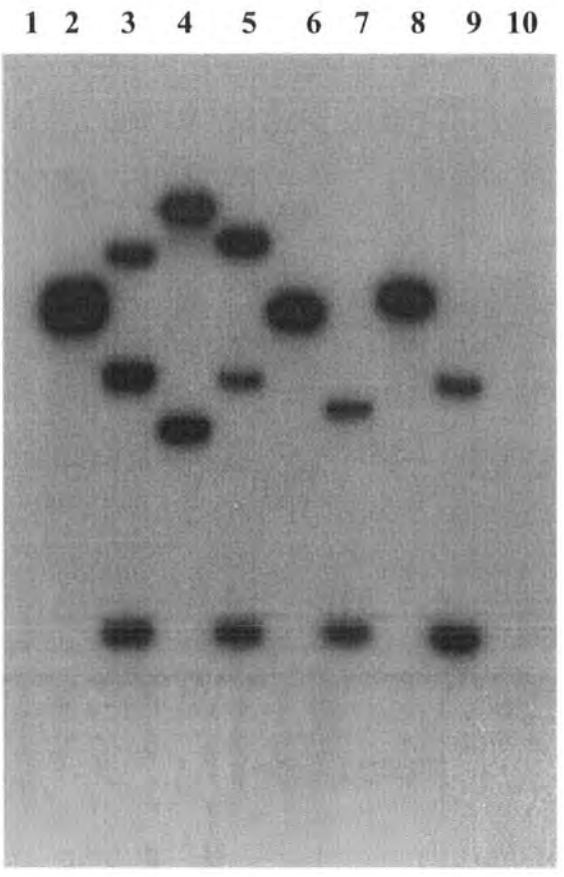


Fig (4.1.8)

Fig. (4.1.7): 1% agarose gel electrophoresis of chromosomal DNA from wild type, intermediate strains and *cheY1* in-frame deletion mutant (cheY1-12) digested by *Hind*III and *EcoR*V. Lanes 1, 10: λDNA/*EcoR*I:*Hind*III, lanes 2, 4: intermediate strains digested with *Hind*III, lanes 3, 5: intermediate strains digested with *EcoR*V, lanes 6, 7: *A.tumefaciens* mutant digested with *Hind*III and *EcoR*V respectively, lanes 8, 9: *A.tumefaciens* (wild type) digested with *Hind*III and *EcoR*V respectively.

Fig. (4.1.8): Subsequent Southern blot analysis of strains digested in fig. (4.1.6) using *cheY1*-deleted fragment containing upstream and downstream flanking sequence of the *cheY1* as a probe.

Digestion of wild-type DNA with *Hind*III gave a single fragment of 4.1Kb after probing with the *cheY1* probe. Digestion with *EcoR*V gave fragments of 0.9Kb and 2.7Kb after probing. Digestion of intermediate strain chromosomal DNA with *Hind*III gave two fragments of 7.8Kb and 2.2Kb. Also digestion with *EcoR*V gave fragments of 0.9Kb, 6.0Kb and 3.0Kb and showed the expected fragments size of intermediate A. Digestion of mutant chromosomal DNA with *Hind*III gave a fragment of 3.7Kb and digestion with *EcoR*V gave two fragments of 0.9Kb and 2.3KB.

4.2. Mutagenesis of *cheY2*

The plasmid pELW1 was digested with *Bgl*II/ *Mlu*I and a 957bp fragment containing the *cheY2* gene, and 363bp upstream and 628bp downstream flanking sequences was isolated from the gel. After separation by agarose gel electrophoresis fragment was made blunt ended and ligated into pBluescriptSK+ plasmid which had been digested with *EcoR*V, to produce the sk+/cheY2 plasmid. This construct was checked by digestion with appropriate restriction enzymes and has subsequently verified by DNA sequencing. To delete the *cheY2* gene, two primers were designed as follows:

cheY2a (forward): 5'- GGACT CCATGG GAG AGA CAT TAG TCA GCA- 3'

cheY2b (reverse): 5'- GGACT CCATGG TGA TGG AAG CTG CGG CCA- 3'

The forward primer contains upstream flanking sequence, the first 9bp of *cheY2* gene, plus *Nco*I site (CCATGG) and five extra bases (GGACT) at the 5' end to use for cloning of PCR fragment. The reverse primer contains downstream flanking sequence, the last 3bp of *cheY2* gene, plus an *Nco*I site and 5 extra bases at 5' end.

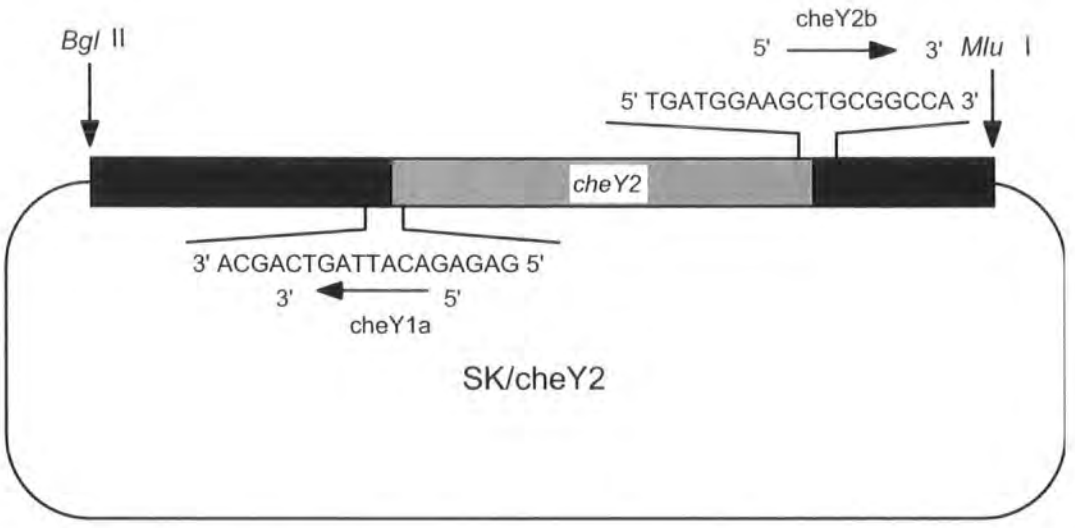


Fig. (4.2.1) Diagram of Sk/cheY2 plasmid showing sequence traces across deletion junctions and position of cheY2a and cheY2b primers.

Polymerase chain reaction was carried out using *sk+/ cheY2* as template DNA.

After reaction 5µl of PCR product was separated by electrophoresis on 0.8% agarose gel and the expected fragment (~ 3.4Kb) was isolated from the gel.

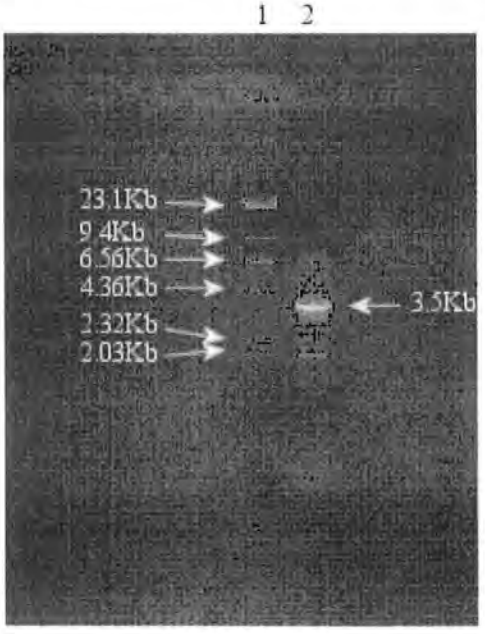


Fig. (4.2.2): 0.8% agarose gel electrophoresis showing the expected PCR product

fragment for *cheY2* in-frame deletion. Lane1: λDNA/*Hind*III lane2: in-frame deleted fragment (SK/delY2).

The PCR product was digested with *Nco*I, purified by agarose gel electrophoresis, religated using T4 DNA ligase and transformed into *E. coli* (DH5α). The plasmid isolated from bacterial clones was subjected to DNA sequencing. The result of sequencing showed that the PCR product contained only the upstream flanking sequence of *cheY2*; that is, the 3' flanking sequence had been deleted. In order to make the desired in-frame deletion of *cheY2*, it was therefore decided to ligate the *Rsa*I fragment of pELW1, containing the downstream flanking sequence of *cheY2*, into the new construct from the previous step. The plasmid containing the PCR product was digested with *Sma*I and the 590bp *Rsa*I fragment, containing downstream flanking sequence of *cheY2*, purified by agarose gel electrophoresis, was ligated to pBlueskriptSK to make new construct, sk+/ delY2.

Sk+/delY2 was transformed into *E. coli* and the resulting plasmid was checked by restriction enzyme digestion and confirmed by sequencing as well. At the next step the sk+/ delY2 plasmid was digested with *Hind*III/*Xba*I and the expected fragment containing upstream and downstream flanking sequence of *cheY2* was ligated into similarly cut pK18*mobsacB* to produce pK18/ delY2 plasmid. The new construct was transformed into *E. coli*, reisolated from the bacterial strain and checked by restriction enzyme digestion. The construct was confirmed by DNA sequencing as well.

The *E. coli* strain contain pK18/delY2 was used as a donor cell to transfer the plasmid into *A. tumefaciens* during the conjugation process.

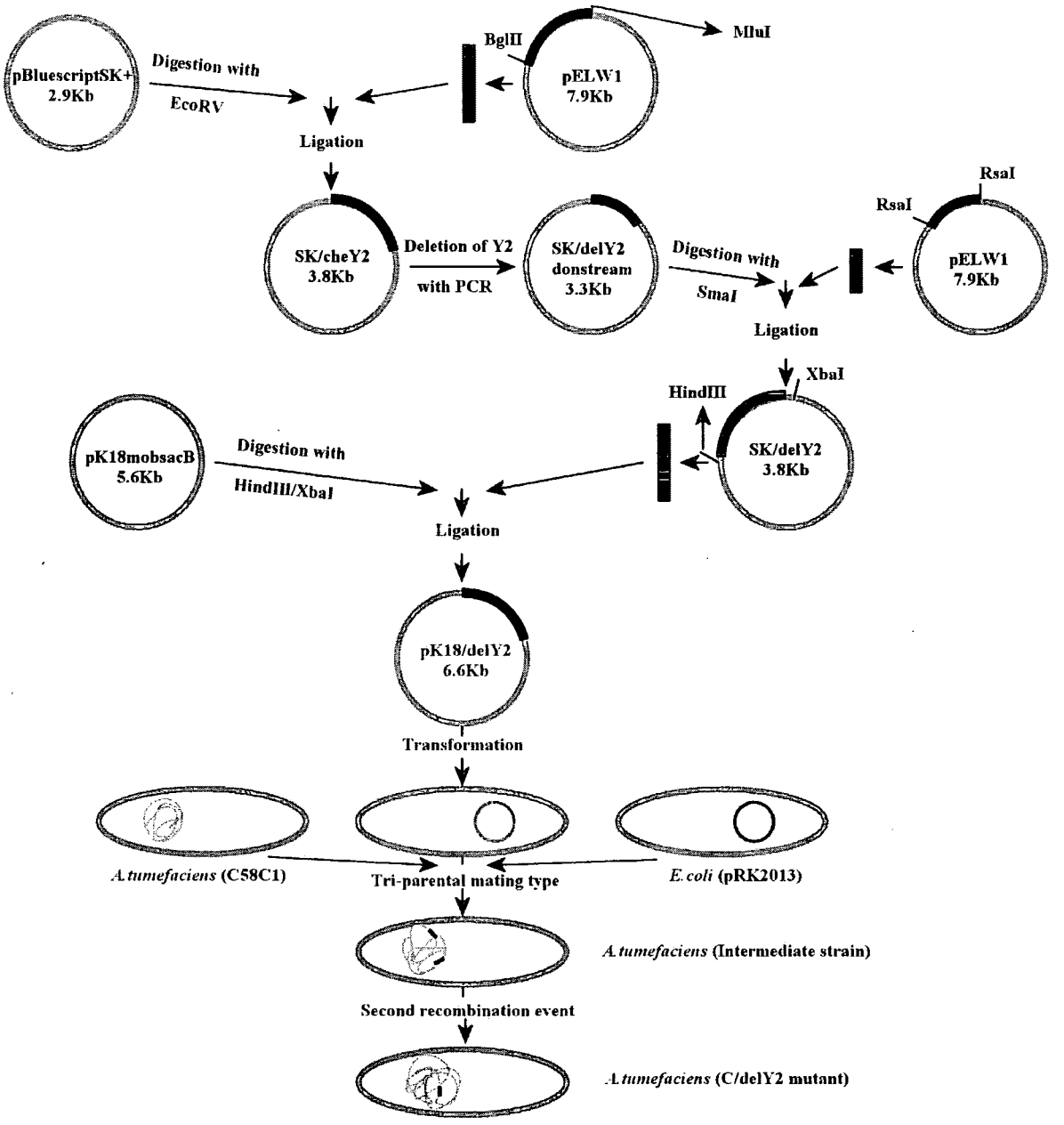


Fig. (4.2.3): Diagram showing schematic steps for construction of *cheY2* in-frame deletion mutant of *A. tumefaciens*.

Tri-parental mating: was performed as previously described. After overnight incubation of the mating mixture on a filter, several dilutions were made and plated on LA media containing Rifampicin and Kanamycin. The plates were incubated at 28-30°C, and after 3-4 days several colonies were picked and restreaked on the same media to obtain colonies showing stable antibiotic resistance. These intermediate strains containing both the wild type and *cheY2* genes were grown overnight in liquid LB media containing Rifampicin only to hence the second recombination event. After the overnight growth, serial dilutions were made and plated on MinA media containing 10% sucrose. The number of colonies grown from different dilutions were as follows:

dilution	Number of cells
Neat	>2000
10^{-1}	>1000
10^{-2}	>800
10^{-3}	>500
10^{-4}	~200
10^{-5}	~50
10^{-6}	20

Several colonies from 10^{-6}, 10^{-5} and 10^{-4} dilutions were picked and checked for sensitivity to kanamycin. All kanamycin-sensitive strains were checked by swarming plate assay on 0.2% LA. Some strains that showed a deficiency in swarming

behaviour, in comparison with wild type strain, and were checked by PCR. Some intermediate and wild type strains were checked by PCR as well.

Polymerase chain reaction for mutant verification: Two primers were designed as follows:

cheY21 (forward):	5'- CGG TGA GCG TCA TTT ACA GA- 3'
cheY22 (reverse):	5'- CTC TGC AAG CTG CAT TGG AA- 3'

The chromosomal DNA was used as a template. After the PCR reaction 5µl of PCR product was electrophoresed.

Fig. (4.2.4): 0.8% agarose gel electrophoresis of PCR products from resulting amplification of chromosomal DNA showing expected fragments resulting of PCR for each wild type, intermediate and mutant strains of *A. tumefaciens*. Lanes 5, 6, 7: mutant, lanes 3, 4: intermediate strains, Lane2: wild type and lane1: λDNA/*EcoR*I: *Hind*III

As shown in Fig 4.2.4 lanes 5, 6 and 4 showed the expected fragment (delY2~933bp), lanes 3, 4 showed the expected fragments for intermediate strains (~ 933bp and ~ 1321bp) and lane 2 (wild type) showed the expected fragment for wild type only (~1321bp).

Southern blot analysis of mutants:

Intermediate strains and mutants that had shown the expected fragments in PCR were checked by Southern blot. The wild type strain (C58C1) was included as a control. The *cheY2* deleted fragment (containing upstream and downstream flanking sequences of *cheY2*) was used as a probe. The chromosomal DNA of intermediate strains, possible mutants and wild type was digested with *Hinc*II and *Hind*III. The southern blot was done as discussed in materials & methods.

The following diagram showed the expected fragments of each intermediate, mutants and wild type strains.

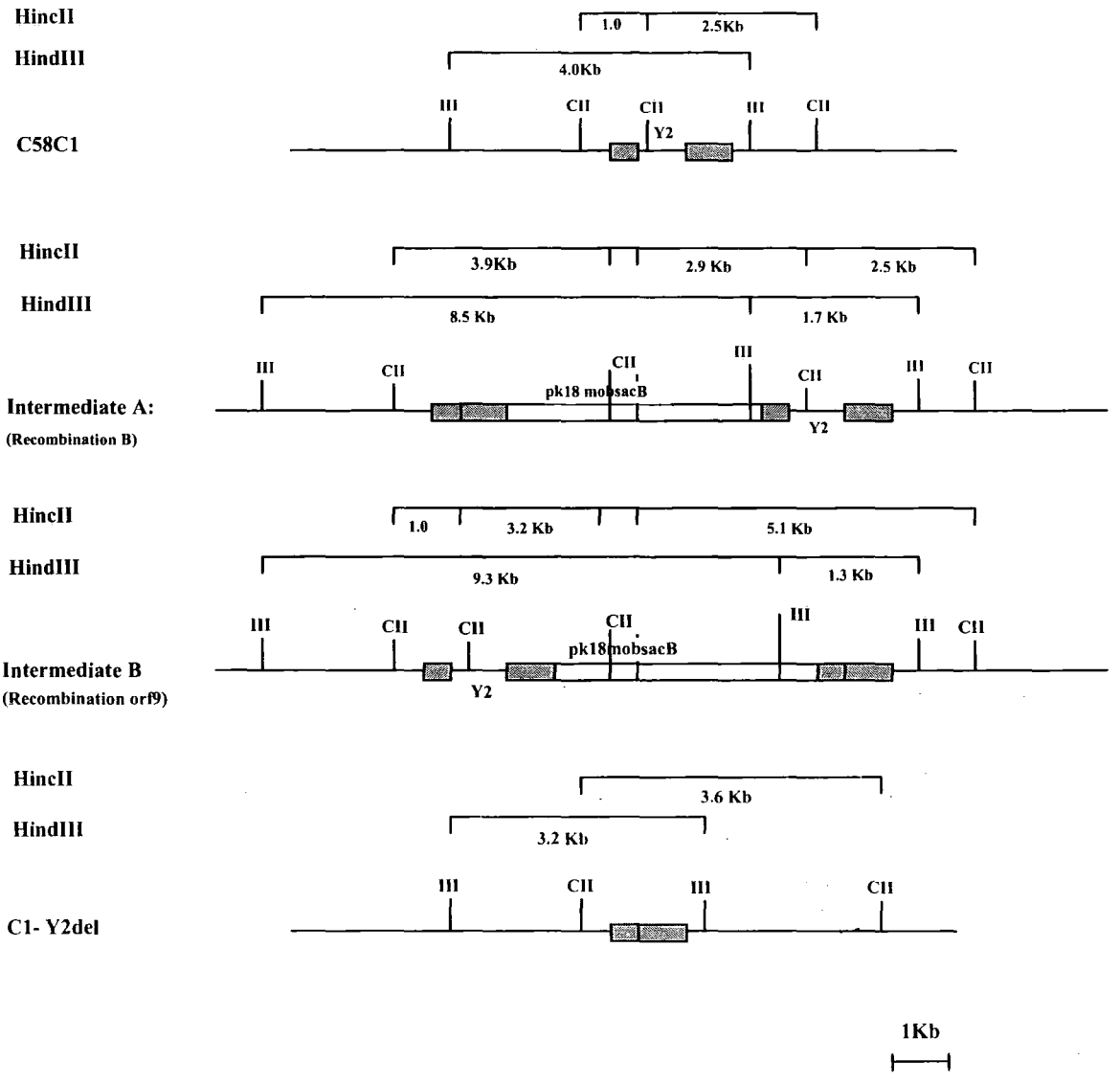
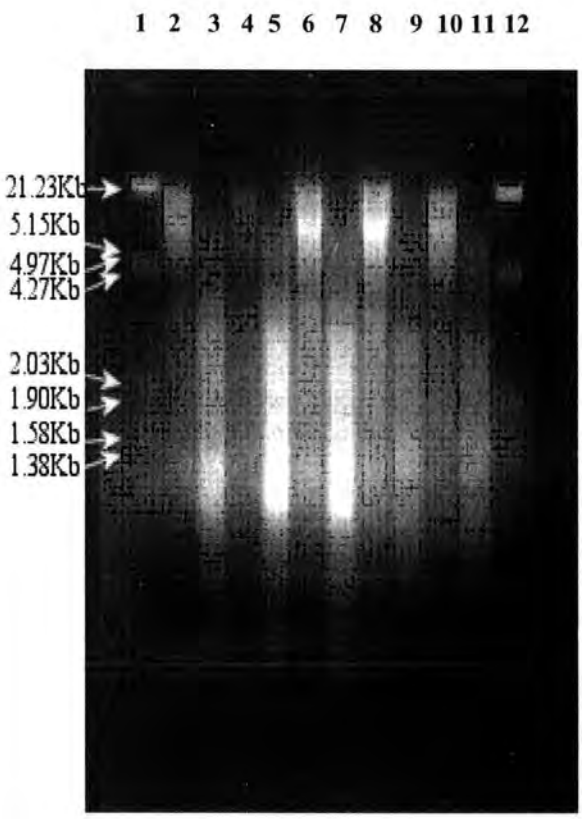


Fig. (4.2.5): Diagram showing the position and expected size of the *Hinc*II and *Hind*III fragments for each *A. tumefaciens* wild type, two possible intermediate and the *cheY2* in-Frame deletion mutant strains.



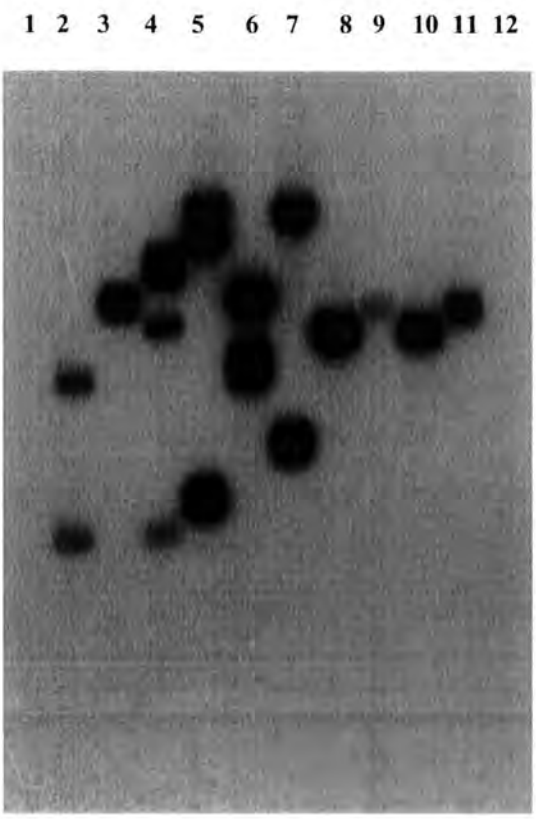


Fig. (4.2.6) **Fig. (4.2.7)**

Fig. (4.2.6): 1% agarose gel electrophoresis of wild type, intermediate strains and *cheY2* in-frame deletion mutant chromosomal DNA digested by *Hind*III and *Hinc*II. Lanes 1, 12: λDNA/*EcoR*I: *Hind*III, lanes 4, 6: intermediate strains digested with *Hinc*II, lanes 5, 7: intermediate strains digested with *Hind*III, lanes 9,11: *A. tumefaciens* mutant digested with *Hind*III, lanes 8, 10: *A.tumefaciens* mutant digested with *Hinc*II, lanes 2, 3: *A. tumefaciens* (wild type) digested with *Hinc*II and *Hind*III.

Fig. (4.2.7): Subsequent Southern blot analysis of strains digested in gel in fig. (4.2.6) using deleted fragment (containing upstream and downstream flanking sequences of *cheY2*) as a probe.

Digestion of wild-type chromosomal DNA with *Hind*III gave a single fragment of 4.0Kb after probing with the *cheY2* deleted fragment probe. Digestion with *Hinc*II gave fragments of 1.0Kb and 2.5Kb after probing. Also, digestion of intermediate strains chromosomal DNA with *Hind*III for some strains gave fragments of 8.5Kb and 1.7Kb, and digestion with *Hinc*II gave fragments of 3.9Kb, 2.9Kb and 2.5Kb after probing. Digestion of some of the other intermediate strains DNA with *Hind*III gave fragments of 9.3Kb and 1.3Kb. Digestion with *Hinc*II gave fragments of 1.0Kb, 3.2Kb and 5.1Kb after probing with *cheY2* deleted fragment. Digestion of mutant chromosomal DNA with *Hind*III gave a single fragment of 3.2Kb and digestion with *Hinc*II gave a single fragment of 3.6Kb after probing.

The result of the Southern blot showed that the size of fragments produced by wild type and mutants for both enzymes are correct. In addition lanes 4&5 show the correct size for intermediate B and lanes 6&7 indicated the correct size for intermediate A. The mutants were named C1/delY2 and used to investigate the phenotypic properties of mutant.

4.3. Mutagenesis of *cheB* gene

To make template DNA for the PCR-based deletion, plasmid pELW1 was digested with *EcoR*V and *Ehe*I, separated by agarose gel electrophoresis, and the 1777bp fragment containing *cheB* gene plus 399bp upstream and 326bp downstream flanking sequences was isolated from the gel. This fragment was ligated into pBluescriptSK+ digested with *EcoR*V to produce SK+/cheB plasmid. The new construct was transformed into *E. coli*, the plasmid was isolated from the bacterial strain and checked by digestion with restriction enzyme. The sequencing confirmed the result. SK+/cheB was then used as template DNA in PCR reaction for deleting the *cheB* gene.

Polymerase chain reaction: Two primers were designed as follow:

B1 (forward): 5'- GG CCATGG TGC GCT CAT GCC TTC CTC- 3'

B2 (reverse): 5'- GG CCATGG TGA CTA ATG TCT CTC GCA G- 3'

The forward primer contains the upstream flanking sequence, the first 9bp of *cheB* gene, an *Nco*I site (CCATGG) and two extra bases (GG) at the 5' end to use for cloning of PCR product.

The reverse primer also contains the downstream flanking sequence, the last 3bp of *cheB* gene, an *Nco*I site and two extra bases at the 5' end.

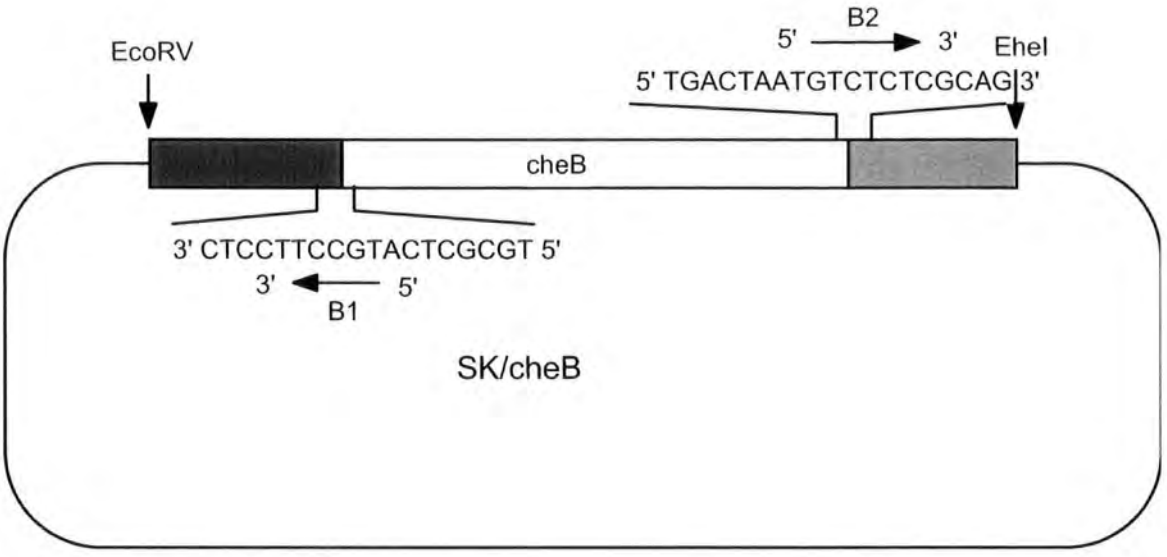


Fig. (4.3.1) Diagram of Sk/cheB plasmid showing sequence traces across deletion junctions and position of B1 and B2 primers.

After the reaction, 5µl of PCR product was electrophoresed and the expected fragment (~3.6Kb) was isolated from the gel.

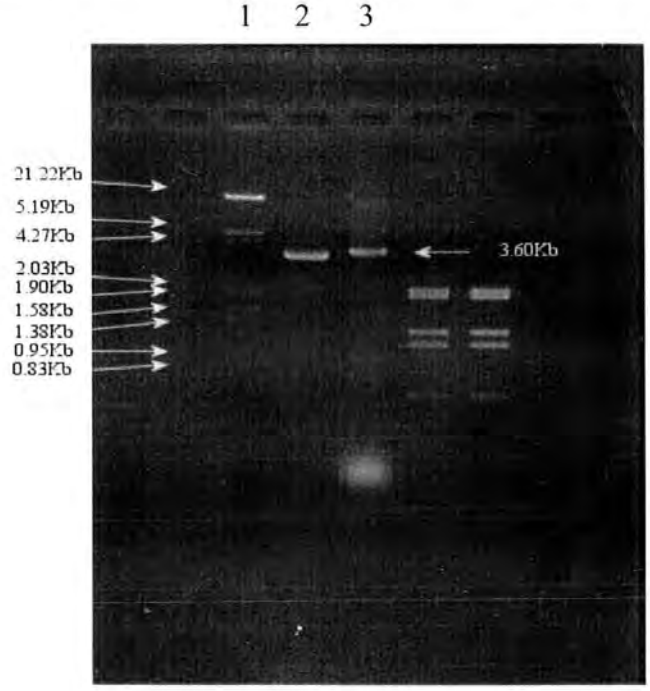


Fig. (4.3.2): 0.8% agarose gel electrophoresis showing the expected fragment for *cheB* in-frame deletion. Lane 1: λDNA/*Hind*III:*Eco*RI lane 3: in-frame deleted fragment (SK/delB).

To religate the PCR product two methods were used:

In the first, the PCR product was made blunt ended by treatment with T_4 DNA polymerase prior to ligation. The mixture was incubated at 16°C for 2 days then 5µl was transformed into *E. coli* (DH5α) directly.

In the second method, the PCR product was restricted with *Nco*I. After overnight incubation at 37°C, the digested fragment was electrophoresed, isolated from the gel and the single stranded ends were religated together using T_4-DNA ligase. The resulting plasmid was transformed into *E. coli*. Both methods gave successful results. The new construct plasmid, SK+/delB, was isolated from transformed cells and checked by restriction enzyme digestion. The correct construct was confirmed by sequencing as well.

The entire insert fragment from SK+/delB was excised with *Hind*III/ *Xba*I and ligated into similarly cut pK18*mobsacB* to generate pK18/ delB plasmid. After cloning of new construct into *E. coli*, the plasmid was reisolated from bacterial cells and checked once by digestion with restriction enzyme. Plasmid pK18/delB was confirmed by sequencing as well.

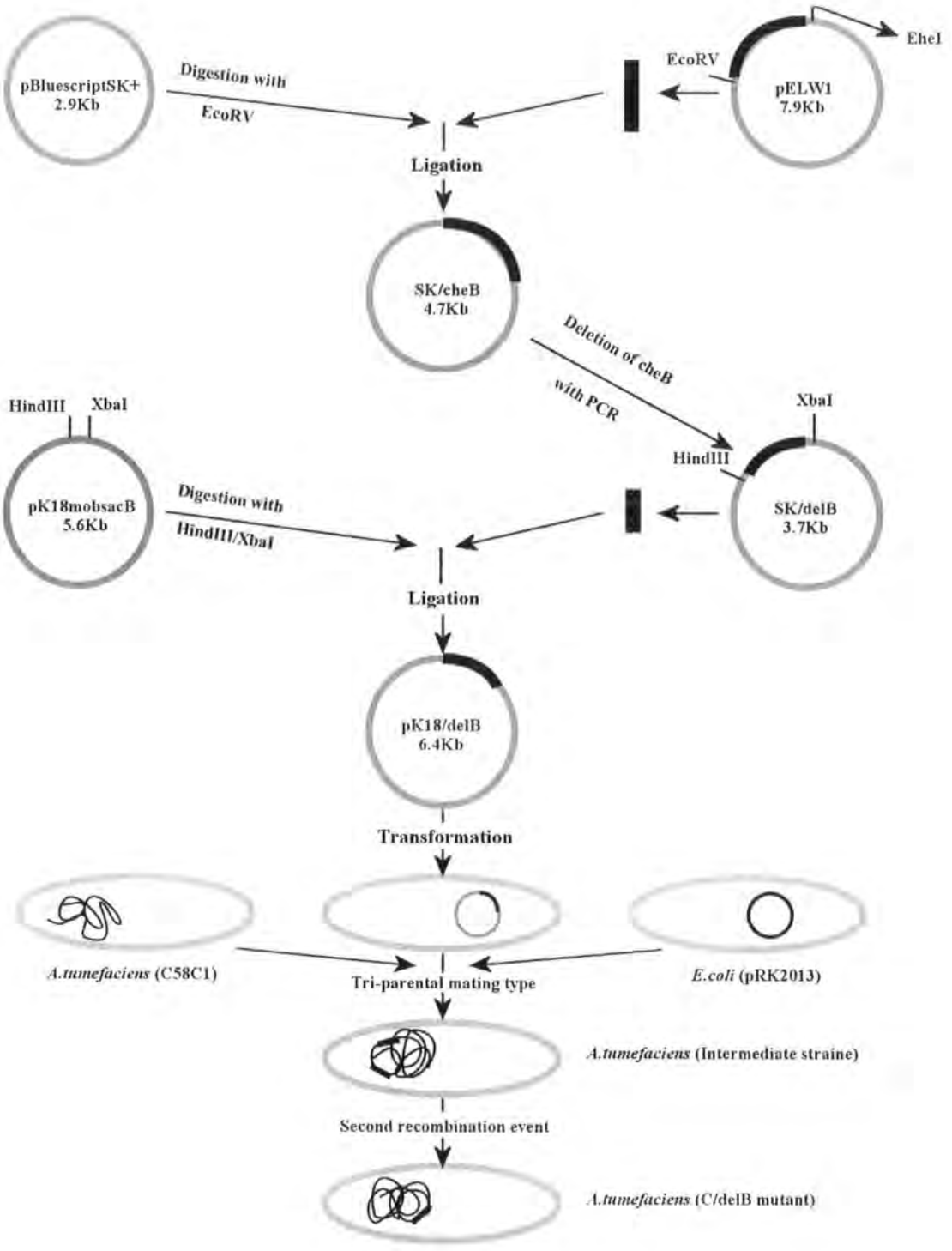


Fig. (4.3.3): Diagram showing schematic steps for construction of *cheB* in-frame deletion mutant of *A. tumefaciens*.

Tri-parental mating was done as before. The *E.coli* (pK18/delB) was used as a donor cell. Mating mixture was plated onto selective media (LA+ rifampicin & kanamycin) and incubated at 28-30°C for 3 days. Several colonies were picked up, restreaked on the same media to give the stable resistant colonies and grown overnight in LB media containing Rif+ Kam. Chromosomal DNA was isolated from these strains and integration of pK18/delB plasmid into the bacterial chromosome was checked by PCR using chromosomal DNA as a template and two following primers:

cheBa (forward): 5'- TAC GAT GAC AAC GCG CTT GA- 3'

cheBb (reverse): 5'- TGC GCG GTG AGA ATG ATG AA- 3'

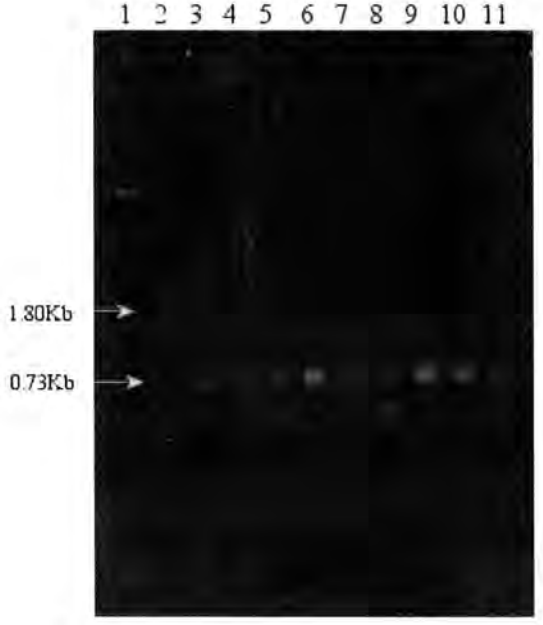


Fig. (4.3.4): 1% agarose gel electrophoresis of PCR products of intermediate strains of *A.tumefaciens* Lane 1, λDNA/*EcoR*I:*Hind*III, lanes 3, 4 showed the correct size for the intermediate strains.

The PCR product was electrophoresed. As shown in Fig. 4.3.4 two strains (lanes 3 & 4) produced the expected fragment for intermediate strains in comparison with lane 2, wild type strain, C58C1. These two strains were used in a second recombination event. To promote the second recombination event, the intermediate strains were growth overnight in LB media containing Rifampicin only, then serial dilutions were made and plated on MinA media plus 10-15% sucrose. After 3-4 days incubation at 28-30°C the number of colonies grown were as follows:

dilution	number of colonies
Neat	> 500
10^{-1}	> 200
10^{-2}	~ 30
10^{-3}	9

Several colonies from the lower dilutions were picked up, restreaked on LA+Rifampicin plates and checked for sensitivity to kanamycin on LA media containing Rif+ Kam. The chromosomal DNA was isolated from kanamycin-sensitive strains and checked by PCR under the same conditions as used for intermediate strains. As shown in fig 4.3.5 two strains (lanes 2, 3) produced the expected fragment of mutant only.

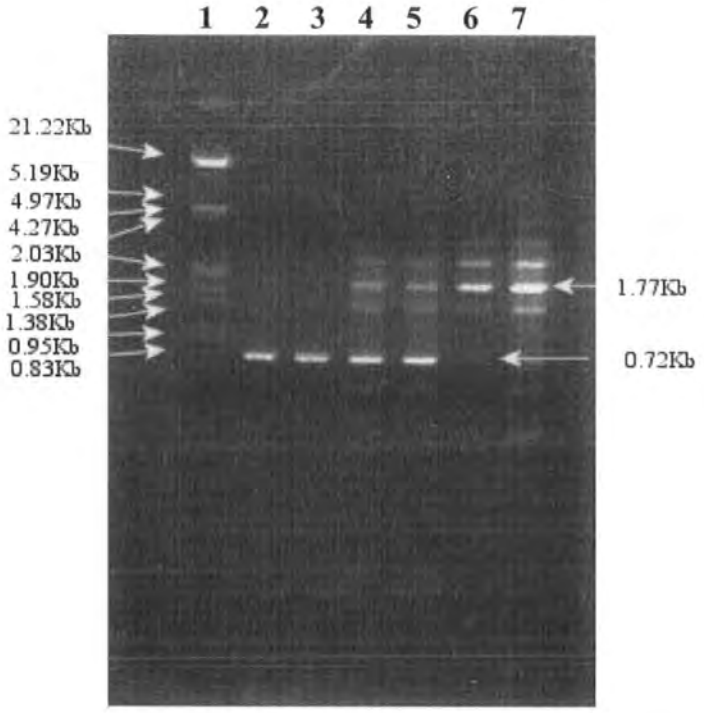


Fig. (4.3.5): 0.8% agarose gel electrophoresis showing expected fragments resulting from PCR for each wild type, intermediate and mutant strains of *A.tumefaciens*. Lanes 2, 3: mutant, lanes 4, 5: intermediate strains, lane6: wild type and lane1: λDNA/ *EcoR*I:*Hind*III.

Southern blot analysis of mutant: Chromosomal DNA of wild type, intermediate strains and possible mutants were digested with *Pst*I and *Hinc*II. The *cheB* deleted fragment (containing upstream and downstream flanking sequences of *cheB*) was used as a probe.

The following diagram shows the expected fragment size for each wild type, intermediate and mutant strains.

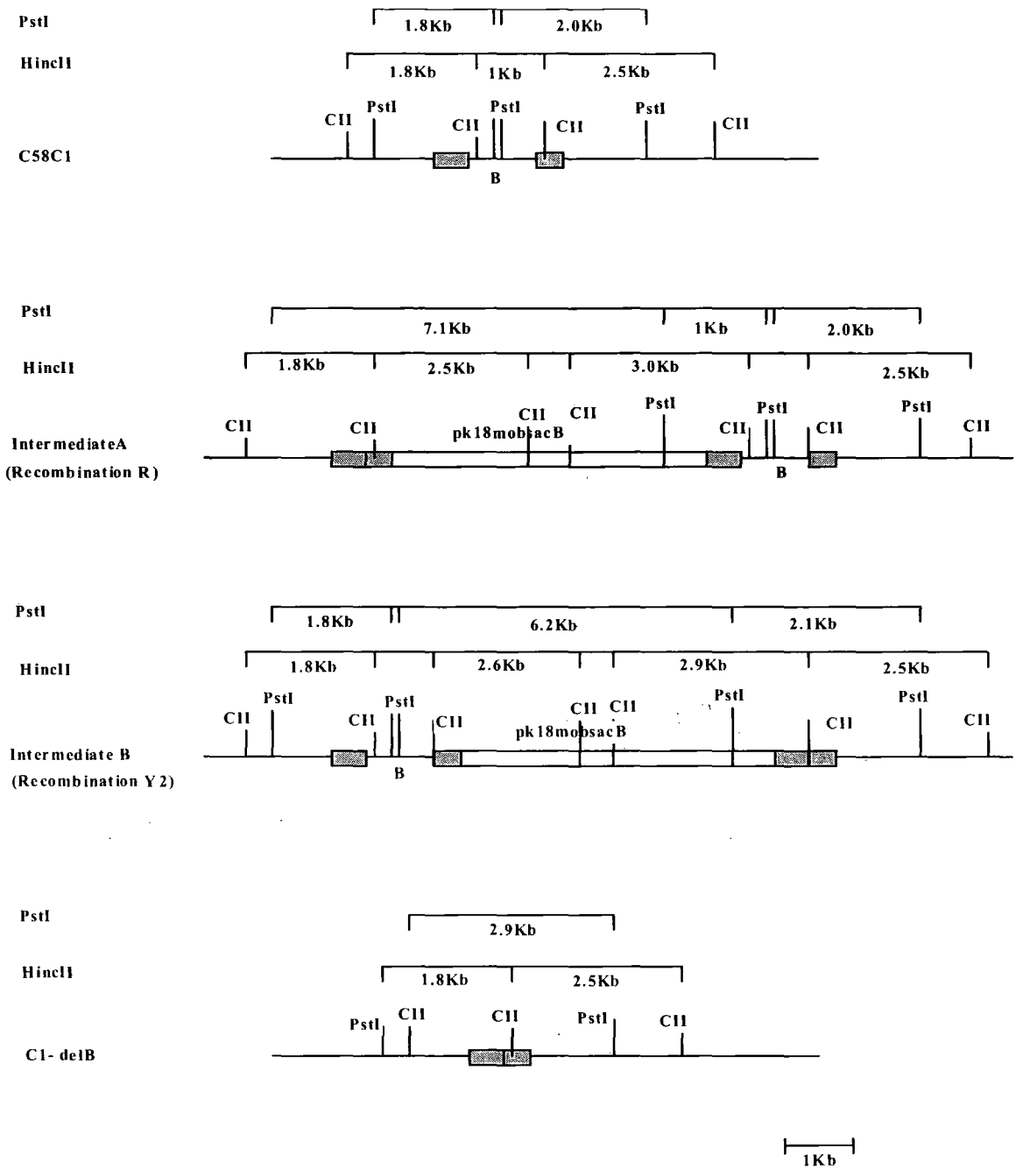


Fig. (4.3.6): Diagram showing the position and expected size of the *Hinc*II and *Pst*I fragment for each *A. tumefaciens* wild type, two possible intermediate and the *cheB* in-Frame deletion mutant strains.

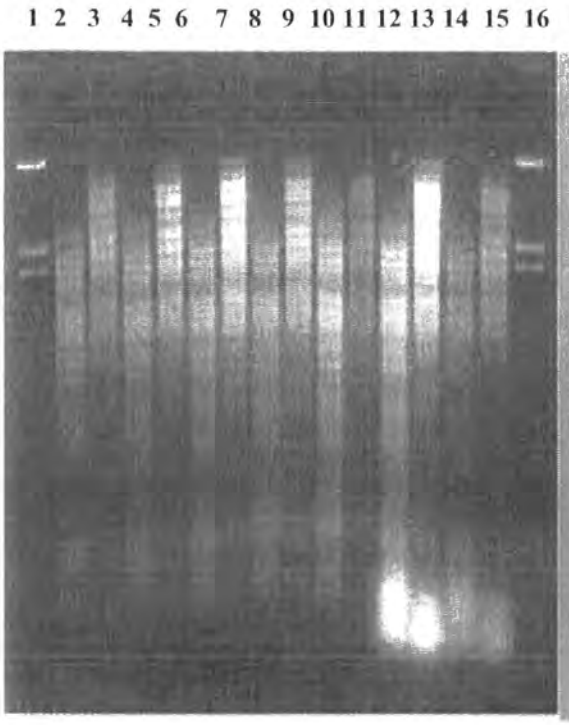


Fig. (4.3.7)

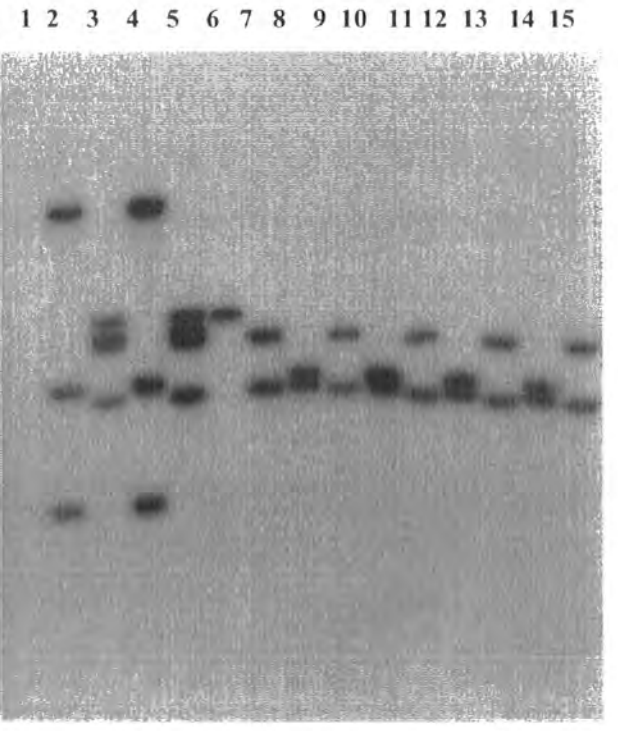


Fig. (4.3.8)

Fig. (4.3.6): 1% agarose gel electrophoresis of wild type, intermediate strains and *cheB* in-frame deletion mutant chromosomal DNA digested by *Pst*I and *Hinc*II. Lanes 1, 16: λDNA/*EcoR*I:*Hind*III, lanes 3, 5: intermediate strains digested with *Hinc*II, lanes 2, 4: intermediate strains digested with *Pst*I, lanes 4, 6, 8, 10, 12: *A. tumefaciens* mutant digested with *Pst*I, lanes 7, 9, 11, 13: *A. tumefaciens* mutant digested with *Hinc*II, lanes 14, 15: *A. tumefaciens* (wild type) digested with *Pst*I and *Hinc*II.

Fig. (4.3.7): Subsequent southern blot analysis of strains digested according to fig. (4.3.6) using *cheB* deleted fragment (containing upstream and downstream flanking sequences of *cheB*) as a probe.

Digestion of wild-type chromosomal DNA with *Pst*I gave fragments of 1.8Kb and 2.0Kb after probing with the *cheB* deleted fragment probe. Digestion with *Hinc*II gave fragments of 1.0Kb, 1.8Kb and 2.5Kb after probing. Also digestion of intermediate strain chromosomal DNA with *Pst*I gave fragments of 1.0Kb, 2.0Kb and 7.1Kb. Digestion with *Hinc*II gave fragments of 1.8Kb, 3.0Kb and 2.5Kb after probing. Digestion of mutant chromosomal DNA with *Pst*I gave a single fragment of 2.9Kb and digestion with *Hinc*II gave fragments of 1.8Kb and 2.5Kb after probing.

Southern blot indicated that the fragments size for wild type and mutant strains for both enzymes are correct. The fragments size for intermediate strain are correct as well and confirmed the intermediate A type of recombination.

4.4. Mutagenesis of the *cheR* gene

To make template DNA for the PCR- based deletion method, plasmid pELW1 was digested with *Pst*I, separated by agarose gel electrophoresis and the 1827bp fragment containing *cheR* gene plus 200-500bp upstream and downstream flanking sequences was isolated from the gel. This fragment was ligated into pBluescriptSK+ digested with the same enzyme to produce SK+/cheR plasmid. The resulting construct was transformed into *E. coli*, plasmid was isolated from bacterial strain and checked by digestion with *Pst*I enzyme again. DNA sequencing confirmed the correct fragment had been cloned. SK+/cheR was used as template DNA in PCR reaction for deleting the *cheR* gene.

Polymerase chain reaction: Two primers were designed as follow:

cheR1 (forward): 5'-GCATGC TGC TGC CAT TGG ATA TCC GC - 3'

cheR2 (reverse): 5'-GCATGC TGA GCG CAC TCG CAC GGG TC - 3'

The forward primer contains the upstream flanking sequence, the first 9bp of *cheR* gene, and a *Sph*I site (GCATGC) at the 5' end to use for cloning of PCR product.
The reverse primer also contains the downstream flanking sequence, the last 3bp of *cheR* gene and *Sph*I site in 5' end .

After The PCR reaction, 5µl of PCR product was separated by electrophoresis and the expected fragment (~3.8Kb) was isolated from the gel.

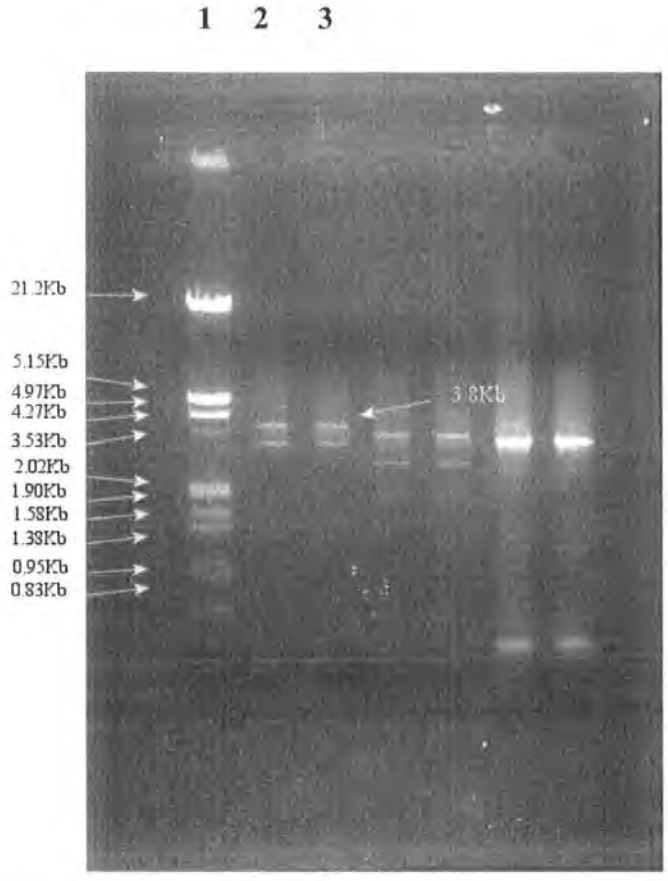


Fig. (4.4.1): 0.7% agarose gel electrophoresis showing the expected fragment for *cheR* in-frame deletion. Lane 1: λDNA/*Hind*III lanes 2 & 3: in-frame deleted fragment (SK/delR).

This fragment was digested with *Sph*I and religated with T_4 DNA ligase to transform into *E.coli.* The procedure was performed several times with different conditions but no recombinant plasmids were obtained. The in-frame deletion of *cheR* was therefore made according to the following procedure.

The SK+/cheR plasmid was digested with *EcoR*V/*Hinc*II. After electrophoresis two fragments, 3427bp containing vector backbone plus the immediate downstream flanking sequence of *cheR* and 401bp fragment containing sequence immediately upstream of *cheR,* were isolated from the gel and religated together, making SK+/delR plasmid. This new construct was transformed into *E.coli* (DH5α), and, after the isolation of plasmid from the bacterial strain, checked with

digestion by *EcoRI/Xba*I. To confirm the result and to ensure that *cheR* is in-frame correctly, SK+/delR was subjected to DNA sequencing as well.

SK+/cheR was digested with *EcoRI/Xba*I and the 900bp fragment containing in-frame deleted fragment of *cheR* was ligated into the suicide vector, pK18*mobsacB*, and digested with the same enzymes to make pK18/delR plasmid. This new construct was transformed into *E.coli* and after isolation from the bacterial strain was checked with enzyme digestion again. The *E.coli* containing this new construct used as a donor cell and introduced into *A. tumefaciens* by tri-parental mating.

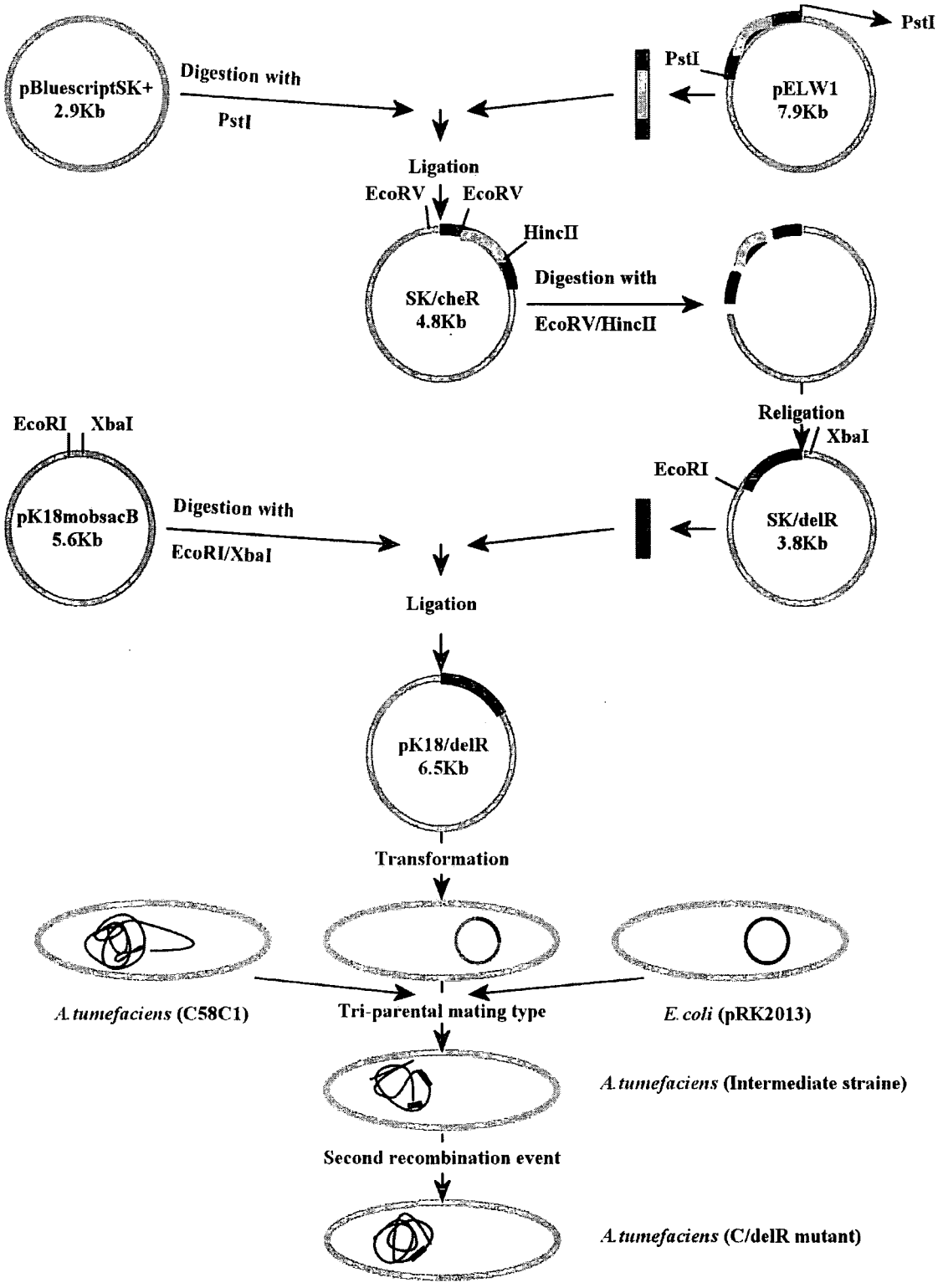


Fig. (4.4.2): Diagram showing schematic steps for construction of *cheR* in-frame deletion mutant of *A. tumefaciens*.

To carry out the tri-parental mating 300µl of recipient cell, 150µl of donor cell and 150µl of helper plasmid were mixed, and after collection of mating mixture from the filter, to reduce the unwanted background colonies, 100µl of the mixture was incubated overnight in 10ml liquid media containing kanamycin and rifampicin. Then, serial dilutions of this culture were plated onto selective media (LA+ rif./ kan.).
After 2-3 days incubation at 28°C several colonies were picked up and resreaked on the same media to get the pure colonies. LB media plus rifampicin and kanamycin was inoculated with these strains and after overnight incubation at 28-30°C, the chromosomal DNA was isolated to check the intermediate strains by PCR or southern blot.

Polymerase chain reaction: Two primers were designed as follows:

delR1 (forward): 5'-ATC CAC TCC TTC GGT TCC AA-3'

delR2 (reverse): 5'-CCG ATA TTC GCT CAT CGG AA-3'

The PCR was performed as before. The chromosomal DNA of possible intermediate strains and wild type were used as DNA template. No PCR product was detected in this reaction, neither in wild type nor in intermediate strains, in both a standard reaction and after modifying the PCR conditions. Despite this problem, it was decided to attempt to produce the desired mutant.

Second recombination event: To promote the second recombination event, several possible intermediate strains from previous step were grown overnight in LB

media containing Rifampicin only. Serial dilutions were made and plated on MinA media plus 10-15% sucrose. After 3-4 days incubation at 28-30°C, the number of colonies grown were as following table:

dilution	number of colonies
Neat	> 1000
10^{-1}	> 500
10^{-2}	~ 100
10^{-3}	30
10^{-4}	9

Several colonies from the lower dilutions were picked, restreaked on LA+Rifampicin plates and checked for sensitivity to kanamycin on LA media containing Rif+Kam. The chromosomal DNA was isolated from kanamycin- sensitive strains and checked by Southern blot, with intermediate strains and wild type as control.

Southern blot analysis was performed as described in the Material & Methods. The chromosomal DNA of the strains was digested with *Pst*I and *Eco*RV. The deleted *cheR* fragment (containing only the upstream and downstream flanking sequences of *cheR*) was used as a probe. The following diagram shown the expected fragments size for each wild type, intermediate and mutant strains.

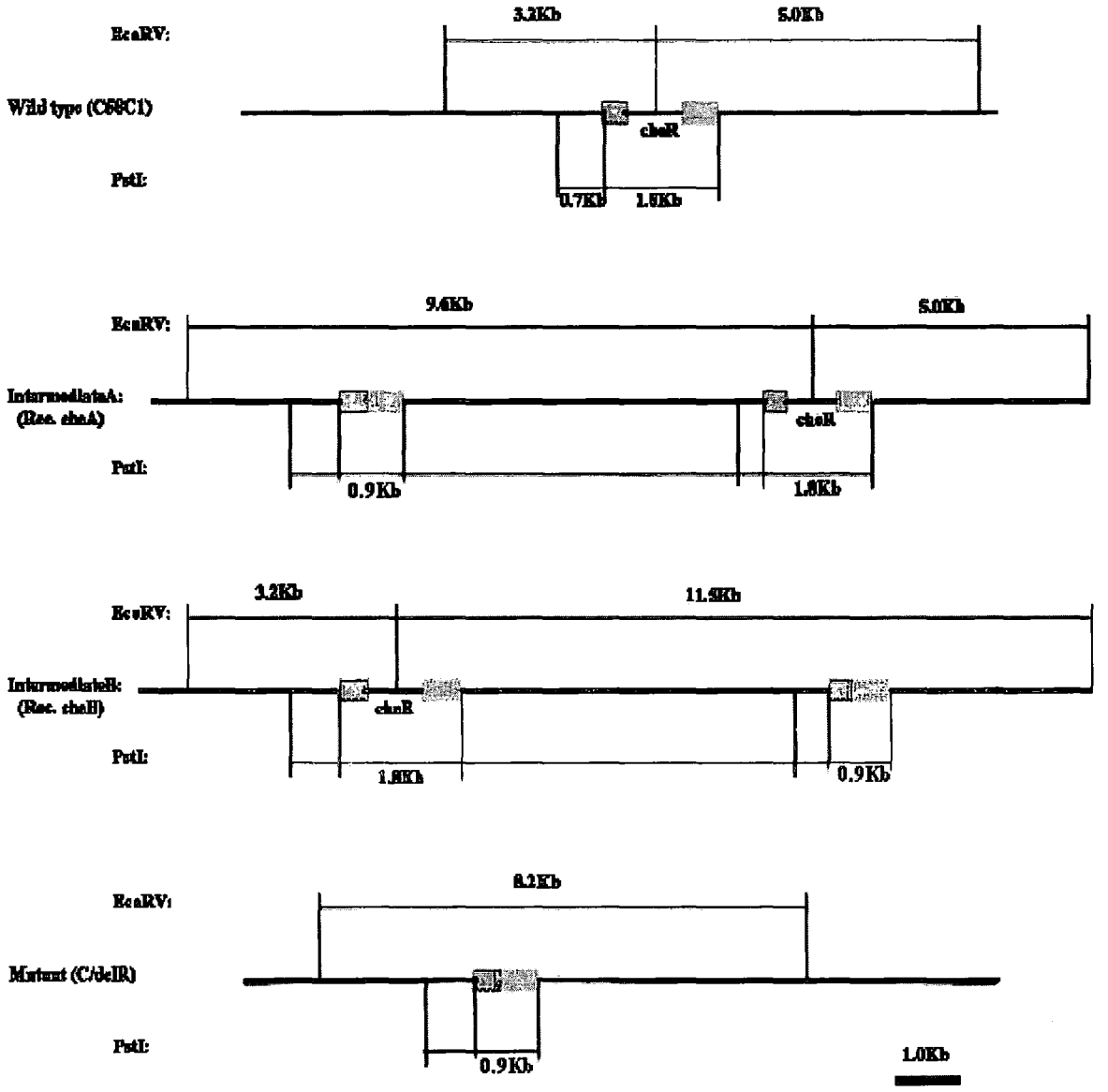


Fig. (4.4.3): Diagram showing the position and expected size of the *Eco*RV and *Pst*I fragment for each *A. tumefaciens* wild type, two possible intermediate and the *cheR* in-Frame deletion mutant strains.

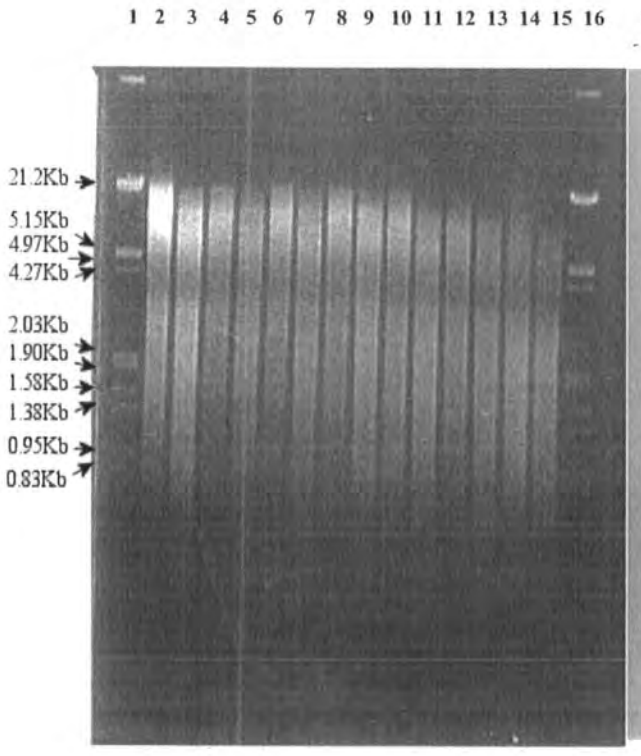


Fig. (4.4.4)

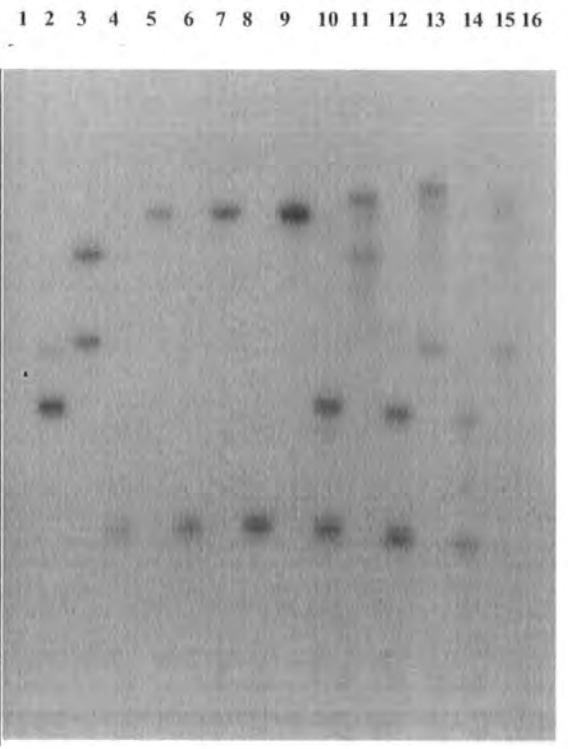


Fig. (4.4.5)

Fig. (4.4.4): 1% agarose gel electrophoresis of wild type, intermediate strains and *cheR* in-frame deletion mutant chromosomal DNA digested by *Pst*I and *EcoR*V. Lanes 1, 16: λDNA/*EcoR*I:*Hind*III, lanes 10, 12, 14: intermediate strains digested with *Pst*I, lanes 11,13,15: intermediate strains digested with *EcoR*V, lanes 4, 6, 8: *A. tumefaciens* mutant digested with *Pst*I, lanes 5, 7, 9: *A. tumefaciens* mutant digested with *EcoR*V, lanes 2, 3: *A.tumefaciens* (wild type) digested with *EcoR*V and *Pst*I.

Fig. (4.4.5): Subsequent southern blot analysis of strains digested according to fig. (4.4.4) using deleted *cheR* fragment (containing only upstream and downstream flanking sequences of *cheR*) as a probe.

Digestion of wild-type chromosomal DNA with *Pst*I gave a single fragment of 1.8Kb after probing with the *cheR* deleted fragment probe. Digestion with *EcoR*V gave fragments of 3.2Kb and 5.0Kb after probing. Digestion of intermediate strain chromosomal DNA with *Pst*I gave fragments of 1.8Kb and 0.9Kb. Digestion with *EcoR*V gave fragments of 3.2Kb and 11.5Kb after probing. Digestion of mutant chromosomal DNA with *Pst*I gave a single fragment of 0.9Kb and digestion with *EcoR*V gave a single fragment of 8.2Kb after probing.

The result of Southern blot confirmed the expected fragments for both wild type and mutant strains. The fragments produced by chromosomal DNA of the intermediate strains confirmed the intermediate B type of recombination. The mutants renamed as C1/delR.

Chapter 5

Phenotypic properties of *A.tumefaciens* mutants

5. Phenotypic properties of the *A. tumefaciens* mutants

5.1. Swarming behaviour assay

The migration of bacterial cells is usually studied in semi-solid agar plates. The concentration of agar is sufficiently low (0.2-0.35%) to allow to bacteria to swim in this medium. The behaviour is complex; swimming involves transport, metabolism and growth, as well as motility (Berg, H. C., 2000).

If the swarming plate contains nutrients which the bacterium can metabolise and to which it is tactically responsive, the growing population of cells swarms outward from the centre, following the gradient which it has created. This method provides a powerful means for screening of motility and taxis in wild type and mutant bacterial strains.

During swarming, different types of swarming pattern can be formed that are chemotactically inert. The type of pattern depends on the amount of nutrition and energy source that is uniformly distributed in the petri dish. Earlier studies (Adler, 1973) demonstrated that the bacteria migrate in the form of a ring, and, depending on nutrition and energy source, the number of rings is differ.

In Adler's experiment, rings formed when cells of *E. coli* are placed in an environment containing substances (oxygen, amino acids, etc.) that the bacteria both consume and respond to chemotactically. The consumption of the substrate generates an attractant gradient, which provokes chemotaxis. The net response is a ring of cells moving outwards from the centre in the petri dish.

Budrene and Berg (1995) found conditions in which more complex patterns can form. In contrast to Adler's experiment, the environmental conditions induce the

bacteria to excrete an attractant toward which the cells undergo chemotaxis. These conditions produce patterns that are more complex.

More investigation has shown that migration of bacteria through semi-solid agar occurs in the absence of many of the genes known to be required for chemoreception and chemotactic signal processing. The swarming pattern for such strains vary in size and in pattern, depending on whether or not the flagellar motor in the bacterial strain is able to spin both clockwise and counter-clockwise. Experiments by Wolf and Berg (1989) indicate that in *E.coli*, formation of the wild type band structure requires the complete set of chemotaxis proteins. Non-chemotactic cells that retain the ability to tumble as well as to run migrate faster than cells that can only run. Cells with ability to run only get trapped in the agar.

5.1.1: Effect of the deletion of *cheY1* and *cheY2* genes

The swarming behaviour of wild-type and mutants of *A. tumefaciens* was compared. Usually 3µl sample of cells grown in LB medium were placed on swarm plates and incubated at 28-30°C for up to three days. The swarm-ring size was measured after 10, 24, 48 and 56hr. The effects of the deletion of *cheY1* and *cheY2* genes are shown in Fig. (5.1.1) and Fig. (5.1.2).

When incubated, both wild-type and mutants for the same period of time C1/delY1 formed swarms approximately 30% reduced in size compared to those formed by *A. tumefaciens* (C58C1) wild-type. Also C1/delY2 inhibited swarming behaviour and it swarmed to approximately 60% the size of swarms formed by wild-type. The data suggest that *cheY1* and *cheY2* have different effects on the tactic response, it seems CheY2 is the main response regulator (Fig. 5.1.2., compare No 2. with No 3 & 4).

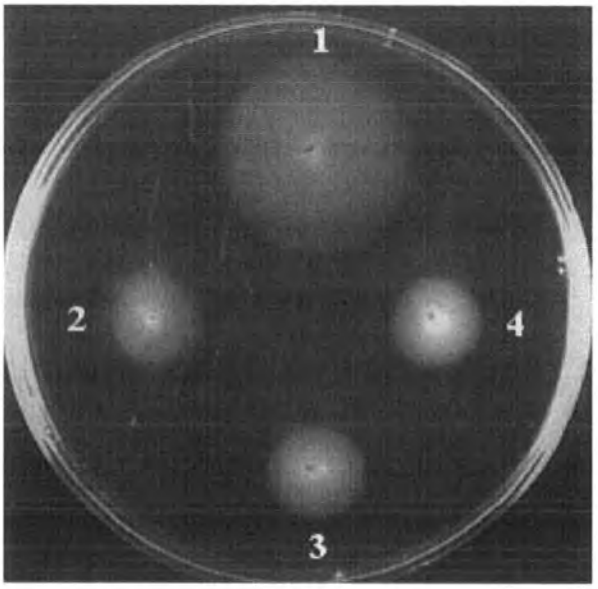


Fig. (5.1.1): Swarm test of *A. tumefaciens* wild type (1) and chemotaxis mutant (2-4) C1/delY2 (Photograph after 48hr).

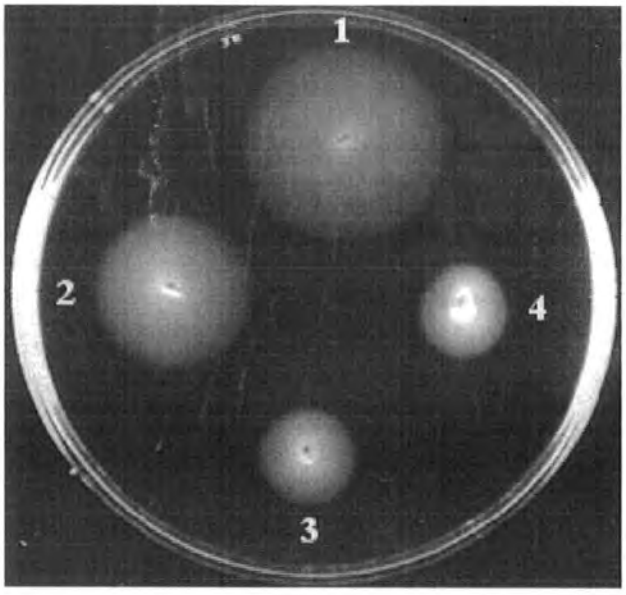


Fig. (5.1.2): Swarm test of *A. tumefaciens* wild type (1) and chemotaxis mutant (2) C1/delY1 and (3& 4) C1/delY2 (Photograph after 48hr).

Wild-type cells (C58C1) formed a swarm ring, and their displacement increased with time (See Fig. 5.1.3).The first migration appeared about 2.5 hr after inoculation and it increased with time. Wild-type cells produced swarm of approximately 3cm in diameter after 56hr. The mutant cells, C1/delY1, produced a swarm of approximately 2.2cm in diameter after the same length of time. The edge of swarm produced by *cheY1* mutant moved outward at a rate about 60% of the wild-type swarms (Fig. 5.1.3).

The mutant cells, C1/delY2, produced swarm approximately 1.2cm in diameter after 56hr. This mutant produced a growth pattern of high density. The edge displacement swarm was at a rate about 30% of the wild type swarms.

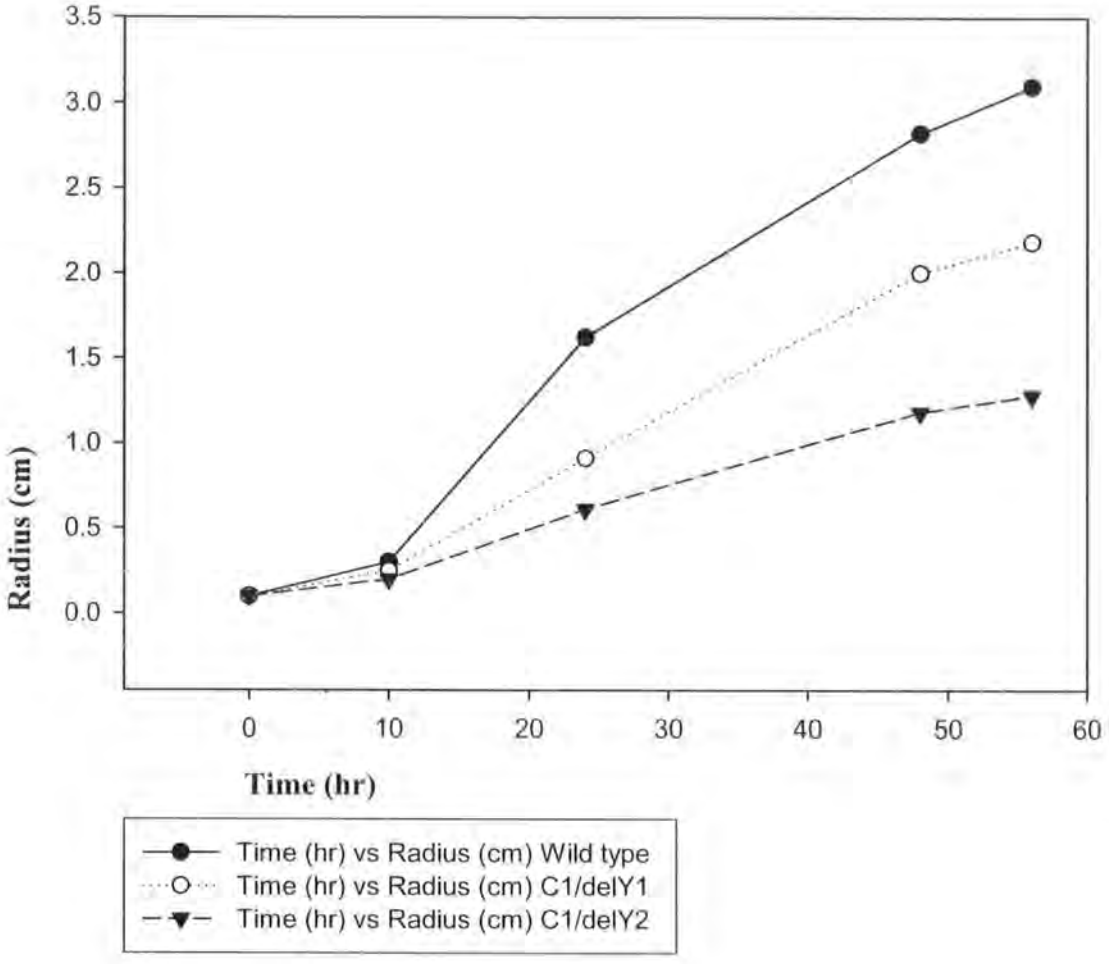


Fig. (5.1.3): Displacement of the edge of swarms produced by cells of *A. tumefaciens* (wild type) and the mutant strains, C1/delY1 and C1/delY2.

5.1.2: Effect of the deletion of *cheB* and *cheR* genes

The predicted amino acid sequences of CheR and CheB were compared with the equivalent proteins from *E. coli*. The CheR and CheB have 41% and 48% identity, respectively, with the corresponding *E. coli* proteins and therefore designated homologues of these adaptation enzymes.

>ref|NP_416397.1| response regulator for chemotaxis (cheA sensor); protein

```
          methylesterase [Escherichia coli K12]
         Length = 349

 Score =  305 bits (781), Expect = 3e-84
 Identities = 168/345 (48%), Positives = 229/345 (66%), Gaps = 11/345 (3%)

Query: 6   RVLVVDDSPTMRGLISAVLKADPEVEVVGQAGNAMEARAAIKQLNPDVVTLDIEMPEMNG 65
           RVL VDDS  MR +++ ++ +  ++E+V  A + + AR  IK+ NPDV+TLD+EMP M+G
Sbjct: 5   RVLSVDDSALMRQIMTEIINSHSDMEMVATAPDPLVARDLIKKFNPDVLTLDVEMPRMDG 64

Query: 66  LEFLEKIMRLRPMPVIMVSSLTHRGADASLAALEIGAFDCVGKPAPGDARPF----GDLA 121
           L+FLEK+MRLRPMPV+MVSSLT +G++ +L ALE+GA D V KP  G           +A
Sbjct: 65  LDFLEKLMRLRPMPVVMVSSLTGKGSEVTLRALELGAIDFVTKPQLGIREGMLAYNEMIA 124

Query: 122 DKVKXXXXXXXXXXXXTRPETAAAPQPVPMSEYRAGRKVVAIGSSTGGVEALIAVLQKFP 181
           +KV+                T     P+  SE     K++AIG+STGG EA+  VLQ  P
Sbjct: 125 EKVRTAAKASLAAHKPLSAPTTLKAGPLLSSE-----KLIAIGASTGGTEAIRHVLQPLP 179

Query: 182 ANCPPTVITQHMPPTFTKSFAERLNRICAPVVEEATDGARLQTGKIYLAPGGERHLQIAN 241
            + P  +ITQHMPP FT+SFA+RLN++C   V+EA DG R+  G  Y+AP G+RH++++
Sbjct: 180 LSSPALLITQHMPPGFTRSFADRLNKLCQIGVKEAEDGERVLPGHAYIAP-GDRHMELSR 238

Query: 242 RSAPC-CRLLDRDPVNGHRPSVDVLFDSVAELAGRNAVGVILTGMGRDGAAGLLKMRHAG 300
             A    ++ D   VN HRPSVDVLF SVA+ AGRNAVGVILTGMG DGAAG+L MR AG
Sbjct: 239 SGANYQIKIHDGPAVNRHRPSVDVLFHSVAKQAGRNAVGVILTGMGNDGAAGMLAMRQAG 298

Query: 301 ARTVGQNEKTCVVYGMPRVAYELGAVEQQLPLASIGEEILKLTTA 345
           A T+ QNE +CVV+GMPR A  +G V + + L+ + +++L   +A
Sbjct: 299 AWTLAQNEASCVVFGMPREAINMGGVCEVVDLSQVSQQMLAKISA 343
```

Fig.(5.1.4): Alignment of the *CheB* from *A. tumefaciens* with *E. coli* (K-12).

```
Score =  174 bits (442), Expect = 5e-45
Identities = 102/246 (41%), Positives = 144/246 (58%), Gaps = 9/246 (3%)

Query: 32  IAAMIYADAGIYLNDTKASLVYSRLSKHIRNLGLSGFREYCALVSSSEGAQPRREMLSHL 91
           I+ +IY  AGI L D K  +VY+RL + +R+LGL+ F  Y  L+ S++ +   +  ++ L
Sbjct: 30  ISQLIYQRAGIVLADHKRDMVYNRLVRRLRSLGLTDFGHYLNLLESNQHSGEWQAFINSL 89

Query: 92  TTNFTRFFRENHHFEHLRDEVLPGLIARAKSGGRVRIWSAACSDGQEPYSIALTVLAMFP 151
           TTN T FFRE HHF  L D       AR +S G  R+WSAA S G+EPYSIA+T LA
Sbjct: 90  TTNLTAFFREAHHFPLLADH------ARRRS-GEYRVWSAAASTGEEPYSIAMT-LADTL 141

Query: 152 NAADYDFKILATDIDPKILAQARAGVYDDNALETVSPAMRKQWFTEVDAGGRRKFRIDDK 211
             A   +K+ A+DID ++L +AR+G+Y    L+ ++P   +++F          R+  +
Sbjct: 142 GTAPGRWKVFASDIDTEVLEKARSGIYRHEELKNLTPQQLQRYFMRGTGPHEGLVRVRQE 201

Query: 212 VKRLITFNELNLMT-QWPFKGNFDVIFCRNVVIYFDEPTQVRIWSRFAGLLPEGGHLYIG 270
           +   + F  LNL+  Q+   G FD IFCRNV+IYFD+ TQ  I  RF  LL   G L+ G
Sbjct: 202 LANYVDFAPLNLLAKQYTVPGPFDAIFCRNVMIYFDQTTQQEILRRFVPLLKPDGLLFAG 261

Query: 271 HSERVS 276
           HSE  S
Sbjct: 262 HSENFS 267
```

Fig.(5.1.5): Alignment of the *CheR* from *A. tumefaciens* with *E. coli*

The swarming behaviour of wild-type and mutants of *A. tumefaciens*, C1/delB and C1/del/R, were compared. Usually a 3µl sample of cells grown in LB medium were placed on swarm plates, or the bacterial cells were stab inoculated, and plates were incubated at 28-30°C for up to three days. The swarm-ring size was measured after 10, 24, 48 and 56hr. The effects of the deletion of *cheB* and *cheR* genes are shown in Fig. 5.1.6 and Fig. 5.1.7.

When both wild-type and mutants were incubated for the same period of time both C1/delB and C1/delR displayed a strong effect on swarming behaviour. *A. tumefaciens cheB* mutant had inhibited swarming behaviour and their swarm ring was approximately 60% reduced in comparison with those formed by wild-type, C58C1. *A. tumefaciens cheR* mutant formed swarms approximately 36% of the diameter of wild-type swarms after incubation for the same period of time.

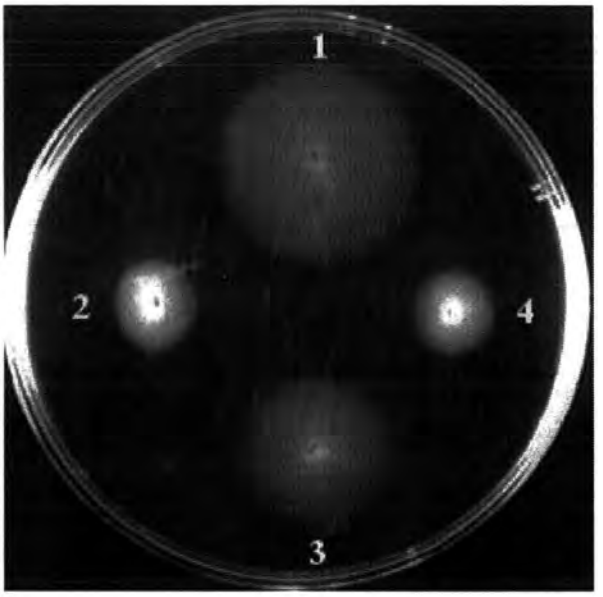


Fig. (5.1.6): Swarm test of *A. tumefaciens* wild type (1) and chemotaxis mutant (2 & 4) C1/delB and (3) C1/delY1 (Photograph after 48hr).

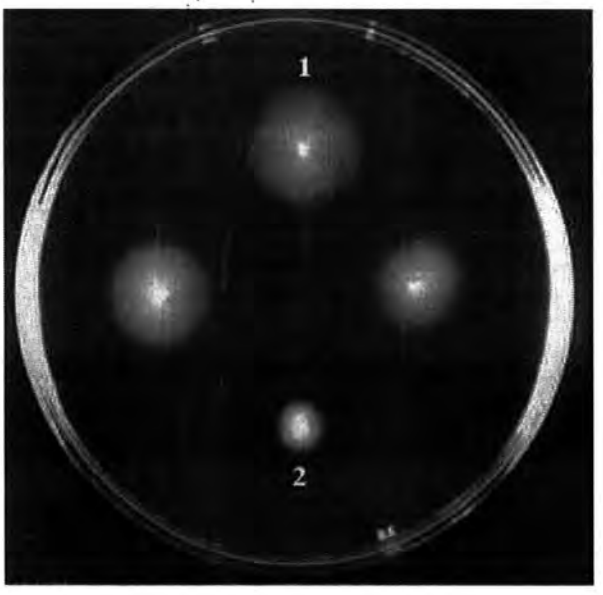


Fig. (5.1.7): Swarm test of *A. tumefaciens* wild type (1) and chemotaxis mutant (2) C1/delR. Another two strains are resulting from mutagenesis those changes to phenotypic properties of wild type (photograph after 24hr).

Wild-type cells (C58C1) formed swarms and their displacement of the edge was clearly different from that produced by *cheB* and *cheR* mutants (See Fig. 5.1.8). Wild-type produced swarm of approximately 3cm in diameter after 56hr. The mutant cells, C1/delB, produced swarm of approximately 0.9cm in diameter after the same length of time. The edge of swarm produced by *cheB* mutant moved outward at a rate of only about 20% of that of the wild-type swarm (see Fig. 5.1.6). The mutant cells, C1/delR, produced swarm approximately 0.8cm in diameter after 56hr; however, this mutant produced a growth pattern of high density. The edge displacement swarm was at a rate about 20% of the wild type swarm.

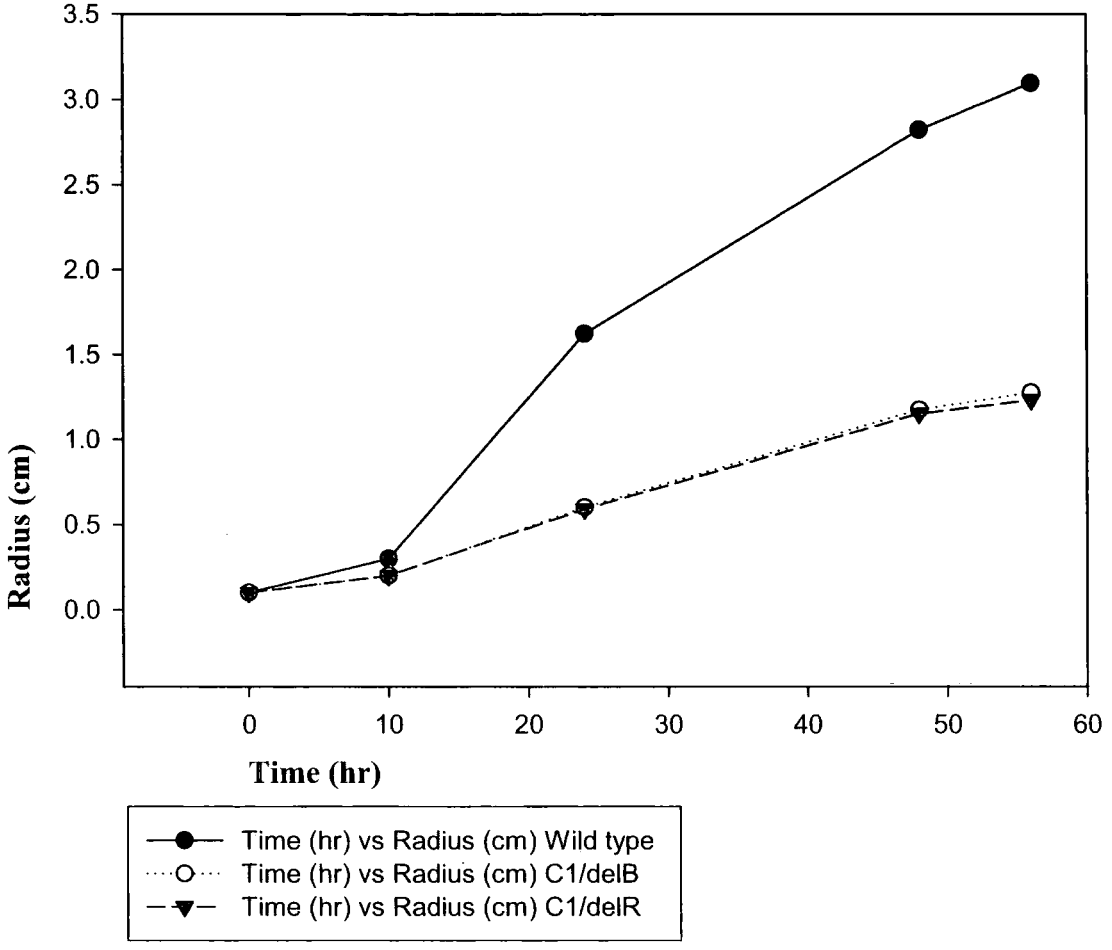


Fig. (5.1.8): Displacement of the edge of swarms produced by cells of *A. tumefaciens* (wild type) and the mutant strains, C1/delB and C1/delR.

5.2. Blindwell assay

5.2.1. Effect of the deletion of *cheY1* and *cheY2* genes:

Results were presented based on the number of bacterial cells attracted to acetosyringone and also in the form of the Chemotaxis index (CI). Data from the chemotaxis assays show that *cheY1* and *cheY2* have effects on the tactic response. According to Fig. (5. 2. 1) *A. tumefaciens* (C58C1) wild-type cells showed a weak response to acetosyringone in the concentration range of 10^{-4} M to 10^{-8} M and the maximum response observed in the concentration of 10^{-7} M.

Compared to wild-type strain, taxis of the *A. tumefaciens cheY1* mutant was 75% reduced at 10^{-7} M concentration of attractant, and 27% increased at 10^{-5} concentration indicating decreased sensitivity of CheY2 alone to chemotaxis signalling (see Fig. 5.2.1). The decrease response of *cheY1* mutant indicates the importance of CheY1, which is essential for the full tactic response. Compared to *cheY1,* mutant data from the chemotaxis assay indicated that *cheY2* gene has greater effect on the tactic response. At the peak concentration (10^{-7} M), the response of wild-type strain, taxis of the *cheY2* mutant was minimum (98% reduced) (Fig. 5.2.1).

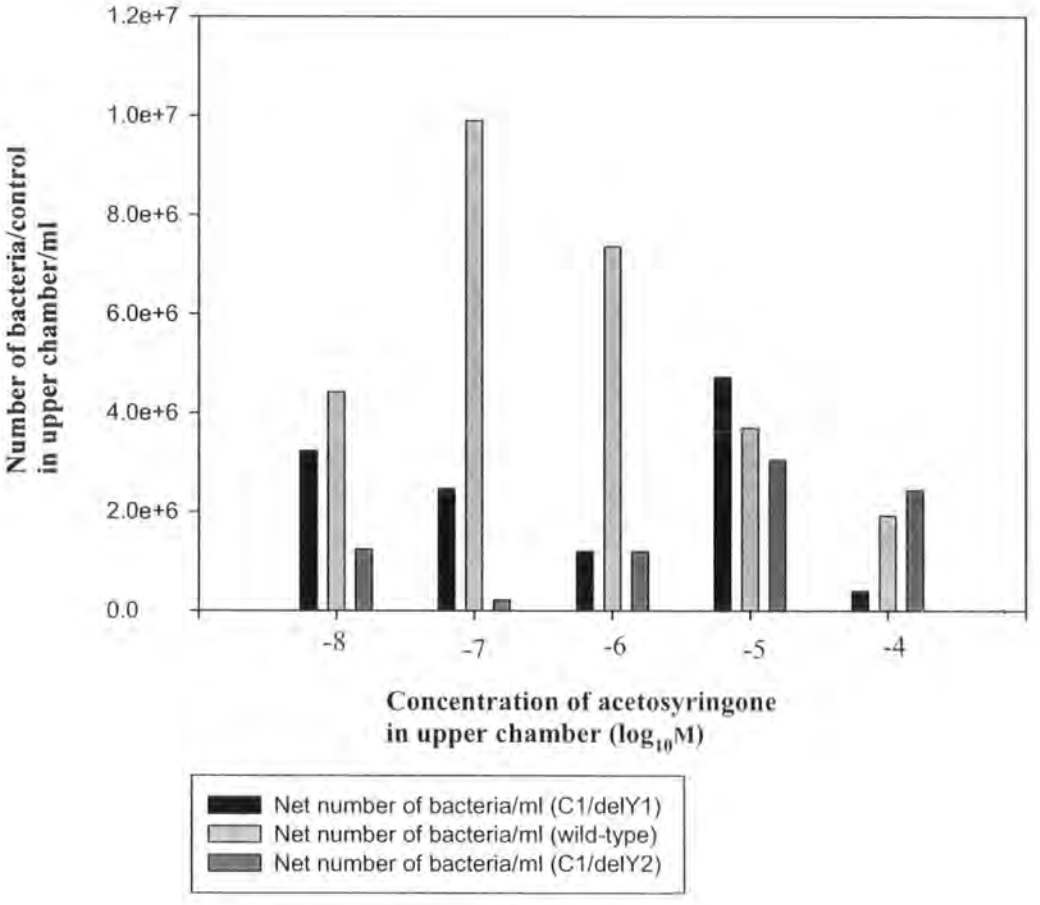


Fig. (5.2.1): Concentration- response chart showing the net number of *A. tumefaciens* wild type and chemotaxis-mutant strains, C1/delY1 and C1/delY2, cells attracted toward acetosyringone.

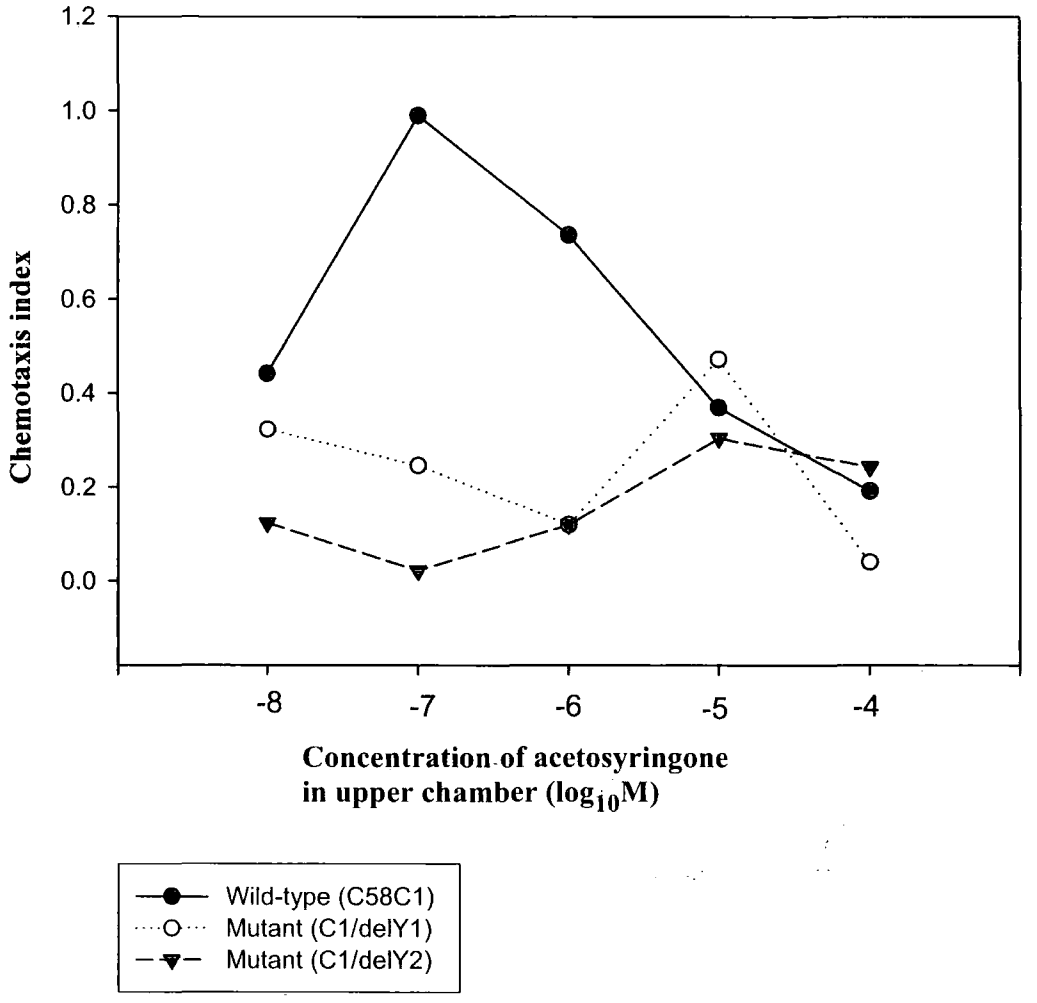


Fig. (5.2.2): Concentration-response curves for *A. tumefaciens* wild type (C58C1) and Chemotaxis mutant strains, C1/delY1 and C1/delY2, in blindwell assay. Each curve represents the mean of two experiments, each with duplicate readings, after background subtraction.

5.2.2. Effect of the deletion of *cheB* and *cheR* genes:

Chemotaxis assays were performed on the *cheB* and *cheR* mutant strains using acetosyringone as a chemoattractant. The results show that *cheB* and *cheR* mutants have the same phenotype indicating that both genes are essential for tactic response. At the peak concentration response of wild-type (10^{-7} M) only small number of *cheR* mutant cells (~1- 1.2% of wild-type) were able to response to attractant (Fig. 5.2.4). Compared with wild-type, taxis response of *cheB* mutant strain is 94% reduced at 10^{-7} M, but the maximum reduction occurred at the 10^{-6} M (~ 4% of wild-type cells) (see Fig. 5.2.3).

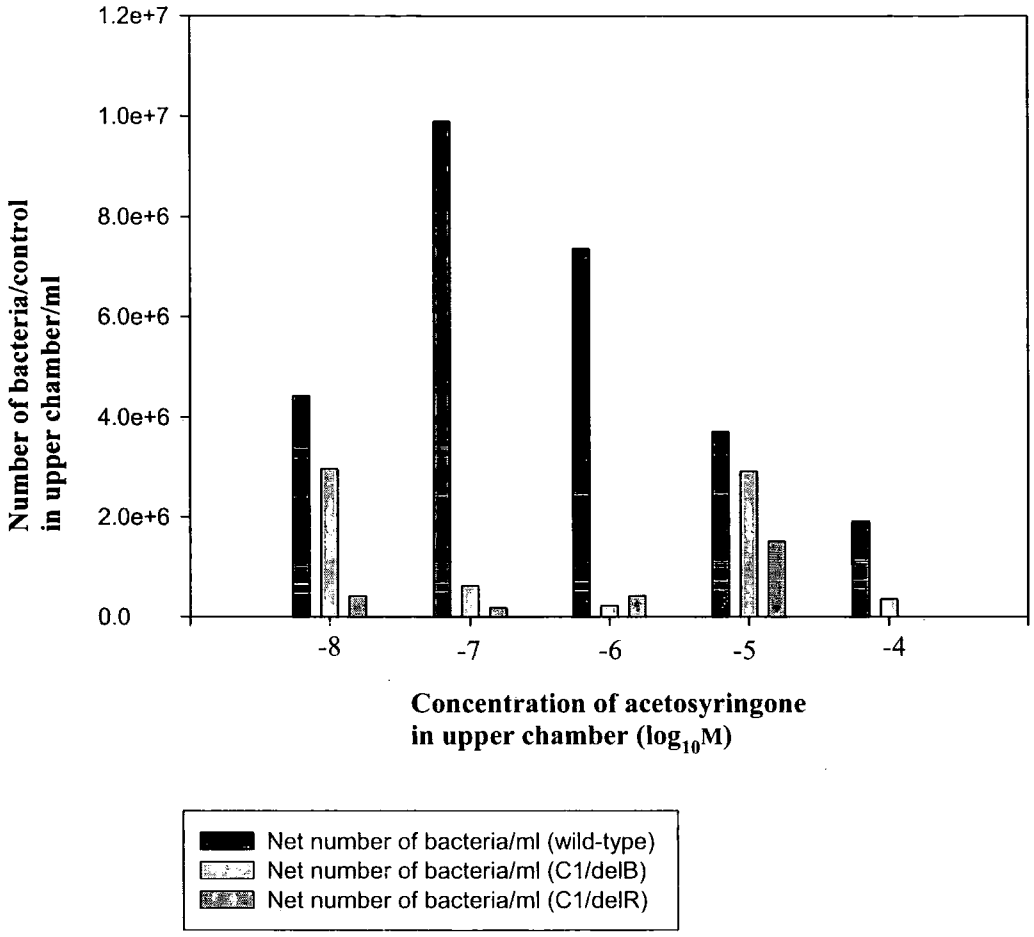


Fig. (5.2.3): Concentration- response chart showing the net number of *A. tumefaciens* wild type and chemotaxis-mutant strains, C1/delB and C1/delR, cells attracted toward acetosyringone.

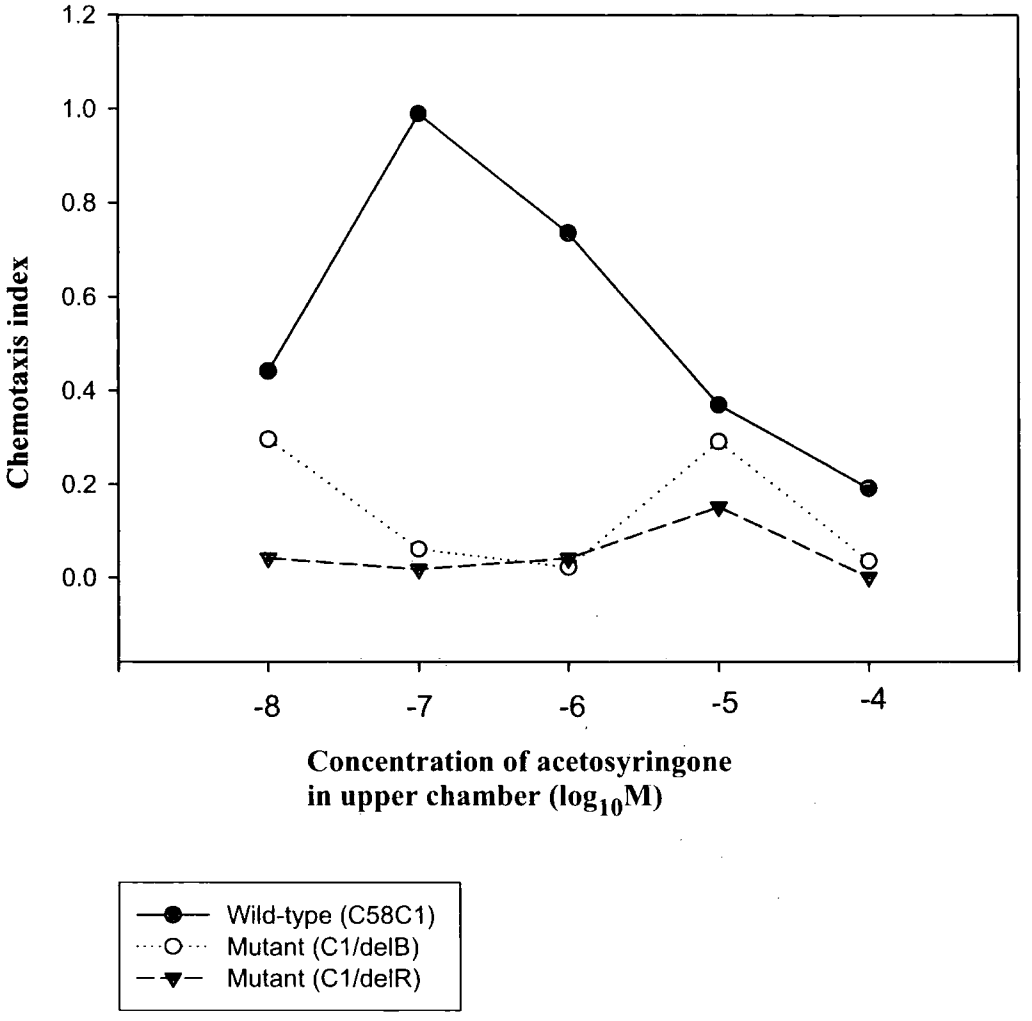


Fig. (5.2.4): Concentration-response curves for *A. tumefaciens* wild type (C58C1) and chemotaxis mutant strains, C1/delB and C1/delR, in blindwell assay. Each curve represents the mean of two experiments, each with duplicate readings, after background subtraction.

Chapter 6

Discussion

Discussion:

Agrobacterium tumefaciens has a chemotaxis operon containing *orf1*, *orf2*, *cheY1*, *cheA*, *cheR*, *cheB*, *cheY2*, *orf9* and *orf10*. A common feature of chemotaxis operon found in this bacterium the same as other members of α–subgroup of *proteobacteria* is two homologues of *cheY* gene, named *cheY1* and *cheY2*, and also the lack of *cheZ* gene homologues.

In *E. coli*, CheW links to the signalling domain of a MCP, the histidine protein kinase, CheA, which phosphorylates response regulator, CheY, or methyltransferase, CheB, in response to an attractant.

In this study, mutations were made in four genes of the chemotaxis operon genes previously identified (*cheY1*, *cheY2*, *cheB* and *cheR*). Phenotypic studies showed that each, except *cheY1* mutant, have strong effect on motility and chemotaxis behaviour of *A. tumefaciens*. The *cheY2*, *cheB* and *cheR* mutant strains showed impaired chemotactic capabilities in swarming behaviour assay.

As discussed earlier, the central part of chemosensory pathway is methyl-accepting chemotaxis protein (MCP), and the other part of this study was to find possible MCP gene (s) in *A. tumefaciens*.

The role of *cheY1* and *cheY2*:

Here, we have demonstrated that both CheY1 and CheY2 are required for the chemotactic behaviour in *A. tumefaciens*. Data from chemotaxis assays show that CheY1 and CheY2 have different effects on the tactic response. CheY2 alone mediates larger decrease (60%) in swarming behaviour to amino acids, suggested that

CheY2 is the main response regulator. The effect of the *cheY1* mutant on phenotypic behaviour of *A. tumefaciens* indicates the importance of functional CheY1 that is essential for the full tactic response.

In *Sinorhizobium meliloti*, it has been showed that signal termination is brought about by one of the two CheYs (SourjiK, V., and R. Schmitt, 1996). In this bacterium both CheY1 and CheY2 are phosphorylated by CheA, and it is suggested that CheY1 assumes the role of a phosphatase of CheY2-P by acting as a sink for phosphate that is shuttled from CheY2-P back to CheA. The protein database analysis of *A. tumefaciens* CheY1 and CheY2 showed 90% and 89% identity to CheY1 and CheY2 proteins of *S. meliloti*, suggesting the same phosphotransfer reaction between CheY1, CheY2 and CheA in *A. tumefaciens*.

```
>ref|NP_384744.1| CHEMOTAXIS REGULATOR PROTEIN [Sinorhizobium meliloti]
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 Score =  224 bits (571), Expect = 5e-59
 Identities = 110/121 (90%), Positives = 118/121 (97%)

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           MKK+VLTVDDSRTIRNMLLVTLNNAGFETIQAEDG+EGLE L+ +NPDVIVTDINMPRLD
Sbjct: 1   MKKRVLTVDDSRTIRNMLLVTLNNAGFETIQAEDGVEGLEKLDTANPDVIVTDINMPRLD 60

Query: 61  GFGFIEGVRRNEKYRAIPILVLTTESDAEKKNRARQAGATGWIVKPFDPAKLIDAIERVT 120
           GFGFIEGVR+N++YRA+PILVLTTESDAEKKNRARQAGATGWIVKPFDP KLIDAIERVT
Sbjct: 61  GFGFIEGVRKNDRYRAVPILVLTTESDAEKKNRARQAGATGWIVKPFDPTKLIDAIERVT 120

Query: 121 A 121
           A
Sbjct: 121 A 121
```

Fig.(6.1): Alignment of the CheY1 from *A. tumefaciens* **with** *S. meliloti*

```
>ref|NP_384749.1| CHEMOTAXIS REGULATOR PROTEIN [Sinorhizobium meliloti]
          Length = 129

 Score =  225 bits (573), Expect = 3e-59
 Identities = 116/129 (89%), Positives = 121/129 (93%)

Query: 1   MSLAEKIKVLIVDDQVTSRLLLSDALTQLGFKQITSAGDGEQGLKIMEQQPHHLVISDFN 60
           MSLAEKIKVLIVDDQVTSRLLL DAL QLGFKQIT+AGDGEQG+KIM Q PHHLVISDFN
Sbjct: 1   MSLAEKIKVLIVDDQVTSRLLLGDALQQLGFKQITAAGDGEQGMKIMAQNPHHLVISDFN 60

Query: 61  MPKMDGLGFLHAVRANPTTKKAAFIILTAQGDRALVQKAAQLGANNVLAKPFTIDKMRAA 120
           MPKMDGLG L AVRANP TKKAAFIILTAQGDRALVQKAA LGANNVLAKPFTI+KM+AA
Sbjct: 61  MPKMDGLGLLQAVRANPATKKAAFIILTAQGDRALVQKAAALGANNVLAKPFTIEKMKAA 120

Query: 121 IEAVFGSLK 129
           IEAVFG+LK
Sbjct: 121 IEAVFGALK 129
```

Fig.(6.2): Alignment of the CheY2 from *A. tumefaciens* with *S. meliloti*

A. tumefaciens CheA protein was shown to have 48% identity to *E. coli* CheA protein. The main region of variation between the two CheA proteins was shown to be between the *E. coli* CheA region containing the P2 domain, which has CheY-binding capability. Differences in the sequence of the domain in the *A. tumefaciens* CheA might be because it may interact with both CheY1 and CheY2 proteins. On the other hand, because a CheZ-like phosphatase has not been detected in *A. tumefaciens*, and CheY1 was found to be essential for the full tactic response (Fig. 5.1.3), it is possible that CheY1 is a competitor of CheY2 for phosphorylation by CheA.

The same result was obtained for *Sinorhizobium meliloti* when its chemotaxis measured against proline (Sourjik, V., and R. Schmitt, 1996). Phosphotransfer reactions assay using radiolabeled recombinant proteins, CheA, CheY2 and CheY1 showed that CheY1 has the role of a phosphatase of CheY2-P by acting as a sink for phosphate when unphosphorylated CheA is present. Phosphotransfer is from CheY2-P via CheA to CheY1 (Sourjik, V., and R. Schmitt, 1996).

To find the exact role of *cheY1* and *cheY2* it seems that more testing of chemotactic capability is required perhaps by performing the swarming plate assay

using specific chemoattractants; also, either blind well or capillary assays could be performed using a similar range of compounds.

To find the mechanism of signal transduction in *A. tumefaciens* it is necessary to investigate the phosphotransfer reactions between CheA/CheY1/CheY2. Also, monitoring changes of the rotation rate of flagella in tethering experiments and analysis of free-swimming cells in both wild type and each *cheY1*, *cheY2*, *cheY1/cheY2* mutant strains and in combination with cheA indicated the correct relationships between CheA, response regulators (CheY1 and CheY2) and the flagellar motor.

The role of *cheB* and *cheR*:

CheR is a methyltransferase, which transfers methyl groups from s-adenosylmethionine molecules to the cytoplasmic domain of the receptors during adaptation to positive stimuli. CheB is also a methylesterase that removes methyl groups from receptors during adaptation to negative stimuli. Several observations have led to the suggestion that methylation-dependent adaptation may have a role in *A. tumefaciens* chemotaxis. *A. tumefaciens* met-auxotroph does not display chemotaxis unless supplemented with methionine. Partial chemotaxis can be restored by supplementation with moderate substrate for s-adenosyl methionine synthatase (Shaw, C.H., 1996).

In this study we also examined the adaptation enzymes by genetic and some biochemical approaches. Our data from chemotaxis assay and swarming plate assay shown that both *cheR* and *cheB* have strong effect on the tactic response of *A. tumefaciens* mutant strains, C1/delB and C1/delR. The deletion of either *cheR* or *cheB*

resulted in a nonchemotactic phenotype both on swarm plates and in chemotaxis assays under conditions tested.

Comparative protein sequence analysis of *A. tumefaciens* CheR revealed that it has a CheR-specific N-terminal domain, the catalytic domain, and a β–subdomain. This part of the protein show 41% identity to *E. coli* CheR protein. The functional role of the β–subdomain is interaction with MCPs. It also has homology with α_2 helix of *E. coli* CheR that is involved in the recognition and methylation of MCP. This information suggest that chemotaxis is mediated by chemotaxis operon genes in *A. tumefaciens* and is dependent on a methyl-accepting chemotaxis protein.

```
Score =  174 bits (442), Expect = 5e-45
Identities = 102/246 (41%), Positives = 144/246 (58%), Gaps = 9/246 (3%)

Query: 32  IAAMIYADAGIYLNDTKASLVYSRLSKHIRNLGLSGFREYCALVSSSEGAQPRREMLSHL 91
           I+ +IY  AGI L D K  +VY+RL + +R+LGL+ F  Y  L+ S++ +   +  ++ L
Sbjct: 30  ISQLIYQRAGIVLADHKRDMVYNRLVRRLRSLGLTDFGHYLNLLESNQHSGEWQAFINSL 89

Query: 92  TTNFTRFFRENHHFEHLRDEVLPGLIARAKSGGRVRIWSAACSDGQEPYSIALTVLAMFP 151
           TTN T FFRE HHF  L D       AR +S G  R+WSAA S G+EPYSIA+T LA
Sbjct: 90  TTNLTAFFREAHHFPLLADH------ARRRS-GEYRVWSAAASTGEEPYSIAMT-LADTL 141

Query: 152 NAADYDFKILATDIDPKILAQARAGVYDDNALETVSPAMRKQWFTEVDAGGRRKFRIDDK 211
             A   +K+ A+DID ++L +AR+G+Y    L+ ++P   +++F          R+  +
Sbjct: 142 GTAPGRWKVFASDIDTEVLEKARSGIYRHEELKNLTPQQLQRYFMRGTGPHEGLVRVRQE 201

Query: 212 VKRLITFNELNLMT-QWPFKGNFDVIFCRNVVIYFDEPTQVRIWSRFAGLLPEGGHLYIG 270
           +   + F  LNL+  Q+   G FD IFCRNV+IYFD+ TQ  I  RF  LL   G L+ G
Sbjct: 202 LANYVDFAPLNLLAKQYTVPGPFDAIFCRNVMIYFDQTTQQEILRRFVPLLKPDGLLFAG 261

Query: 271 HSERVS 276
           HSE  S
Sbjct: 262 HSENFS 267
```

Fig.(6.3): Alignment of the CheR from *A. tumefaciens* with *E. coli*

In experiments on the *A. tumefaciens cheB* mutant, chemotaxis does not occur because of the lack a methylation-independent adaptation system. This hypothesis is supported by the following observations: (i) the full sequence of *A. tumefaciens* chromosomal DNA indicates that there are no other cheB homologues genes (ii) unlike *A. tumefaciens,* in some bacteria such *Bacillus subtilis,* two other proteins,

CheC and CheD, have a role in adaptation pathway. Then, alternative path could allow *B subtilis* to achieve chemotaxis.

The swarming behaviour assay indicated 60% size reduction of C1/delB and 64% size reduction of C1/delR in comparison with *A. tumefaciens* wild type strain (Fig.5.1.6). Results of chemotaxis assay shown that both *cheR* and *cheB* genes were essential for normal chemotaxis; compared to the wild type, *cheB* mutant showed a 94% decrease in response to acetosyringone, and the *cheR* mutant indicated 99% decrease in response to acetosyringone at 10^{-7}M concentration (see Fig.5.2.3).

As discussed in Chapter 3, genetic and some previous biochemical data indicated that *A. tumefaciens* has a DNA fragment containing an orf that has a role, coupled with CheR and CheB, in the chemotactic response of this bacteria.

To investigate and find the exact role of *cheR* and *cheB* additional experiments should be performed. As discussed in Chapter 1, CheB act as a response regulator and phosphoryl group acceptor from CheA. Therefore performing a phosphotransfer assay could indicate the relationship between CheA and CheB. Methanol release after methylation of MCPs is a common reaction during chemotactic response and therefore a methanol release experiment could show another aspects of chemotaxis pathway and determine the role of both *cheR* and *cheB* genes.

It is necessary to check the swarming and chemotaxis behaviour of *cheR* and *cheB* mutant strains against more specific chemoattractants and to analyse free-swimming cells in both wild type and mutants, by measuring the rotation rate of flagellar motor in tethering experiment, which could indicate the influence of *cheR* and *cheB* genes on flagellar motor rotation and bacterial cell movement.

Identification of the MCP-like gene:

The presence of a protein antigenically related to the *E. coli* Trg protein in *A. tumefaciens* has been reported (Morgan, D.G., et al., 1993). Our results provide evidence for the existence of a MCP-like gene in *A. tumefaciens* C58C1. Positive hybridisation to a probe from MCP3.2.7P of *Rhizobium leguminosearum* and sequence similarity between this fragment and the deduced amino acid sequences for MCPs suggest that this gene does code for MCP protein. The highest alignment scores occurred with *McpA* gene recently identified from *A. tumefaciens*.

The amino acid sequences indicate this gene contains all the characteristic features of MCPs, such as methylation sites, signalling domain and transmembrane domains (Fig. 3.2.4). Transmembrane domains have been predicted in McpA using a transmembrane helix prediction program, the TMHMM programme (Krogh, A., et al., 2001). This predicts two transmembrane regions, the first TMD from amino acid 20 to 42 and the second from amino acid 205 to 224. This prediction suggests the N-terminal domain of McpA is located in the periplasmic space and probably functions as a sensor domain. In some bacteria, such as *E. coli* and *S. typhimurium*, it has been shown that a pentapeptide sequence (NWETF) is present at the carboxyl terminus of some chemoreceptors (the high-abundance receptors), as is a similar motif in McpA (GWEDF, GFEDF). This pentapeptide provides a docking site for the methyltransferase and greatly enhances methylation of these receptors (Barnakov, A.N., et al., 1999). Such a docking site is absent in *A. tumefaciens* McpA indicating that methyltransferase may be docking to other specific sites in this protein.

Previous investigations argue that *A. tumefaciens C58* is attracted to a group of phenolic compounds that have been identified as *vir* gene inducers. Acetosyringone

and related compounds elicited chemotaxis in two different assays. According to these experiments the range of concentrations active in inducing chemotaxis was quite narrow and chemotaxis required the Ti-plasmid, and specifically the regulatory genes *virA* and *virG* (Shaw, C. H., et al., 1989).

Palmer, A., et al. (1992) reported that non-phosphorylatable, mutant VirA and VirG proteins are incapable of replacing their wild type counterparts in conferring the ability to respond chemotactically to nanomolar concentrations of vir-inducing phenolics such as acetosyringone. It seems chemotaxis in *A. tumefaciens* involves phosphorylation of VirA and VirG.

Also Ashby, A. M., et al. (1988) have described the phenolic compounds that have different effect on virulence region and have different roles to acting as chemoattractants as well. They categorized the acetosyringone as a strong inducer of the virulence region and a chemoattractant for Ti-plasmid-harbouring strain only (Ashby, A.M., et al., 1988).

Results of another group failed to detect chemotaxis toward acetosyringone at any concentration (Hawes, M.C., and L. Y. Smith, 1989); however, another group reported that acetosyringone did not elicit chemotaxis in *A. tumefaciens A348* and that chemotaxis toward related compounds did not require the Ti-plasmid (Parke, D., et al. 1987).

Our results in this project show that acetosyringone is a weak chemoattractant for *A. tumefaciens* wild type *(C58C1)* and at the 10^{-7}M concentration (peak concentration of attraction) about 1% of bacterial cells are attracted to this phenolic compound (Fig.5.2.1). Comparing the response to acetosyringone of the wild type and *che* mutant strains produced in this thesis indicates that this phenomenon is mediated by the chemotaxis operon genes.

Compared with previous results discussed above, the results of this study indicate that *A. tumefaciens* can express two separate receptor systems for chemotaxis toward acetosyringone: (i) a Ti plasmid-encoded system, (ii) a chromosomally encoded system. The *virA* and *virG* are the Ti plasmid functions responsible for the specific chemotactic response toward acetosyringone. At low concentrations of attractant they mediate chemotaxis and at higher concentration they effect *vir* induction.

Our results demonstrate that chromosomally encoded system is mediated by chemotaxis operon genes. Both systems gave a definite dose response, indicating maximal attraction at 10^{-7}M concentration of acetosyringone. The two systems differed markedly in the number of bacterial cells attracted towards acetosyringone. The chemotactic response mediate by chemotaxis operon genes showed a smaller number of bacterial cells attracted towards attractant.

The positive chemotactic response of *A. tumefaciens* to acetosyringone and several other aromatic compounds raises the question as to whether these compounds are attractants or whether they are converted to metabolite (s) that interact with one or more chemoreceptors. The full chromosomal sequence indicates that *A. tumefaciens* contains several genes that appear to code for distinct methyl-accepting chemotaxis proteins. It appears more likely that some of them are acting as chemoreceptors for these phenolic compounds.

In order to study the chemotactic response of *A. tumefaciens* in-frame deletions should be made of the remaining *che* operon genes. Also, further analysis of these effects could be come from performing overexpression studies of each of the proteins transcribed by these genes.

A. tumefaciens has a unique perspective on which further investigations should be carried out. This is the possible interactions between Ti-encoded virulence system and chromosomally-encoded chemotaxis system. Strong chemotaxis activity to plant wound phenolics has been shown to require *virA/virG* system from Ti-plasmid. It is therefore possible that this system interacts with some elements of chemotaxis operon genes. To investigate interactions between *vir* and *che* systems it could be possible to use the Ti-containing *che* mutant strains constructed in this work, using wound exudates as a chemoattractants.

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