THE MOLECULAR BIOLOGY OF CHEMOTACTIC SIGNAL TRANSDUCTION IN RHODOBACTER SPHAEROIDES

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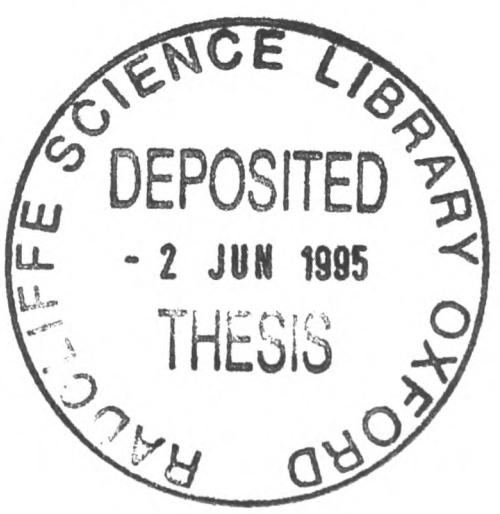

ABSTRACT

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This study has succeeded in identifying, cloning and sequencing three genes previously unknown in *R. sphaeroides*, *che* W, *che* R and *che* Y2. These genes are homologous to genes recently discovered in *Rhizobium meliloti* and also show a lesser homology to genes in other organisms including the well characterised enteric chemotaxis genes.

A comparative analysis of the deduced proteins has been made to determine structural and functional similarities between the *R. sphaeroides* genes and their homologues in *R. meliloti* and in *E. coli.* Considerable conservation of functionally important amino acid residues was revealed.

In vivo complementation was done using the *R. sphaeroides* Che W protein to complement Che W $^-$ strain of *E. coli.* The *R. sphaeroides* protein complemented the *E. coli* strain empirically proving similarity of function of the protein between these widely divergent genera.

Homologous recombination using transposon-interrupted genes and internal gene fragments was attempted and met with limited success because *R. sphaeroides* proved permissive to the suicide vectors used.

The most significant result of this study has been not in the similarities revealed between the chemotaxis systems of *R. sphaeroides* and the enterics, but in the differences discovered. It is now known that *R. sphaeroides* possesses two Che Y proteins, Che Y and Che Y2, and that whereas the *R. sphaeroides* Che Y2 protein is closely related to the *E. coli* Che Y protein, the *R. sphaeroides* Che Y protein shows considerable evolutionary divergence from it's enteric homologue. The significance of a second copy of Che Y protein suggests that these two response regulators act independently at the motor/switch complex and that they represent the final elements of two functionally distinct chemotactic sensory transduction pathways. This dual pathway system is not present in the enteric bacteria (a member of the γ-group of proteobacteria) but, we propose, may be present in a large group of environmentally important bacteria, the α-group of proteobacteria. *Caulobacter*, *Agrobacterium*, *Rhizobium* and *Azospirillum* species (α-group proteobacteria) all show behavioural similarities, and there are genetic clues to suggest that these organisms possess a dual chemotactic sensory transduction similar to the one we have found in *R. sphaeroides.*

Apart from being fundamentally important, the dual sensory pathway hypothesis explains the wild-type behaviour of *R. sphaeroides*, as well as the difficulty in obtaining behavioural mutant phenotypes using methods developed during the investigation of *E. coli.*

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1. INTRODUCTION

1.1

Historical Perspective

The study of bacterial behaviour has a venerable history. From the earliest microscopic studies Leewenhoek recognised the ability of microorganisms to propel themselves through their environment, and reasoned that they must possess organs for this function, which were too small to be resolved by his microscope. In 1838, Eherenberg described the eukaryotic flagellum, and it was supposed that smaller motile organisms, including bacteria might also propel themselves with a similar organ. In the 1880's Englemann discovered oxygen taxis by bacteria (Englemann, 1883) and Pfeffer, who had recently described chemotaxis by the sperm of ferns and mosses, published the first article on the behaviour of microorganisms, in which he described the accumulation of bacteria at the mouth of a capillary tube containing a chemical attractant (Pfeffer, 1884). The early work of Pfeffer and Englemann did not initiate widespread investigation and, except for a brief flurry of work by Clayton in the 1950's and 1960's, was consigned to become a historical footnote (Clayton, 1953a, 1953b, 1953c, 1955a, 1955b, 1957, 1958 and 1964). It was not until nearly eighty years later when Julius Adler began his work on bacterial chemotaxis and signal transduction that the field began its rapid expansion (Adler, 1969, 1973 and 1988). Adler chose *Escherichia*

coli as his primary research organism because it was relatively well defined physiologically and biochemically defined and because of the availability of genetic techniques which had recently been developed using this species. Adler reasoned that the chemotactic signal transduction pathway might be investigated using similar sorts of algorithms to those which had proved so effective in the dissection of biochemical pathways; but first, assays had to be developed to quantify the chemotactic response.

Adler initially converted Pfeffer's qualitative capillary tube method into a quantitative assay (Adler, 1969; Adler, 1973) which revealed that many amino acids and simple sugars acted as potent attractants and that accumulation occurred at certain attractant concentrations which were then defined as being "optimal". Semi-solid agar "swarm" plates, in which cells produce and respond to nutrient gradients as they grow (see methods), were used to enrich for behavioural mutants by serially picking cells from the centre of a swarm and reinoculating into a fresh swarm plate. Using this technique Adler, Armstrong and Dahl isolated the first chemotaxis mutants (Armstrong *et al*, 1967; Adler, 1969). This original procedure was laborious, producing a very low mutant yield, many of which simply possessed motility or growth defects; also a large number of the cells picked from the centre of the swarm, even after numerous passages, proved to be genetically wild-type. This was due to a high proportion of genotypically normal cells which had suffered some physiological insult, such as temporary loss of flagella,

and therefore had become phenotypically, chemotactically abnormal. It was the development of "miniswarm" methods, in which semi-solid agar was seeded with bacteria, producing several hundred separate colonies per plate, that eventually facilitated the efficient screening and isolation of chemotaxis mutants. Mutagenesis, using transposons carrying antibiotic resistance markers, used in conjunction with miniswarm screening, has been used successfully to isolate mutants deficient in various chemotaxis components, and most of the known elements of the signal transduction pathways of a variety of genera have been discovered using the products of such experimentation (Parkinson, 1987).

In the 1960's, prior to the work by Adler, it was generally felt that bacteria did not sense attractants *per se*, but responded to a more general change in internal energy levels related to the general metabolic state of the cell, probably via sensing of the concentrations of various high-energy intermediates (Clayton, 1964). This theory held wide appeal since it would produce an organism which did not need to make dedicated sensory receptors, but which could efficiently and sensitively recognise and respond to any substance or environment which was bioenergetically favourable. It was Adler who demonstrated the existence of dedicated chemoreceptors for sensing attractants (Adler, 1969) and also showed, by use of a capillary tube assay using one *E. coli* strain that could not metabolize methionine and another that could not transport galactose, that neither metabolism nor uptake of the

effector was necessary to produce a chemotactic response. By virtue of this phenomenon he inferred, and later proved, that the receptor spanned the plasma membrane, transducing a signal from the periplasmic space into the cytoplasm (Hazelbauer *et al*, 1971).

It should be noted that the existence of dedicated chemoreceptors does not invalidate Clayton's concept of general energy sensing and there is compelling recent evidence to suggest that there is a general mechanism underlying and interacting with components of the dedicated system. A global signal is thought to exist which is likely to be a phosphate donor, possibly acetyl phosphate (McCleary *et al*, 1993). Considerable attention has also been given to intracellular fumarate, which has been shown to act in conjunction with a component of the chemotaxis signal transduction system, at the motor, to produce a pseudo-tactic effect (Eisenbach and Barak, 1992). It is also interesting to note, in retrospect, that Clayton's chosen experimental organism was the purple non-sulphur bacterium, *Rhodospirillum rubrum*. Theories developed with *R. rubrum* were extrapolated and applied to *E. coli*, which is now known to be significantly different in terms of its taxis machinery (see later). Energy sensing taxis experiments using *E. coli* seem to have yielded few encouraging results, and once dedicated sensory receptors had been discovered, research into energy sensing taxis systems became marginalised and unfashionable.

The discovery that methionine (and specifically a metabolic product of

methionine, s-adenosylmethionine, which is a methyl-group donor) is essential for this transducer-dependent taxis led in the mid-seventies to the identification of the methyl-accepting chemotaxis proteins (MCP's) as the initial membrane spanning receptor/transducer component in the chemotaxis signal transduction pathway (Kort *et al*, 1975). To date, five MCP's are known in *E. coli* and *S. typhimurium* (Tar, Tsr, Trg, Tap and Tcp), each associated with a different set of chemoeffectors (Macnab, 1992). Methylation and demethylation at specific glutamate residues on the cytoplasmic side of the MCP was found to be correlated with an adaptive response (Aswad *et al*, 1975). It was observed that an increased attractant concentration or decreased repellent concentration was associated with an increase in methylation. A decrease in attractant concentration or an increase in repellent was associated with demethylation (and ester hydrolysis) at the MCP. The degree of methylation (and esterification) was shown to be related to the change in chemoeffector concentration (Silverman *et al*, 1977). The proteins responsible for mediating adaptation were identified as a glutamyl methyltransferase, Che R (Springer *et al*, 1977; Kirsch *et al*, 1993) and an s-adenosylmethionine : glutamyl methylesterase, Che B (Stock *et al*, 1978; Stewart, 1993).

By 1972, Howard Berg and others had elucidated the "biased random walk" strategy of tactically controlled bacterial locomotion based on an alteration in the frequency of changes in swimming direction (Berg *et al*, 1972; Macnab *et al*, 1972). In 1974 Silverman and Simon were able to tether the bacterial flagellum to a glass slide by anti-flagellin

antibody and observe the rotation of the bacterial body, thereby demonstrating that unlike the eukaryotic flagellum, the bacterial flagellum was powered by a unique rotary motor (Silverman and Simon, 1974) as had been hypothesized a year earlier by Berg and Anderson (Berg and Anderson, 1973). Silverman and Simon proposed that alteration in swimming direction was related to flagellar switching frequency and in that same year it was experimentally demonstrated, again using tethering experiments, that chemotaxis results from an alteration in the frequency of directional change which is based on an alteration of the frequency of flagellar rotational reversals (Larsen *et al*, 1974). These observations begged the questions: what controls the frequency of rotational switching? what is the signal? and how is the signal relayed from the MCP to the motor?

The study of mutants of *E. coli* and *S. typhimurium* that were deficient in directional changing ("smooth swimmers") or that changed direction with a pathologically high frequency ("tumbly") led to the identification of just six genes involved in signal transduction: *che* A, *che* B, *che* R, *che* W, *che* Y and *che* Z. Deletion of any one of these genes produces a cell which is fully motile but unable to respond to chemoeffector gradients (Parkinson *et al*, 1982; Stock, 1989). Mapping revealed that genes associated with disparate but related aspects of behaviour were clustered. Silverman and Simon identified the "Mocha" operon (Silverman *et al*, 1976) which contains genes for MCP production, motor (Mot) components, chemotaxis signal

transduction (Che) proteins and some of the structural flagellar proteins. Other functionally related genes of the flagellar and chemotaxis system have since been found to be similarly arranged in operons (Fig.01), and transcribed polycistronically (Macnab, 1992).

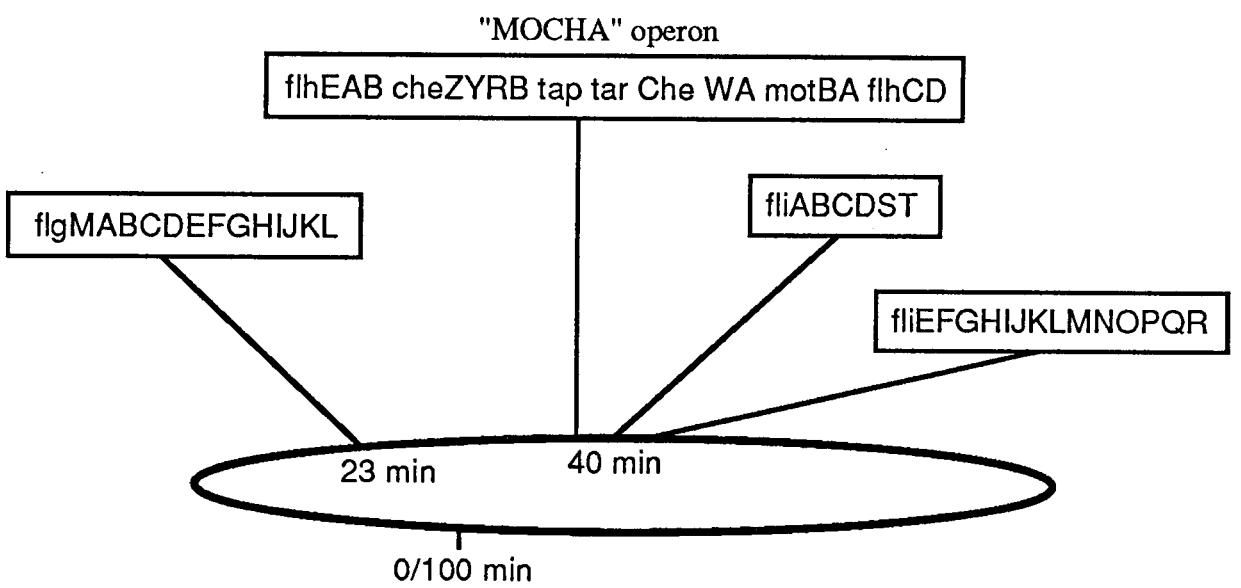


Fig.01: Chromosomal organization of the flagellar, chemotaxis and related genes of *E. coli* (adapted from Macnab, 1992).

The Mocha operon in *E. coli* is under the control of a single promoter and so produces a single, polycistronic transcript providing concomitant expression of functionally related genes and maintaining stoichiometry between components. The Che system has since been shown to be sensitive to variations in component stoichiometry (Gegner *et al*, 1992) and signal transduction is severely impaired by overexpression of the Che proteins (Stewart *et al*, 1988; Stock *et al*,

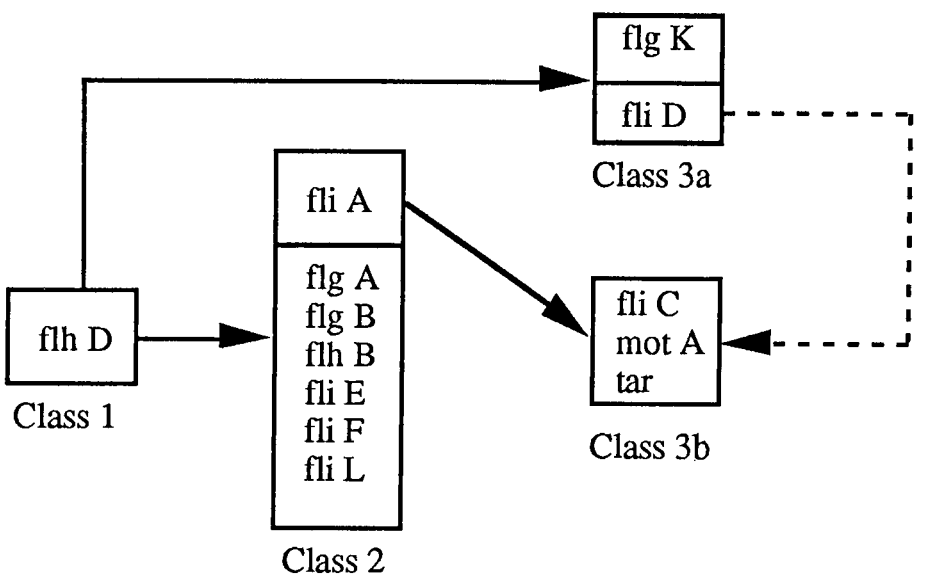


Fig.02: Hierarchy of flagellar gene expression. Flh D codes for a sigma factor required for the expression of the class 2 and class 3a operons. Fli A codes for a sigma factor required for transcription of the class 3b operon.

1991). Expression of operons controlling flagellar synthesis and bacterial behaviour was found to be hierarchical, involving alternative sigma factors, positive and negative regulated gene expression (Hellman *et al*, 1988; Macnab, 1992). Within the last few years, sequences of the *mot*, *fla*, *che* and *mcp* genes have been deduced, proteins have been purified and functions ascribed to them (Bollinger *et al*, 1984; Yamaguchi *et al*, 1986; Wolfe *et al*, 1987; Stock *et al* 1985 and 1988; Macnab, 1992). The flagellum and transmembrane transducer proteins are now relatively well defined. Areas presently under the greatest scrutiny and promising the greatest reward are those dealing with the interactions between the Che proteins themselves, between proteins within the Che A/Che W/Che Y/MCP complex and between Che proteins and the flagellar motor. A valuable resource in

these studies is the availability of genera which are chemotactic but inherently different from enterics, functionally and therefore possibly structurally; such differences can be compared and exploited to reveal areas of conservation and elucidate the functions of non-common components.

1.2

Elements of Motility: The Bacterial Flagellum

The majority of motile bacteria are propelled by the rotation of helical, semi-rigid flagella (fig.03) (Weibull, 1948; Macnab, 1992), of which there may be several, as with *Escherichia coli*, or only one, as in the case of *Rhodobacter sphaeroides*. Energy for rotation is provided not by the dephosphorylation of nucleotides such as ATP, but directly from a transmembrane electrochemical gradient (Larsen et al, 1974; Glagolev and Schulachev, 1978); this is usually a proton gradient (the "proton motive force" or "pmf"), but in some alkalophilic species, a sodium gradient is used (Hirota *et al*, 1983). The electrochemical gradient is transduced into kinetic energy via the bacterial flagellar motor/switch complex (fig.03) which in enteric bacteria is a structure of about twenty five different proteins (Macnab, 1992). Five of these proteins (Mot A, Mot B, Fli G, Fli M, Fli N) form the functional core of the motor, responsible for proton translocation and for rotational switching. These five proteins alone can be mutated to produce flagellated, but paralyzed mutants (Blair *et al*, 1991; Sockett and Armitage, 1991). The motor structure functions as a single

macroenzyme embedded within the bacterial membranes (Khan *et al*, 1991).

The flagellum can be structurally and functionally divided into a number of parts. The filament is a polymeric helical structure composed of monomers of a single protein, flagellin, which in enterics, can vary between species from 25 kD to 60 kD (Joys, 1988); for enterics and for *R. sphaeroides* the flagellin molecular weight is about 55 kD. Flagellin subunits produce protofilaments of which there are eleven in the enteric flagellum. Two conformational states are possible for the flagellin subunit, with one of these states producing a shorter protofilament. When two of the eleven protofilaments are in the shorter conformation the functional helical shape of the enteric flagellum results, which is essential if rotation is to produce torque (Macnab, 1988). Assembly of the filament proceeds by the addition of subunits at the distal end. It is believed that the flagellin monomers are translocated via the hollow filament core to the growing end where they self-assemble. This process is dependent on the presence of the capping protein, Fli D, the mechanism of action of which is unknown (Homma *et al*, 1985). Polymerization is entropically favourable and the filament produced is intrinsically thermodynamically stable (Kamiya *et al*, 1982). As in the case of flagellin, all other flagellar proteins (except Flg I and Flg H) lack a peptide signal sequence and therefore must be transported axially to the distal point of assembly (Wichner, 1991). This necessitates prior insertion into the membrane of an export assambly.

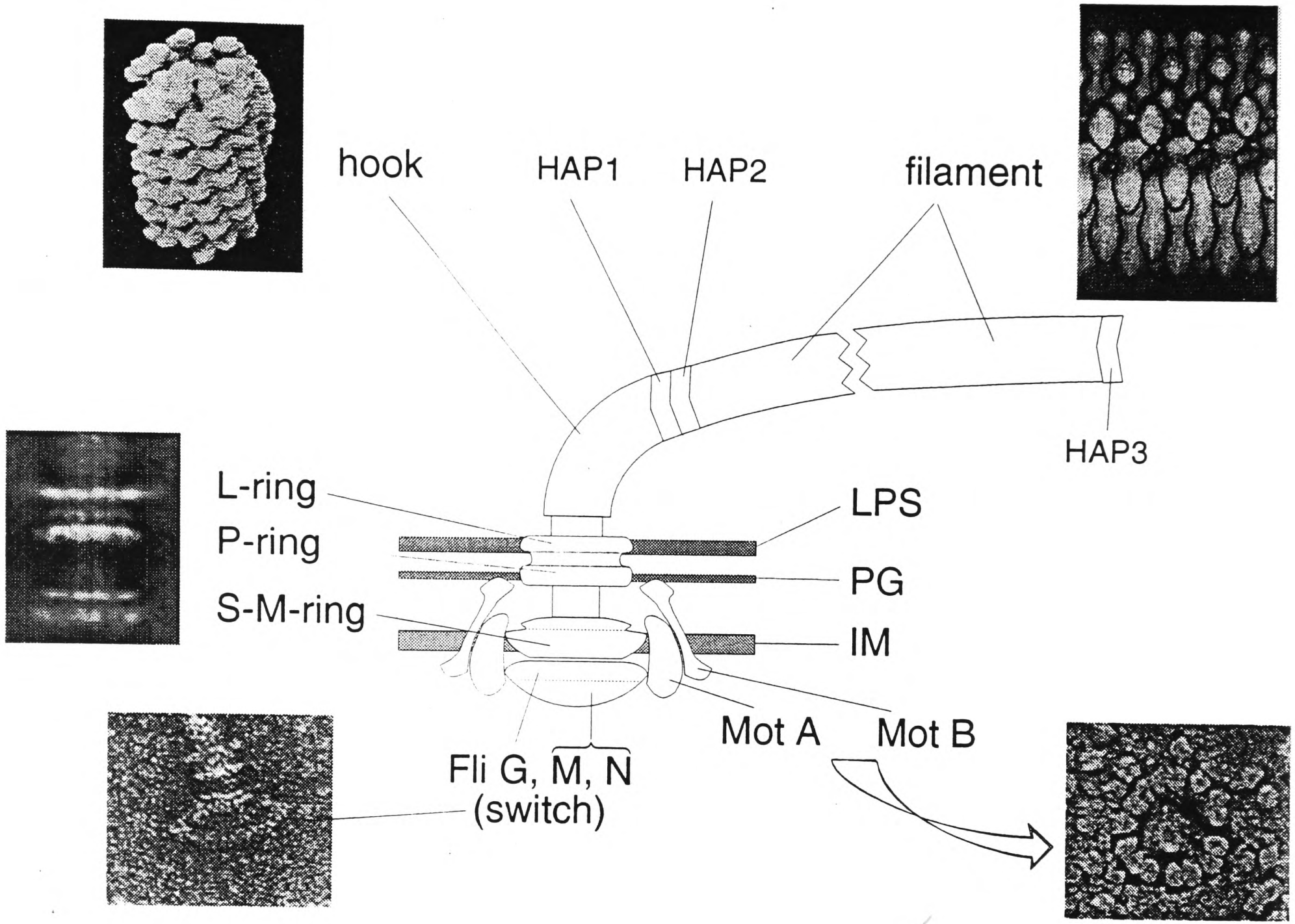


Fig.03: Schematic cartoon of the enteric basal body, filament and hook structures. LPS = lipopolysaccaride layer; PG = peptidoglycan layer; IM = inner (cytoplasmic) membrane. Fli G, Fli M and Fli N are switch proteins. Mot A and Mot B are motor proteins. HAP1 and HAP2 are hook associated linker proteins that join the hook to the filament. HAP3 is the filament cap protein. The width of the filament is about 20 nm; the length of the filament is about 10 μm. (Armitage, 1992)

Monomers of the Fli F protein become embedded the plasma membrane and self-assemble to form the MS ring. Fli G, Fli M and Fli N proteins, which comprise the switch complex, are the next elements to assemble on the cytoplasmic side of the MS ring. The Fli protein complex mediates a change in the direction of rotation of the flagellar motor in response to an interaction with phospho-Che Y, probably at the Fli M protein (Hazelbauer *et al*, 1993; Irikura *et al*, 1993; Meyers *et al*, 1992). Fli G and Fli N are also thought to play a role (together with Mot A and possibly Mot B) in proton conductance (Irikura *et al*, 1993). Switch assembly is followed by assembly of the export apparatus proteins which include Fli I. Fli I is located in the cytoplasm, below the MS ring and the switch apparatus and is thought to bind and hydrolyze ATP, mediating active transport of the other flagellar components. Flg B, Flg C, Flg F and Flg G proteins form the rod structure, which transmits torque from the motor to the hook, which is constructed from a polymer of Flg E proteins and serves as a flexible coupling between the motor and the filament.

E. coli , which is peritrichous, possesses a curved hook; this is in contrast to *R. sphaeroides*, a monoflagellate, in which the hook is straight. The curved hook of enterics may facilitate bundle formation, unnecessary in *R. sphaeroides* (Sockett *et al*, 1991). The L and P rings (Flg I and Flg H) are thought to act as a circular bushing, anchoring the rod and allowing rotation. The flagella of *E. coli* mutants that lack the L and P rings can still rotate, and in Gram positive species, the L and P rings are absent (Blair, 1990). Flg K and

Flg L are hook-filament junction proteins, coupling the hook to the nascent filament. There appears to be no genetic control limiting the length of the filament and it is thought that its length is ultimately limited by mechanical breakage; indeed in *R. sphaeroides* flagellin has been shown to be produced throughout the cell cycle (N. Takahashi, D. Phil thesis, 1995, Oxford). The potential energy of the proton gradient is transduced into the kinetic energy of rotation by the motor proteins Mot A and Mot B (Blair *et al*, 1991). The Mot A and Mot B proteins seem to be complexed together and (in electron-micrographs) appear as a circlet of studs surrounding the MS ring (Stallmeyer *et al*, 1989; Khan *et al*, 1991). The mechanism of energy transduction remains unclear, although it is known that Mot A functions as a proton channel and it is thought that Mot B interacts with both Mot A and the peptidoglycan layer, functioning as a linker, fastening the torque generating machinery to the cell wall (Blair *et al*, 1991; Armitage, 1992; DeRosier, 1994). Both Mot A and Mot B are essential for flagellum rotation but, unlike the switch proteins, are not required for flagellum assembly; this allows the production of paralysed mutants (such as TAB73, see results) by the interruption of the Mot A and/or Mot B genes.

1.3
Elements of Taxis: Motor Rotation, Reversal and Stopping

In the absence of tactic stimuli, prokaryotes tumble with a random frequency, which results in no net population movement. In the

presence of a stimulus gradient however, they will move towards attractants and away from repellents by means of a "biased random walk." This series of biased runs and tumbles (or in some cases stops, eg: *R. sphaeroides and R. meliloti*) ensures a net accumulation of bacteria in areas of optimum chemoattractant concentration.

Enteric bacteria such as *E. coli* and *S. typhimurium*, possess from six to eight flagella which spend the majority of their time (about 95%) rotating counter-clockwise. Counter-clockwise rotation forces the flagella together to form a corkscrew-like bundle which rotates axially, producing thrust. Motor reversal causes a change in the wavelength and handedness of the filaments causing the bundle to fly apart, and the cell to tumble. On resuming counter-clockwise rotation the bacterium continues on a relatively linear course in another, random direction. Certain organisms, such as *R. sphaeroides* (a monoflagelate), only rotate their flagellum in one direction. In this case, reorientation is caused by Brownian motion during rotational stops; the motor does not show reversal. Similarly, *R. meliloti* possesses complex flagella which rotate only clockwise, pausing intermittently to allow directional change (Gotz and Schmitt, 1987).

Although the motor of *R. sphaeroides* is unidirectional, with rotation usually being clockwise, variants have been identified with a counter-clockwise motor, but in both variants, the flagellum pushes the cell forward (Packer and Armitage, 1993). The filament of *R. sphaeroides* can therefore assume a functional, propulsion-generating conformation

when rotating in either direction.

Stopping frequency, rotational stop durations, and translational swimming speeds (swimming speeds between and not including stops) can be highly variable for *R. sphaeroides* (Poole and Armitage, 1988). The flagellum has been shown to stop rotating even under conditions of full pmf and that the extent of 'coupling' between a steady state pmf and the motor can change (Armitage, 1992). This suggests that motor rotation can become partially or completely disengaged from the pmf either by interrupting (or diverting) the flow of protons through the motor, or by uncoupling motor rotation from proton conduction.

1.4
Elements of Taxis: The Biased Random Walk

The essential feature of chemotaxis is an increase in the average length of runs towards an attractant relative to the average length of runs away from an attractant. Three iterative mechanisms are postulated by which this could occur. Firstly, a decrease in the biased (and/or an increase in the anti-biased) stopping frequency would increase the biased run time and therefore the biased run length (fig.04a). Secondly, an increase in the biased (and/or a decreased in the anti-biased) run velocity would increase biased run length (fig.04b). Thirdly, a combination of the two would have the same net effect. The first

fig.04a

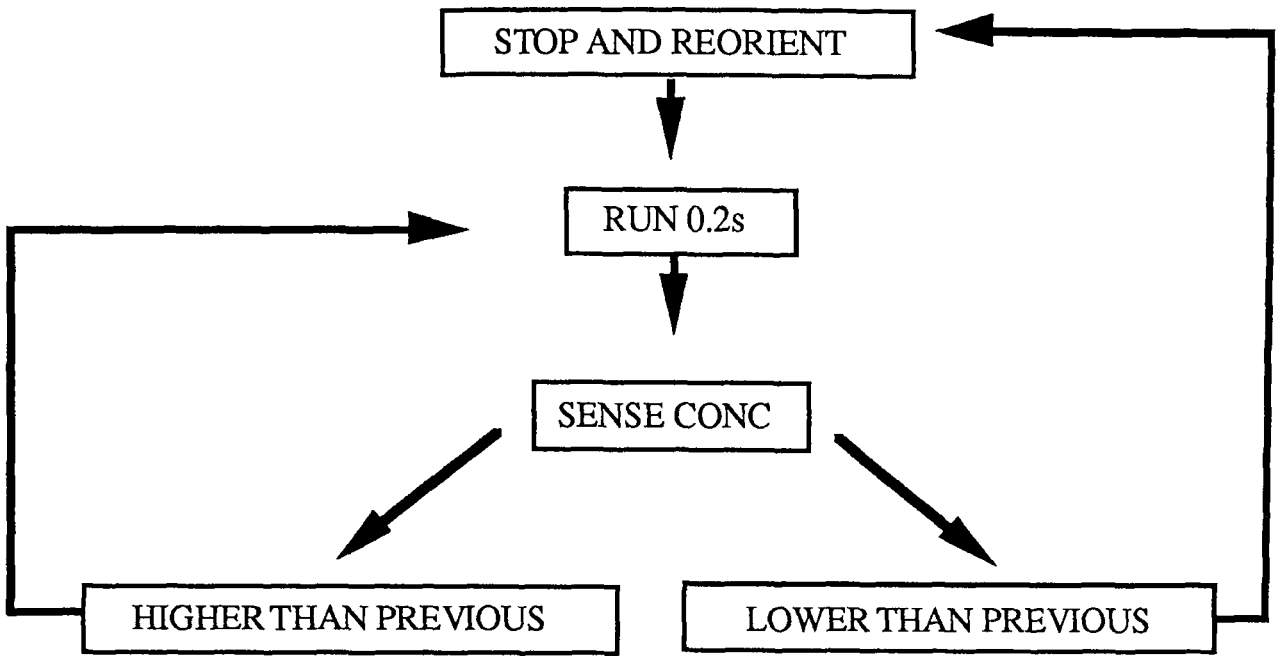


fig.04b

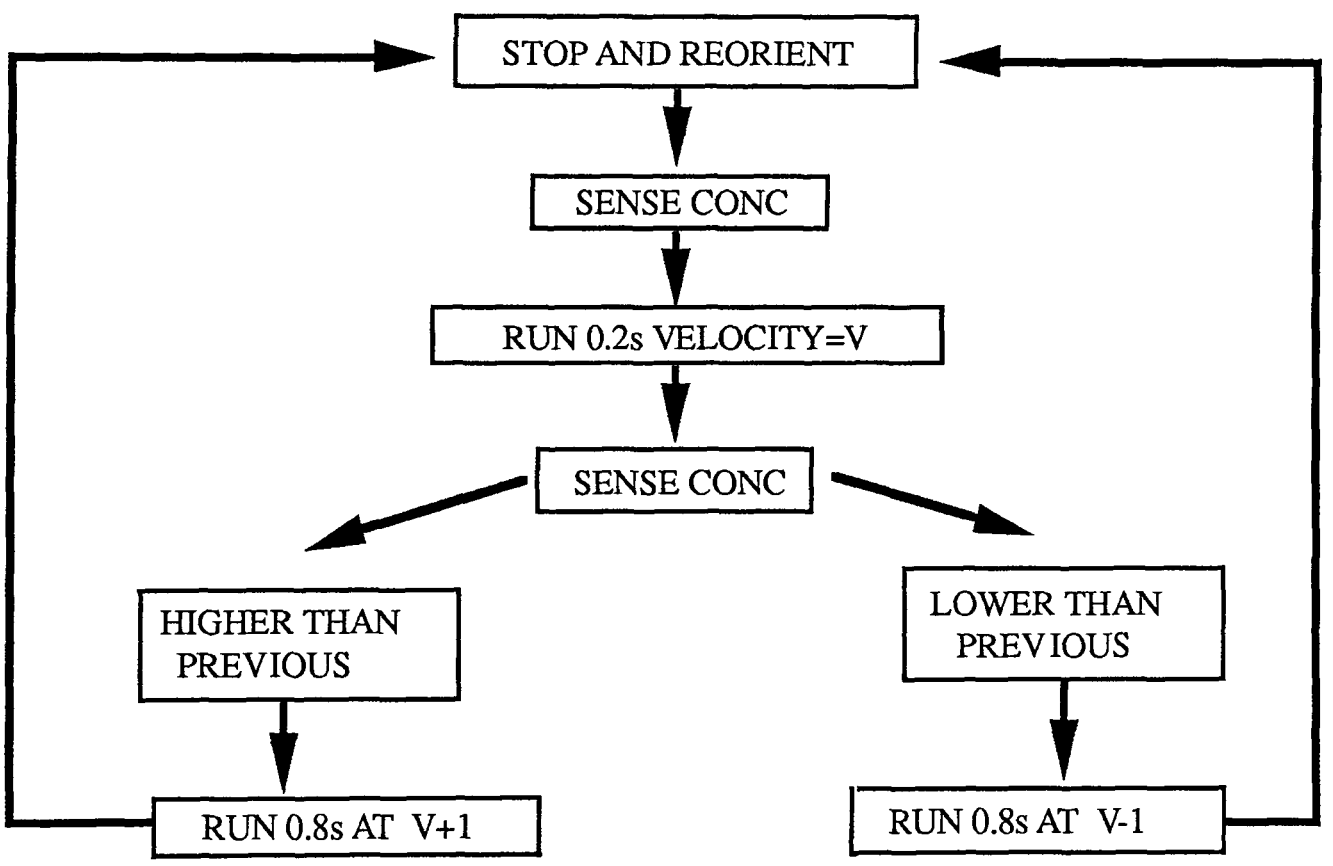


Fig.04a and Fig.04b: Two simple iterative models for biasing run length both of which would result in accumulation of bacteria in an area of high chemoattractant concentration. The top model (fig04a) works solely by variation of stopping probability. The bottom model (fig04b) works solely by variation of run velocity. Both models achieve directed population movement by increasing run length when the bacterium is swimming up a concentration gradient of attractant and by decreasing run length when the bacterium is swimming into an area of lower attractant concentration.

hypothesis is the classic model and has been shown to be the case for *E. coli* which demonstrates a relatively constant swimming speed but variable stopping frequency (Berg and Brown, 1972); it is analogous to Diehn's concept of "klinokinesis with adaptation" (Schnitzer et al, 1992).

Being about two microns long, the average bacterium is too small to sense a concentration gradient across its length; therefore if it is to sense gradients at all, concentrations must be sensed at intervals and compared. A prerequisite for the above models of chemotaxis (figs.04a and 04b) is therefore the ability of the organism to make a temporal comparison of concentrations and it has been demonstrated theoretically that if behaviour does not depend upon earlier events, and speed is a constant, active accumulation is impossible (Schnitzer *et al*,1990).

Thus bacteria must have a "memory" of the concentration of an effector in the immediate past (Armitage, 1992). Since there is no absolute level in these models against which concentrations are measured, adaptation becomes an intrinsic part of the system, with the present concentration only being compared with that of the immediate past. Bacteria will be maintained in an area of optimum chemoattractant concentration because within that area, tumbling frequency (fig.04a) or run speed (fig.04b) will remain unchanged, and run length will not be biased, but once the organism ventures away from that area, tumbling frequency will increase (fig.04a) or run speed will decrease (fig.04b), tending to abate net movement into a non-optimal zone. The above mechanisms are

simplistic in as much as they assume that the effects of diffusion and drift are negligible, the initial distribution of bacteria to be homogeneous and that the mass and momentum of a molecule of water to be insignificant relative to the mass of a bacterium (Schnitzer *et al*, 1992).

The chemotactic model above (fig.04a) does not require any change in swimming speed and is therefore not dependent upon any chemokinetic behaviour. Certain organisms, however, such as *Rhodobacter sphaeroides, and R. meliloti,* can display concomitant chemotactic and chemokinetic behaviour. In such cases, only the net effect is usually considered and is referred to as "chemotaxis", implying both components. On its own, chemokinesis, which is a sustained increase in run speed associated with an increase in flagellar rotation, will tend to act against accumulation, dispersing the bacterial population

1.5
The Chemotactic Signal Transduction Machinery

1.5.1
MCP's and Adaptation

The most thoroughly investigated system of chemotactic signal transduction is in the enteric organisms *E. coli* and *S. typhimurium*, which possess a dedicated sensory and signal apparatus including

methyl-accepting chemotaxis proteins (MCP's) and chemotaxis (Che) proteins. The MCP is a transmembrane protein which transduces a signal, caused by the binding of an effector molecule (which may be coupled to a periplasmic binding protein), through the membrane to the cytoplasmic domain (fig.05). Five types of MCP's have been identified in enterics, each sensitive to a different range of stimuli: Tar senses aspartate and ribose; Tap (in *E. coli* but not in *S. typhimurium*) senses dipeptides; Trg senses maltose and galactose; Tsr senses serine, temperature and pH and Tcp (in *S. typhimurium* but not *E. coli*), senses citrate and phenol. Several hundred copies of each receptor, possibly distributed in patches (Gegner *et al*, 1992; Shapiro, 1993; Maddock and Shapiro, 1993) are present per cell, yet a receptor occupancy change of only four molecules can produce a tactic response, demonstrating the system's remarkable sensitivity. MCP transducers are thought to function as dimers (although this has only been demonstrated *in vitro*) (Kim *et al*, 1992). They are approximately 550 amino acid residues in length and are organized into three distinct domains, each with a specific function (fig.06).

Although almost all of the investigation into MCP's has been done using *E. coli* and *S. typhimurium*-derived proteins, recent studies (Morgan *et al*, 1993) have revealed that proteins antigenically related to MCP's are present in a wide range of bacterial species. Such evidence highlights the versatility and early evolution of this type of transmembrane transducer.

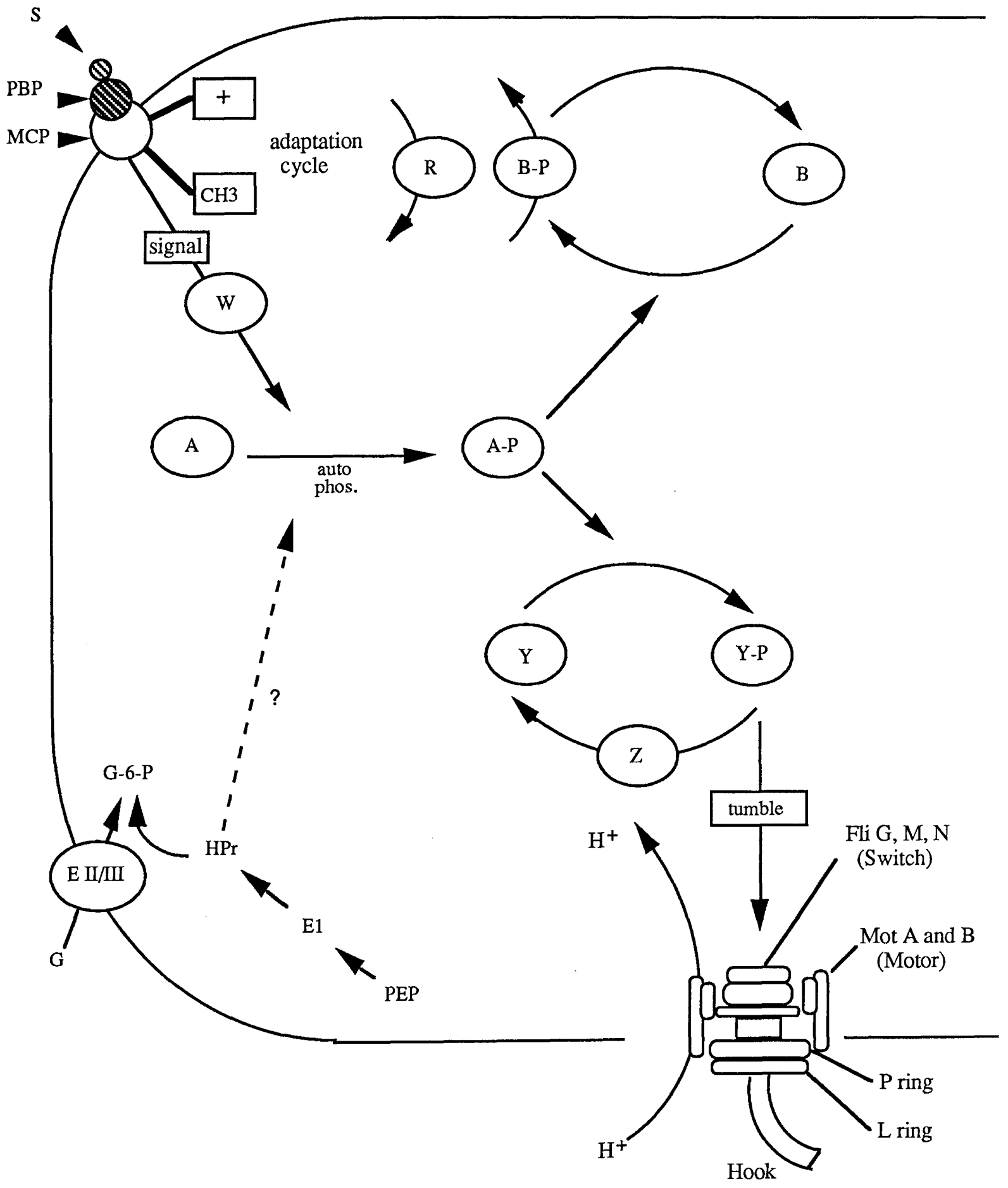


Fig.05: Signal transduction in enteric bacteria. S = substrate molecule. PBP = periplasmic binding protein. MCP = methyl accepting chemotaxis protein. Che A = histidine protein kinase, donates phosphate to Che Y and Che B. Che B = Methylesterase, modifies MCP to cause adaptation to a decrease in attractant concentration. Che R = methyltransferase, modifies MCP to cause adaptation to an increase in attractant concentration. Che W = enhances Che A phosphorylation. Che Y = response regulator, autophosphorylates to produce phospho-Che Y which interacts with switch to cause clockwise rotation. Che Z = antagonizes Che Y function. E I, II and III are enzymes of the PTS system. G = glucose. G-6-P = glucose 6 phosphate.

The greatest structural conservation between MCP's is seen in the C-terminal region which forms the cytoplasmic signalling domain. The mechanics of signal transduction from the MCP to the cytoplasmic Che proteins seems to be identical for each type of MCP, in each case propagating a phosphorylation dependent signal to the flagellar motor. The signalling domain has methylation sites (K1 and R1) that become methylated or esterified in response to a change in chemoeffector concentration, mediating adaptation.

The transmembrane region of the MCP communicates the signal from the receptor domain to the signalling domain by means of a conformational change. Two hydrophobic membrane spanning alpha-helices TM1 and TM2 move relative to one another in response to ligand binding (fig.06). The exact nature of the conformational change and the interaction between helices is unknown; one of the currently prevailing model suggests a "piston" type of action, moving TM1 and TM2 vertically relative to each other, changing interactions between adjacent residues and revealing the functional pocket of the signalling domain (Ames *et al*, 1988; Kim *et al*, 1992).

Other equally likely models suggest a scissoring action in which the fulcrum between the helices is closer to the receptor domain than to the signalling domain thereby amplifying the relative movement between different domains of the molecule (Kim *et al*, 1992 and Hazelbauer *et al*, 1993).

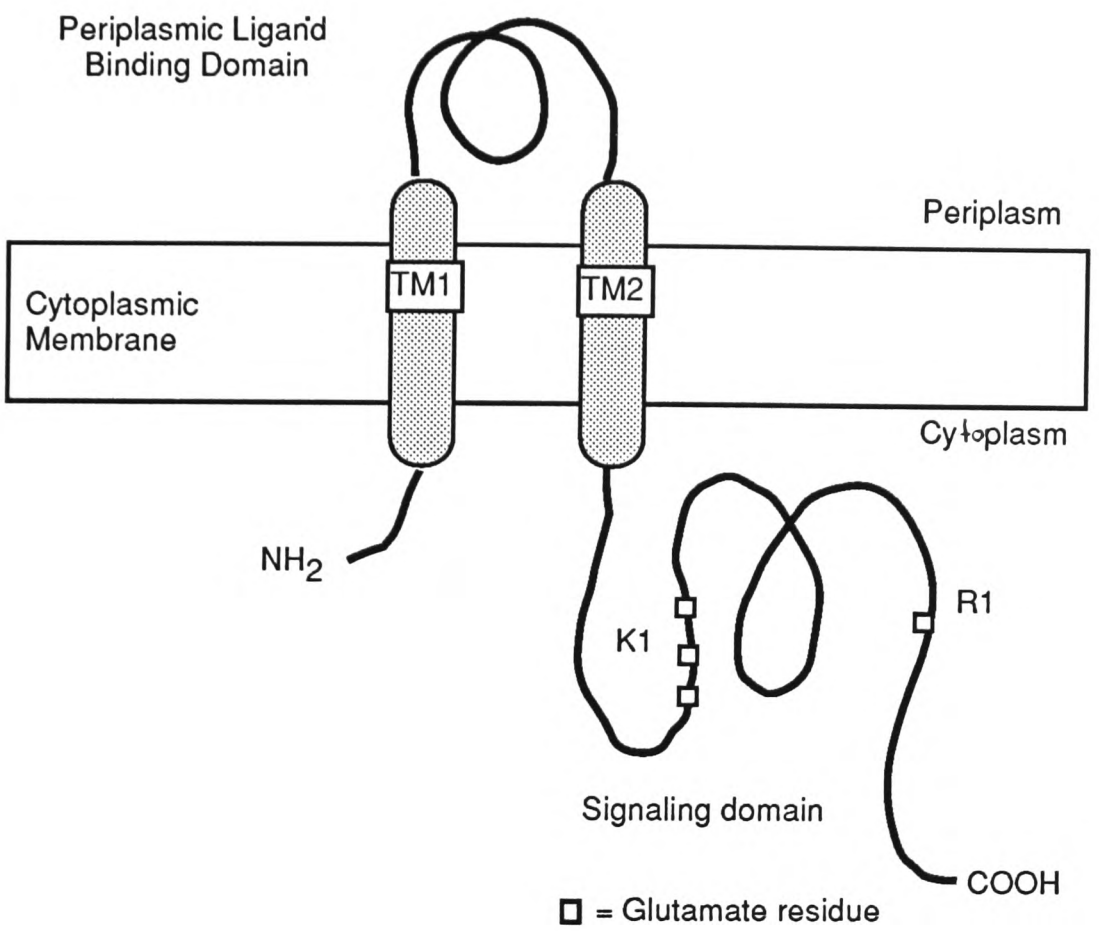


Fig. 06: Schematic cartoon of an enteric methyl accepting protein. TM1 and TM2 are trans-membrane alpha helices which undergo conformational change and transmit a signal across the membrane in response to ligand binding at the periplasmic ligand binding domain. The four glutamate residues present in the K1 and R1 regions of the cytoplasmic domain become methylated or de-esterified by Che R or Che B, mediating an adaptation response. Regions of the cytoplasmic domain interact with Che W, Che A (and possibly with Che Y) to form a complex which mediates signal transduction to the motor/switch complex.

In any case it has been shown (by cysteine substitution) that ligand binding promotes amino-acid interactions both within MCP monomers and between MCP dimers (Armitage, 1992). The result of this conformational change is to induce a structure in the signalling domain

which promotes interaction between the MCP, Che A and Che W, resulting in increased kinetics of Che A autophosphorylation (Gegner *et al*, 1992). It is phospho-Che A that interacts directly both with Che Y, mediating the motor response and with Che B, resetting the MCP methylation level and allowing comparative measurements of effector concentrations to be made. Thus, a reduced phospho-Che A concentration produces smooth swimming.

The adaptation response (without which chemotactic accumulation would be impossible) involves the K1 and R1 regions of the MCP's. Four conserved glutamate residues become methylated in response to effector binding. The degree of methylation is proportional to concentration of periplasmic effector and therefore to the probability of receptor occupancy. An increase in attractant concentration, or decrease in repellent concentration will cause a conformational change exposing glutamate residues which allows increased methylation by the methyltransferase Che R. The activity of Che R is constant and not allosterically regulated, unlike the activity of Che B. A decrease in attractant concentration or an increase in repellent concentration will cause a reduction in methylation and an increase in ester hydrolysis of glutamate residues catalysed by the methylesterase Che B. Both methylation and esterification result in a resetting of the MCP to the pre-signalling conformation. The time course of signal propagation from transducer to motor may be as short as 200 ms whereas the adaptation time course is measurable in minutes (Armitage, 1990; Parkinson, 1993), therefore a change in effector concentration will first

produce the stimulus response, which will then be attenuated by the adaptation (negative feedback) response. It is the difference in time courses that allows this rudimentary form of memory to function and therefore permits the temporal sensing upon which bacterial behaviour is based.

Despite the presence of MCP's across a wide range of genera, not all chemotactic organisms possess such transducers, and *R. sphaeroides, R. meliloti and A. Brasilense* are all thought to lack membrane-spanning MCP's (Armitage, 1992; Greck *et al*, in press; Zhulin and Armitage, 1993).

1.5.2

The "Che" Signal Transduction Proteins - A Brief Functional Overview

The mechanism of signal transduction from MCP to motor is still unclear, but it is known to involve four essential components (Che A, Che W, Che Y and Che Z) and to be phosphorylation dependent. This system will be described briefly before discussing each component in detail. Che A, which functions as a dimer, has autophosphorylation activity which increases with ligand release from the transducer and decreases with ligand binding.

The increase in Che A autophosphorylation is enhanced *in vitro* by the presence of Che W and the MCP (Hazelbauer *et al*, 1990;

McCleary and Stock, 1993). It now appears that a complex is formed between the MCP dimer, the Che A dimer, two molecules of Che W and an unknown number of Che Y molecules (Stock *et al*, 1987; Gegner *et al*, 1992; Parkinson, 1993; Malmqvist, 1993; Hazelbauer *et al*, 1993; Schuster *et al*, 1993; McCleary and Stock, 1993). Addition of ATP causes dissociation of phospho-CheY, which is then presumably free to interact with the Fli (switch) proteins. The binding of Che W alone to Che A does not seem to increase autophosphorylation, but complex formation with Che W and the MCP has been shown to increase autophosphorylation up to 100 times (Stock *et al*, 1992). The sensitivity of the receptor/signal transduction system, which can produce taxis in response to a change in occupancy of only a tiny fraction of MCP receptors, necessitates some form of signal amplification. Such an amplification cascade could easily be envisaged involving the Che proteins with faster phosphorylation kinetics for transducer-proximal components than for transducer-distal components. The phospho-Che A complex donates a phosphate to Che Y which acts at the motor/switch complex to cause rotational switching from counter clockwise to clockwise resulting in tumbling. Che Z promotes Che Y dephosphorylation attenuating the response at the motor end of the system. The activity of Che Z is not known to be modulated and is thought to be constant. Che A also phosphorylates Che B. This phosphorylation enhances Che B methylesterase activity which mediates the adaptation response as described above.

SYSTEM	SPECIES	HISTIDINE PROTEIN KINASE	RESPONSE REGULATOR
Chemotaxis	Ea, Ec, St	CheA	CheY
			CheB
Motility and development	Mx	FrzE	
			SpoOF
Sporulation	Bs	SpoIIJ	
Exoprotein synthesis	Sa		SpoOA
			AgrA
Nitrogen regulation	Bp,Ec,Rm	NR_{II}	NR_1
Phosphogycerate transport	St	PgtB	PgtA
Outer membrane protein expression	Ec,St	EnvZ	OmpR
Virulence	At	VirA	
F Plasmid expression and aerobic metabolism	Ec	CpxA	VlrG
			ArcA
Nitrogen fixation	Rm	FixL	FixJ
Dicarboxylic acid transport	Rm	Dct B	Dct B

Fig.07: Structural and funtional conservation between histidine protein kinases and between response regulators from different systems and different species. ⬭ = Conserved kinase domain of about 100 amino acid residued. ⊘ = Conserved histidine residue. ⬬ = Conserved response regulator domain of about 100 amino acids. At = *A. tumefaciens;* Bp = *B. parasponiae*; Bs = *B. subtilis*; Ea = *E. aerogenes*; Ec = *E. coli*; Mx = *M. xanthus*; Rm = *R. meliloti*; Sa = *S. aureus*; St = *S. typhinurium.*

1.5.3

The Two Component Regulatory System

Che A, Che B and Che Y are members of a two component regulatory family of proteins (Stock et al, 1990; Russo and Silhavy, 1993) and as such, show sequence, structural and mechanistic relatedness to other members of two component families (fig.07). *E. coli* alone is thought to possess more than forty different pairs of these components and so far, more than thirty-nine species of bacteria have been shown to use this system (Parkinson and Kofoid, 1992). Intriguingly, evidence of homology in eukaryotic systems has also recently emerged. These systems include yeast, plant phytochromes and a protein kinase from rat mitochondria (similarities in the latter may not be so surprising). Members of these families are involved in the control of a wide variety of bacterial responses to environmental stimuli and include symbiosis, virulence, nitrogen regulation, sporulation, osmoregulation and chemotaxis.

Mechanistically these systems generally consist of two molecules: a histidine protein kinase and a response regulator. The histidine protein kinase acts as a sensor, often located in the cytoplasmic membrane, and it possesses autophosphorylation activity which is stimulated in response to some environmental signal. Autophosphorylation occurs at a conserved histidine residue, using ATP as the phosphate donor. The histidine protein kinases share a homologous domain of about 100 amino acids generally located near the C-terminus and generally have characteristic hydrophobic membrane spanning regions.

The response regulator becomes phosphorylated at a conserved aspartate residue, receiving a phosphoryl group from the sensor. The phosphorylation site is located within a conserved domain of about 130 residues generally located at the N-terminus. The response regulator is always cytoplasmic and once phosphorylated will mediate a response either by regulating gene expression, as in the case of osmoregulation (Omp R) and nitrogen regulation (NR_I), or by direct protein interaction as in the case of chemotaxis (Che B and Che Y).

Although amplification of the signal has not been demonstrated in these systems, it does present itself as an obvious possibility, supported, in the case of the chemotactic system, by response regulator molecules (Che Y) outnumbering histidine protein kinase molecules by almost five to one (Stock *et al*, 1992). By a simple mass-action effect, and allowing for appropriate kinetics, the signal could be amplified to produce a larger output amplitude at the Che Y branch and (possibly) a smaller output amplitude at the Che B (adaptation) branch. Significant responses are produced by chemoattractant binding at only a few MCP receptors (Lukat and Stock, 1993). This would necessitate either very tight control over phospho-Che Y concentration or signal amplification.

In itself, the two component regulatory system is an over-simplification of *in vivo* control and the system continues to grow steadily more elaborate with the discovery of multiple components, positive and

negative regulatory circuits and communication between elements of different two component regulatory systems. Chemotaxis, for instance cannot be fully explained by interactions between components of the Che system and appears to be underlayed by more physiologically integrated systems involving the sensing of metabolic intermediates such as fumarate (Marwan *et al*, 1990; Armitage, 1992) or phosphate donors such as acetyl phosphate (McCleary *et al*, 1993).

"Crosstalk" is facilitated by homology between components and has clearly been demonstrated between the nitrogen regulation, osmoregulation and chemotaxis systems (Ninfa *et al*, 1988; Igo *et al*, 1989). The purified histidine protein kinase, phospho-Che A, has been shown to phosphorylate the response regulators NR1 and Omp R, producing transcription from the *glu* A and *omp* F promoters respectively. The histidine protein kinase of the porin system, phospho-Env Z, can phosphorylate NR_I and Che Y; and a hyperactive variant of the nitrogen kinase, NR_{II} can be used to phosphorylate Che Y, resulting in tumbling.

Free energies of dephosphorylation have been calculated for target amino acids (histidine in the case of histidine protein kinases and aspartate in the case of response regulators) both as part of the appropriate enzyme and as free amino acids. As free molecules, both amino acids display kinetics strongly favouring the unphosphorylated state; but as parts of their respective enzymes there is a striking

difference, with the histidine residue in the histidine protein kinase enzyme showing a large negative free energy ($\Delta G° = -13$ kcal/mol); and aspartate residue in the response regulator showing a free energy of dephosphorylation much closer to equilibrium ($\Delta G° = +2$ kcal/mol).

This evidence supports the mechanistic hypotheses regarding these species as the histidine protein kinase would need to efficiently and rapidly transduce its phosphate signal whereas the response regulator transmits its effect only in the phosphorylated state and so would want to remain phosphorylated long enough to recognize, attach to and interact with its target molecules, be they a protein complex, as with Che Y or a DNA regulatory sequence as with Omp R and NR1 (Stock, 1990). The above kinetics would also support the theory of signal amplification. With the turnover of phosphorylated histidine protein kinases being rapid, a few molecules of say, Che A could effectively phosphorylate a far larger number of Che Y molecules; and the positive free energy of dephosphorylation would make the phospho-Che Y relatively stable (in fact, *in vivo*, Che Z is present to promote phospho-Che Y dephosphorylation). The rate limiting step would probably be Che A phosphorylation.

1.5.4

The Che A Protein

Most histidine protein kinases seem to be the primary receptors of environmental stimuli in their respective systems; located in the

cytoplasmic membrane they transmit an exterior signal to the cytoplasm. Che A however has no membrane spanning region and exists in soluble cytoplasmic form where it interacts with the primary receptor/transducer molecule of chemotactic signal transduction, the MCP.

A considerable amount of attention has been focused on *E. coli* Che A; its primary structure is known, regions homologous to other histidine protein kinases are well defined, and functional regions have been ascribed. Recently, BIA-core surface plasmon resonance studies (Hazelbauer, 1993; Schuster *et al*, 1993) have determined which other molecules become bound to Che A during complex formation, this gives clues as to how components interact to transmit a signal. The Che A protein consists of 645 amino acids and it uses the gamma phosphate of ATP to autophosphorylate histidine 48 (Hess *et al*, 1988). Interaction with both the MCP and Che W regulate Che A autophosphorylation in a ligand-dependent manner (Gegner and Dalquist, 1991). Che A is thought to act as a dimer (fig.08) and has been shown by surface plasmon resonance to form a complex containing an MCP dimer, a Che A dimer, two Che W molecules and Che Y (Gegnor *et al*, 1992, Hazelbauer *et al*, 1993; Schuster *et al*, 1993). The Che A protein has three distinct functional domains. The carboxyl portion of Che A regulates histidine autophosphorylation and it is at this region that the MCP and Che W are thought to act. Bacteria producing a truncated Che A protein lacking the C terminal display an identical phenotype to mutants lacking CheW, they are smooth-

swimming and not chemotactic. The central portion of the molecule is required for autophosphorylation and is also thought to contain the sites instrumental in dimer formation. The amino-terminal portion of Che A contains the autophosphorylation site (conserved histidine) and has been shown to be essential for Che B and Che Y phosphorylation (fig.08).

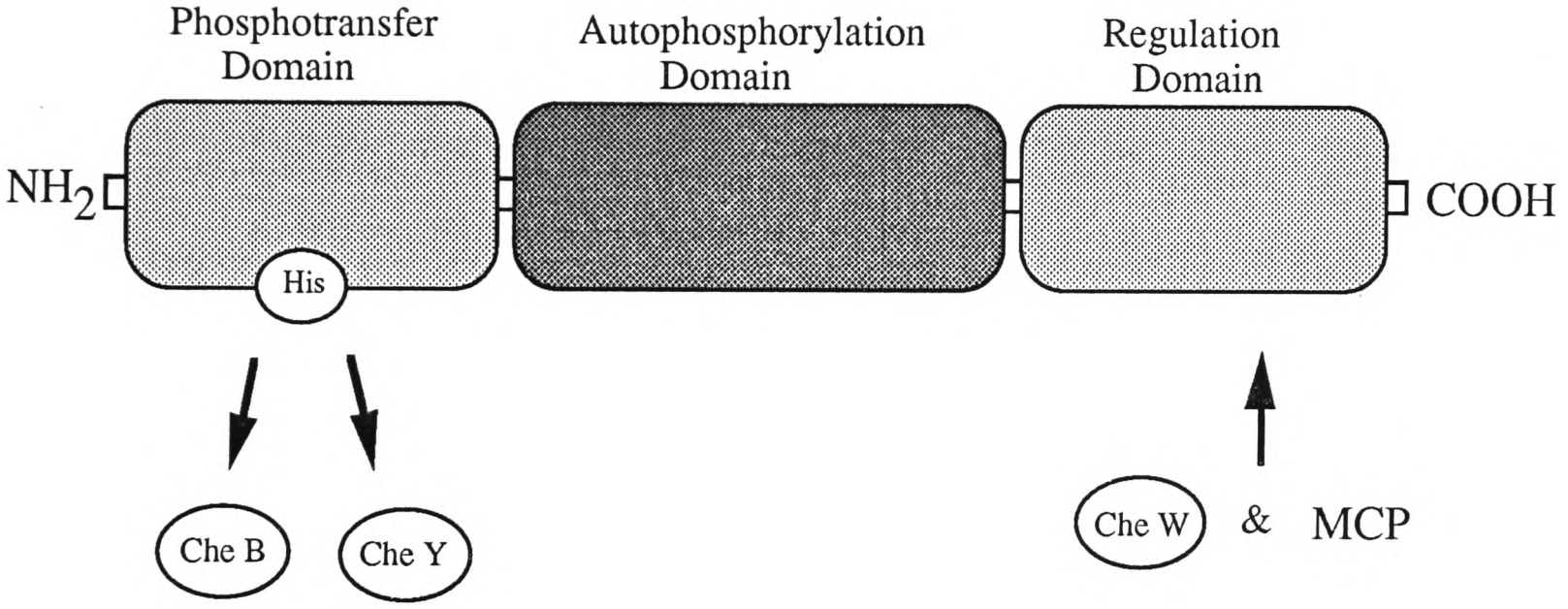


Fig.08: Schematic model of Che A showing functional organization into three distinct domains. The C terminal regulatory domain interacts with the MCP and Che W and in response controls the rate at which the central region catalyzes Che A auto-phosphorylation at the histidine 48 residue. The N terminal functions to transfer the phosphate group to either Che B (which requires Che A kinase activity) or to Che Y (which catalyzes it's own phosphorylation). It is thought that Che A folds so that the N and C termini are in close proximity and interact directly at the time of auto-phosphorylation. The central portion is also essential for dimer formation.

1.5.5

The Che W Protein

Che W has not been as thoroughly investigated as Che A and its mechanistic role remains obscure. It is not a member of a two component regulatory family although it does show conserved homology with proteins across a number of species including *E. coli*, *E. aerogenes*, *B. subtilis*, *M. xanthus* (Dahl *et al*, 1989; Hanlon *et al*, 1992). Che W has been shown to form a complex with both Che A and the MCP. Che W and Che A alone form a short-lived complex with a relatively weak binding affinity (Gegner and Dahlquist, 1991); but a complex which includes Che W, Che A and the MCP in the ratio 1: 1: 1 is relatively thermodynamically stable and long-lived (Gegner *et al*, 1992). Che W may physically link Che A to the receptor inducing a conformation of Che A which enhances phosphorylation kinetics.

Functional sites of Che W have not been precisely ascribed but five residues have been identified which, when altered by single amino acid substitution, give rise to behavioural mutants (Lim and Parkinson, 1991).

The Che W proteins of *E. coli*, *S. typhimurium* and *E. aerogenes* possess a ßaß motif which has been identified with a core fingerprint of Gly-X-Gly-X-X-Gly as a putative nucleotide binding site (Dahl *et al*, 1989, Stock *et al* 1986, Lim and Parkinson, 1991). This motif may bind ATP bringing it into close proximity with the Che A histidine

residue which becomes phosphorylated but no direct evidence of nucleotide binding has yet been obtained. Moreover the ßaß structure does not appear to be present in the Che W homologues of *B. subtilis* or *M. xanthus*. Upon quaternary complex formation with Che W and the receptor (but not with Che W alone), the autophosphorylation activity of Che A is increased up to 100 times (Stock *et al*, 1992) and the level of phospho-Che Y is increased some 300-fold (Borkovich and Simon, 1990), demonstrating at least one element of signal amplification by the chemotactic signal transduction system. The number of molecules of Che W present in an enteric cell is equivalent to Che A, about 2500; this is consistent with their adjacent locations with the Mocha operon. Che W deficient phenotypes present as smooth-swimmers, unable to accumulate in response to a chemoeffector gradient (Hanlon *et al*, 1992), as would be expected if, as in the mutant Che A, a signal cannot be effectively propagated to Che Y and thence to the motor to produce tumbling. Overproduction of Che W produces an identical phenotype to the Che W deficient mutant. Che W is a bivalent molecule with sites of interaction for both Che A and the receptor, therefore at excessive Che W concentrations, Che W molecules may "mop up" the binding sites on Che A and the receptor by monovalent interactions, preventing the formation of the ternary complex and inhibiting signal transduction (Stock and Lukat, 1991).

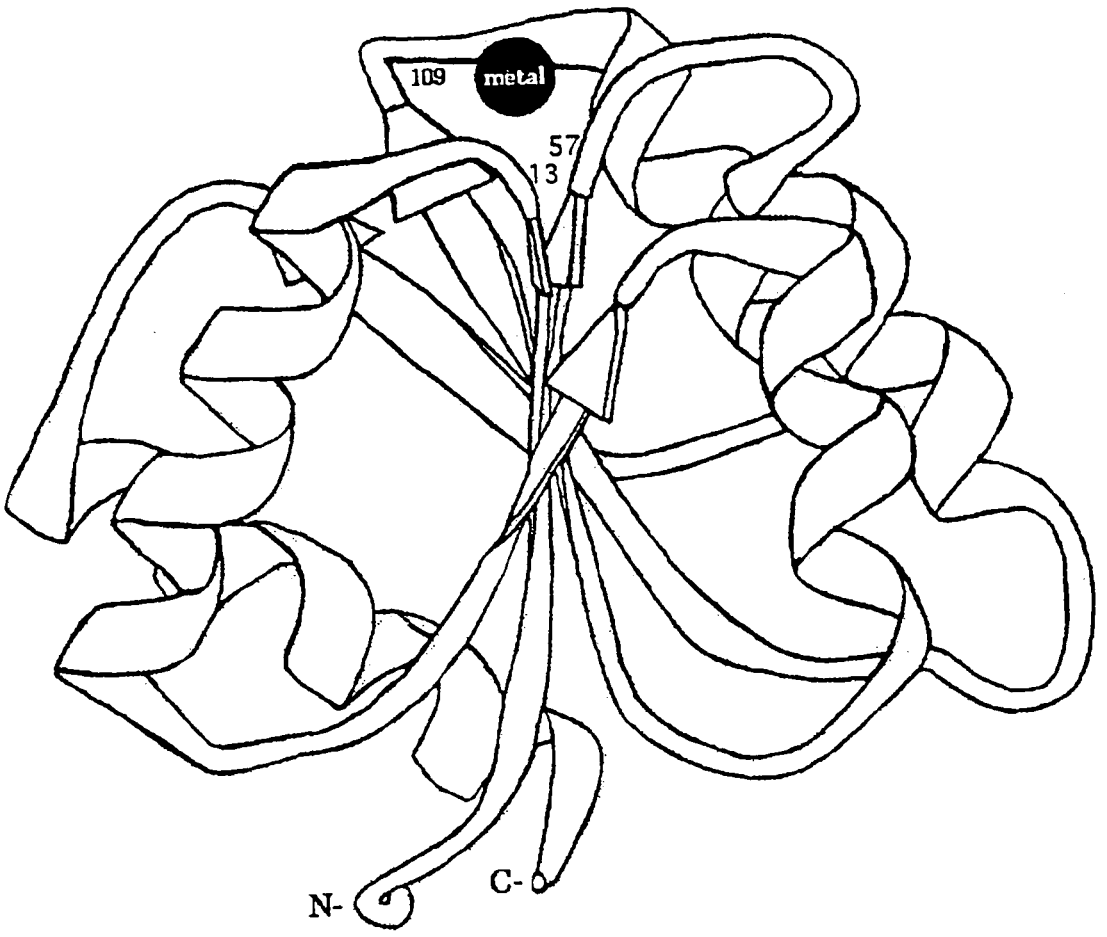


Fig.09: A schematic diagram showing the tertiary structure of Che Y, composed of a five stranded parallel ß sheet surrounded by five α helices. Aspartate residues within the acid pocket are numbered 13, 57 and 109, and the sight of magnesium ion binding is shown by the black disc. Che Y possesses autophosphorylation activity (which is magnesium ion dependant) and receives a phosphate group from phospho-Che A. Phospho-Che Y acts at the motor/switch complex of the flagellum to promote motor reversal and so cause tumbling. It is believed that phosphorylation of Che Y at aspartate 57 stabilizes an alternative protein conformation that reveals a previously masked site which interacts with the flagellar switch. (Adapted from McCleary and Stock, 1993).

1.5.6

The Che Y Protein

Two response regulator species are present in the chemotaxis system, Che Y and Che B. The enteric Che Y has been throughly investigated and a three dimensional structure had been deduced by X-ray diffraction to a resolution on 1.7 angstroms (Stock *et al*, 1990; Stock *et al*, 1992). Che Y is a 14 kD monomer composed of a five stranded parallel ß-sheet surrounded by five α–helices (fig.09). The active catalytic site is located in an acidic pocket containing three aspartate residues, Asp^{12}, Asp^{13} and the phosphoaccepting Asp^{57}. Che Y seems to act as a molecular switch catalyzing its own phosphorylation and dephosphorylation. It is Che Y, rather than Che A which catalyzes the transfer of a phosphoryl group from the kinase histidine residue to the response regulator aspartate residue. Magnesium (Mg^{2+}) ions are required for both phosphorylation and dephosphorylation of Che Y and the site of Mg^{2+} binding is thought to be the acidic pocket (Lukat *et al*, 1990). Prior to phosphorylation, Asp^{57} is located close to Mg^{2+}, but afterwards shifts to coordinate with the phosphate oxygen atoms. This is concomitant with a major conformational change at the active site. NMR studies also suggest a substantial restructuring of Che Y upon phosphorylation, especially at the putative motor interaction face.

Immobilized phospho-Che Y has been used to study interactions with the switch proteins of the flagellar motor Fli G, Fli M and Fli N, and it is apparent that direct interaction occurs between the response regulator

and Fli M (strong binding) and Fli G (weak binding) but not Fli N (Meyers *et al* , 1992 and Hazelbauer *et al*, 1993), implicating Fli M as the major site of interaction.

Che Y is amenable to phosphoryl group donation not only from Che A (and related histidine protein kinases producing "cross-talk") but also from small molecules such as phosphoamidate, carbamoyl phosphate and acetyl phosphate (McCleary and Stock, 1993; McCleary *et al*, 1993; Lukat, 1992). This effect is by no means negligible physiologically and under certain metabolic conditions a significant degree of Che Y phosphorylation can be caused by such donors. Acetyl phosphate is strongly implicated in this sort of control and it has been proposed that it may act as a global regulator, interacting with the response regulators of diverse metabolic and behavioral systems (McCleary *et al*, 1993). It could be argued that such global systems had evolved prior to the kinase-dependent dedicated sensory systems, but because of the algorithms, methods and tools available, the mechanics of the dedicated system have, in recent years, proved a more fruitful research target and so the underlying system has essentially been ignored.

Phosphorylation of Che Y is neither *per se* sufficient nor essential to produce tumbling. Purified Che Y, inserted into fully lysed and re-sealed cell envelopes did not produce an increase in clockwise rotation when the phosphorylation potential was lowered by the inclusion of

ADP. However, when partially lysed cells were used, a three-fold increase in clockwise rotation was observed. Clearly an additional, unidentified cytoplasmic component is involved in producing motor reversal. Unphosphorylated Che Y on its own also appears to produce a small, but significant change in rotation. When compared to fully lysed and resealed cell envelopes containing only buffer, which rotate only counter-clockwise, envelopes containing Che Y showed a 14% clockwise rotation (Barak and Eisenbach, 1992).

Che Y mutants have been produced which shed light on the mechanistic mode action of Che Y as well as on the role of Che Y phosphorylation. Che Y13DK (Bourret *et al*, 1990) is a mutant protein which cannot be phosphorylated, but constitutively causes tumbling *in vivo*; and Che Y109KR (Lukat *et al*, 1991) is a mutant protein which cannot induce tumbling despite the fact that it is highly phosphorylated (due both to defective auto-dephosphorylation activity and to reduced Che Z mediated dephosphorylation).

From the present evidence it appears that effective interaction between phospho-Che Y and the switch proteins requires another, unidentified cytoplasmic component (Barak and Eisenbach, 1992). Also, it seems that phosphorylation of Che Y plays an *indirect* (allosteric) role in Che Y - switch interaction and that the Mg^{2+} dependent phosphorylation of Che Y at Asp^{57} includes a major structural change in Che Y *prior* to interaction at the switch interface (Bourret *et al*, 1993, Lukat *et al* 1990 and 1991, Roman *et al*, 1992, Bourret *et al*, 1989, Stock *et al*, 1988). It appears that phosphorylation of Che Y (at Asp^{57}) induces an

allosteric conformational change producing an architecture in which a previously masked binding site is revealed, enabling interaction with the flagellar switch machinery (McCleary and Stock, 1993).

1.5.7

The Che Z Protein

Che Z functions to accelerate the rate of dephosphorylation of phospho-Che Y (Hess *et al*, 1988; Blat and Eisenbach, 1994). As the free energy of dephosphorylation of phospho-Che Y is positive, it would seem essential to have a mechanism to promote its dissociation, or the cell would tumble indefinitely. This is, in fact, the phenotype of a Che Z^- mutant (Stock *et al*, 1992). As mentioned previously, Che Y catalyses both its own phosphorylation and dephosphorylation and it seems likely that the catalytic activity previously ascribed to Che Z is probably misplaced, and Che Z probably functions as an allosteric regulator of Che Y enzymic activity. Physiological concentrations of Che Z have been shown to cause greater than a 100 fold increase in phospho-Che Y hydrolysis (Stock and Lukat, 1991).

How Che Z functions and whether or not Che Z activity is regulated is unknown. *In vitro* complex formation between Che Z and Che A_S (a short version of Che A, translated from an alternative start site) has been demonstrated, and may be involved in regulation of Che Z activity (Lukat and Stock, 1993). Fli protein alleles have been found that will suppress missense mutations in Che Z, and there is some genetic

evidence to suggest a direct interaction between Che Z and the flagellar switch (Sockett *et al*, 1992), though this has yet to be demonstrated biochemically. It may be that suppression of Che Z mutations by *fli* alleles involves only a resetting of the motor bias and involves no direct Che Z / Fli interaction (Lukat and Stock, 1993).

Che Z has recently been shown *in vitro* to bind Che Y directly, with the probability of binding increased more than two-fold when Che Y is phosphorylated. This binding is enhanced by the presence of Mg^{2+} (Blat and Eisenbach, 1994; Lukat and Stock, 1993; Matsumura *et al*, 1990). The binding of Che Y to Fli M and the binding of Che Y to Che Z do not appear to be co-dependent, neither does the binding of Che Y to Che Z seem to require any of the other components found in the MCP/Che A/Che W/Che Y complex.

Given the known interactions, a reasonable model of Che Z activity could involve Che Z binding to phospho-Che Y cytoplasmically, before phospho-Che Y has had a chance to bind at the switch. Che Z and Fli M would compete for the same binding site on Che Y, with the equilibrium of binding determining the base rate of tumbling for a constant concentration of phospho-Che Y (ie: when chemoeffector concentrations are stable). A Che Y-Che Z complex would be incapable of binding at the switch and Che Z binding would allosterically increase dephosphorylation kinetics of phospho-Che Y; this would lead to a rapid dissociation producing Che Z, Che Y and P_i. If instead, phospho-Che

Y bound at Fli M, tumbling would ensue and phospho-Che Y would then spontaneously dissociate into Che Y and P_i. The survival time of the phospho-Che Y/switch complex is presumably in the region of a few tenths of seconds, since tumbling only lasts this long.

As many as twelve molecules of Che Z have been shown to bind to a single phospho-Che Y molecule *in vitro*. The *in vivo* significance of this, if any, is unclear, though it may be indicative of a mass-action effect involved in phospho-Che Y/Che Z binding; it also may be reflective the polymeric nature of Che Z (Blat and Eisenbach, 1994). Che Z has a unusual quaternary structure, existing *in vitro* as a large homopolymer consisting of over 20 monomers (Stock and Stock, 1987). The functional relevance of this structure is uninvestigated.

Despite the positive free energy of dephosphorylation of (enteric) phospho-CheY, Che Z is not an indispensable component of all chemotactic organisms employing the "Che" system. *B. subtilis* does not possess Che Z (Bischoff and Ordal, 1992) and *R. meliloti* also appears to lack Che Z (Greck *et al*, in press). In these organisms, Che Z could have been made redundant either because i) the structure and thermodynamics of phospho-Che Y is significantly different from that in enterics, or ii) because the binding affinity of phospho-Che Y for the switch is lower than it is in enterics, or iii) because there is competitive binding between phospho-Che Y and another molecule at the switch

Considerable functional and structural analogy has been drawn between the chemotactic signal transduction system of bacteria and the eukaryotic *ras* super-family of GTP binding proteins; and the structures of Che Y and H-ras appear strikingly similar (Lukat *et al*, 1991). The comparison extends to the Che Z protein which can be thought of as functionally analogous to the GTPase activating protein, GAP. The ras protein has intrinsic GTPase activity but its kinetics are slow. GAP accelerates the GTPase activity but does not appear to participate directly in the hydrolysis.

1.5.8

<u>The Che R Protein</u>

Che B and Che R mediate the adaptation response of the chemotactic signal transduction system. Che R mediates adaptation to an increase in chemoattractant concentration or a decrease in repellent concentration. Che B mediates adaptation to a decrease in attractant concentration or an increase in repellent concentration. Both enzymes function to reset the conformation of the MCP to a pre-stimulus architecture, attenuating the stimulus response and allowing the MCP to respond to new changes in extracellular chemoeffector concentration. Che R is not a member of a two component regulatory family and has not yet been throughly characterized. Of all the components of the Che system, Che R shows the least structural conservation between genera (fig.10) (Simms *et al*, 1987). Although it's function and mechanism of action appears to be the same in *S. typhimurium* and *E. coli*

(Subbaramaiah *et al*, 1991; Stock *et al*, 1992) it has recently been suggested that its function in *B. subtilis* is slightly different (Kirsch *et al*, 1993).

Chemotaxis Protein	Amino Acid Changes per 100 residues
Che R	13.6
Che Z	7.0
Che W	6.6
Che B	4.9
Che Y	2.3

Fig.10: Comparison of conservation of primary protein structure of Che proteins between *E. coli* and *S. typhimurium* (adapted from Simms *et al*, 1987). Che R is clearly the least conserved of the Che proteins between these two closely related organisms; this also appears to be the case when the Che proteins of other organisms are compared with those of *E. coli*.

Che R is an s-adenosyl-L-methionine dependent methyltransferase which catalyses the transfer of methyl groups from s-adenosyl-methionine to specific glutamate residues located on the cytoplasmic domain of the MCP. It appears to function as a monomer and in *S. typhimurium*, it has a molecular weight of 33 kD (Simms *et al*, 1987). The number of Che R monomers per cell is only about 200 (this compares with about 12000 receptor dimers) and it has a relatively low processivity, producing about 10 moles of methylester per mole of enzyme per minute, which suggests that under normal physiological conditions, its catalytic capacity is nearly saturated (Stock *et al*, 1992;

Simon *et al*, 1987).

An increase in attractant concentration or decrease in repellent concentration causes a conformational change of the transducer that inhibits functional complex formation between MCP, Che A and Che W which leads to a lowering of the concentration of phospho-Che A, and therefore a decrease in the concentration of phospho-Che Y, resulting in smooth swimming. The same change in receptor conformation may concomitantly expose the four glutamate side chains within the K1 and R1 regions of the MCP (fig.06) to the methyltransferase, Che R, and sequester the methyl-glutamyl groups, sterically hindering the action of the methylesterase, Che B (Stock *et al*, 1992). Methylation of the MCP glutamate residues resets the conformation of the transducer to a pre-stimulus state abating continued (smooth swimming) signal production.

The active sites of Che R are not known although a considerable amount of attention has been given to particular cysteine residues which appear to be essential for enzymatic activity of Che R in *S. typhimurium*. Replacement of cys^{31} with a serine residue in this organism causes an 80% reduction in enzyme activity and it is thought that this residue is located at or very near the adenosyl-methionine binding site (Subbaramaiah *et al*, 1991 and 1992). Cysteine residues have also been identified as important in the functioning of other methyltransferase enzymes involved in the prokaryotic type II restriction modification system (Everett *et al*, 1990; Som and

Friedman, 1991). An examination of the *E. coli* and the *B. subtilis* Che R primary structures, however does not reveal any similarly located cysteine residues. The *B. subtilis* Che R homologue appears to differ functionally from its enteric counterparts. It is a 28 kD protein which displays a 31% homology with *E. coli* Che R. Mutant studies indicate that in *B. subtilis*, Che R functions to mediate adaptation to repellent stimuli, in contrast to the enteric Che R, which mediates adaptation to attractant stimuli. The phenotype of an enteric mutant deleted for Che R is predominantly smooth-swimming, but it will still exhibit some response to effectors (Stock *et al*, 1992). Over-expression of Che R results in a tumbly bias (Stewart *et al*, 1988). These mutants are not functionally chemotactic and cannot form chemotactic swarms in soft agar (Ames *et al*, 1988). In contrast to enterics, the Che R null mutant of *B. subtilis* is predominantly tumbly, suggesting that in this organism, Che R promotes adaptation to an increase repellents or a decrease in attractants (Kirsch *et al*, 1993).

1.5.9

The Che B Protein

Che B is a methylesterase which catalyses the removal of gamma-methyl groups from specific glutamate residues located on the cytoplasmic domain of the MCP, this action also converts glutamines to glutamates (which can then be methylated by Che R) and in effect, Che B functions as an amidase. It is a response regulator member of a two component regulatory family and as such, shares an N-terminus

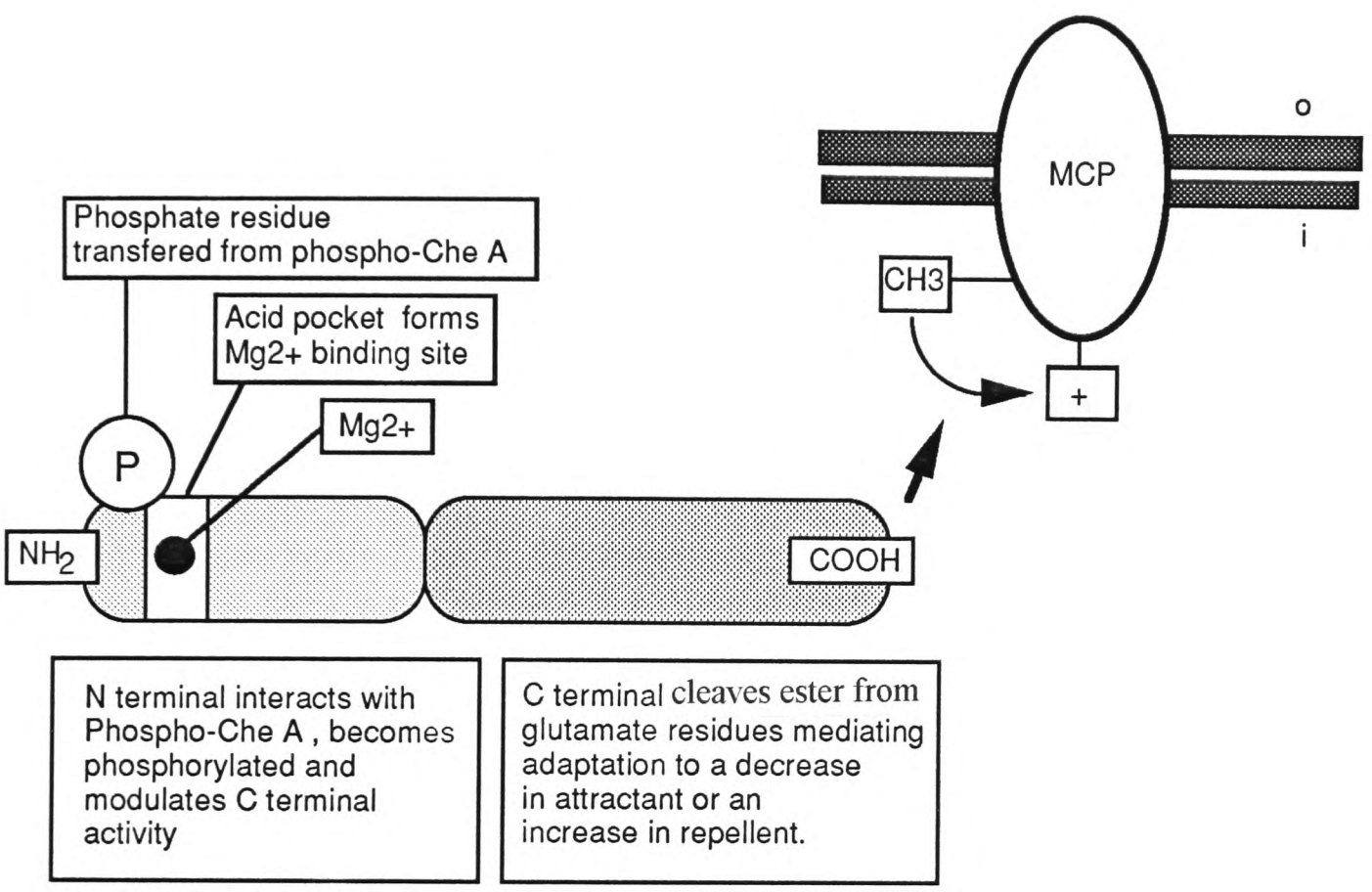


Fig.11: Schematic functional cartoon of the enteric Che B protein. Che B is a methylesterase which mediates behavioural adaptation to decreases in chemoattractant concentration and to increases in repellent concentration. Like Che Y, Che B is a member of the response regulator group of proteins and relieves a phosphate group from phospho-Che A. Like Che Y, Che B catalyzes its own phosphorylation.

homology of about 100 amino acid residues with other response regulators. Unlike many others in this group, Che B displays no common C-terminal homology (Stock *et al*, 1990). Che B possesses two partially characterized functional domains (fig.11); the C-terminal 60% of the protein mediates methylesterase activity, and the N-terminal 40% regulates the function of the C-terminal (Simms *et al*, 1985; Stewart *et al*, 1988; Lupas and Stock, 1989; Stewart *et al*, 1990; Stewart, 1993).

As is the case with Che Y, the other response regulator of the chemotaxis system, Che B catalyses its own phosphorylation, receiving a phosphoryl group from the histidine protein kinase, Che A (Hess *et al*, 1988; Stock *et al*, 1988). Phosphorylation of Che B at the N-terminal is thought to induce an allosteric conformational change which is transmitted to the C-terminal, activating methylesterase activity (Simms *et al*, 1985; Stewart, 1993).

The removal of attractant (or addition of repellent) induces a change in the receptor that promotes complex formation between the MCP dimer, the Che A dimer and Che W, increasing autophosphorylation of Che A. This leads to an increase in both Che Y phosphorylation, producing tumbling, and Che B phosphorylation, mediating adaptation (Hess *et al*, 1988; Stewart, 1993). Concomitantly, the conformational change of the MCP may expose methyl-glutamyl groups in the K1 and R1 domains to the esterase action of the (now active) phospho-Che B

and sequester unmethylated glutamate residues, sterically hindering the action of Che R (Stock *et al*, 1992). The resulting decrease in MCP methylation resets the transducer to a pre-stimulus methylation state, abating continued signal production and preventing indefinite tumbling.

Phospho-Che B is inherently unstable and once phosphorylated, has a half-life of only about five seconds. Whether this instability results from enzymatic or purely thermodynamic considerations is unknown (Stock *et al*, 1990); whichever the case, the ephemeral nature of the molecule is essential to allow the kinetics of adaptation, and therefore taxis, to function effectively.

Although the residues participating directly in phosphorylation and methylation reactions have not been identified, comparison of primary structure between different response regulators does reveal a number of conserved hydrophobic regions and three highly conserved residues. Stewart has recently identified seven residues, the point mutation of which produces aberrant behaviour in *E. coli* . Three of these residues (Asp^{11}, Glu^{58} and Glu^{91}) give rise to methylesterase-activating mutations; the other four (Asp_{10}, Arg^{42}, Arg^{73} and Lys^{107}) produce methylesterase-inhibiting mutations (Stewart, 1993).

In the Che Y molecule, which is the only response regulator whose three dimensional structure has been determined by X-ray crystallography, the conserved hydrophobic regions correspond to a

core which is formed at the intersection of five beta sheets and surrounded by five alpha helices. Of these structural features, three beta sheets (ß1, ß3 and ß4) and three alpha helices (αA, αC and αE) are also found in Che B. Using the Che Y structure as a basis for Che B, five of the seven functionally important residues can be theoretically identified. The three residues, the mutation of which produced methylesterase activation, are all located near the acidic pocket, which in Che Y is the site of Mg^{2+} binding. Mg^{2+} is required for both phosphorylation and dephosphorylation of Che Y (Lukat and Stock, 1993; Lukat *et al*, 1990) and likewise, Mg^{2+} concentration is thought to affect the activity of Che B (Stewart, 1993). Two of the three residues, Arg^{42} and Arg^{73} are located within the αB and αC helices, interactions at these sites could affect the conformation of the acidic pocket.

Enteric deletional mutants of Che B present as tumbly biased phenotypes, although they still respond to changes in chemoeffector concentration (Stock *et al*, 1992) and are able to form normal looking swarms in soft agar (Ames *et al*, 1988). Overproduction of Che B produces a smooth swimming biased phenotype. This is what might be expected since MCP glutamate residues will become de-esterified and methylation will decrease, the concentration of phospho-Che A, and therefore of phospho-Che Y will be reduced leading to reduced tumbling.

The ability of Che B^- mutants to respond chemotactically (forming

normal-looking swarms in plug-plates [Ames et al, 1988]), begs the question: are both Che R and Che B really necessary for taxis? If Che R mediates adaptation to an increase in attractants or a decrease in repellents, then what is the need for a system which mediates adaptation to the opposite? The answer may involve the cell's need for both sensitivity and consistency in its tactic response under a wide range of both intracellular and extracellular conditions. If the adaptation response depended upon the activity of only one type of molecule, especially one with a constant activity (such as Che R), then any changes in the concentration of that molecule, such as may occur during cell growth, would directly effect the cell's ability to adapt. If, however, the adaptation response is controlled by the *relative* activities of two opposing enzymes (whose genes are transcribed polycistronically to produce consistently equimolar protein concentrations [as are *che* R and *che* B]), then the adaptation signal will be independent of the absolute concentration of either Che R or Che B, allowing the response to be consistent despite physiological variations. Sensitivity will be enhanced by actively producing adaptation in response to both positive and negative stimuli.

R. sphaeroides adds an interesting twist to this situation, as the results of this study indicate that *che* B is missing, at least from it's position in the operon as predicted by analogy with the operons of *E. coli* and *R. meliloti* (see results).

1.6

MCP Dependent and MCP Independent Attractants and Repellents

The MCP/Che system described above allows enteric organisms to respond rapidly and sensitively to changes in their environment. Attractant stimuli sensed via this system include amino acids, some sugars, dipeptides and temperature (warmth). Repellent stimuli sensed via this system include weak organic acids, glycerol, pH and temperature (cold). As is the case for the other effectors, temperature changes are thought to induce a conformational change in the receptor, with an increase in temperature producing an effect equivalent to that produced by ligand binding at Trg, and with a decrease in temperature producing an effect equivalent to that of ligand release at Tap (Nara *et al*, 1991).

1.6.1

MCP Independent Taxis

Enteric bacterial behaviour is not exclusively reliant upon MCP-dependent signal reception and transduction, and MCP-independent taxis is well documented, though not so thoroughly investigated. MCP independent attractants of enterics include oxygen, PTS sugars and proline (Armitage *et al*, 1990). Osmolarity and the orientation of electric fields has also been shown to induce accumulation independent of MCP's and Che proteins (Adler *et al*, 1988a; Adler *et al*, 1988b). Metal ions, indole, and leucine are known to act as repellents to enterics and in these cases, no direct MPC/Che involvement has been found, thought indirect effects may be responsible for such responses.

E. coli mutants lacking the MCP/Che system have been shown to respond positively to certain organic acids which provoke a repellent response in wild-type cells; this response is dependent on the presence of acetyl coenzyme A (Wolfe *et al*, 1988). In other cases, motor reversal and cell tumbling (but not chemotaxis *per se*), has been shown to be induced in systems lacking key elements of the signal transduction system. Fumarate, for instance, has been shown to restore flagellum motor switching to envelopes of *E. coli* and *S. typhimurium* which contained Che Y, but no other cytoplasmic constituents (Barak and Eisenback, 1991). In this case, although MCP's and Che Y are present, the intermediate signal transduction molecules are absent, and fumarate is thought to act directly at the motor/switch complex. The nature of this interaction is not understood, but fumarate, being an electron acceptor in the Krebs cycle is well positioned as an indicator of general cellular metabolic activity and of redox poise. Acetyl phosphate has been shown to induce flagellar switching in Che A deleted mutants by acting as a phosphate donor for Che Y and may function as a global signal, donating phosphate groups to response regulators of diverse systems (McCleary *et al*, 1993).

1.6.2

PTS Sugar Taxis

The sugars maltose and galactose are sensed via the Trg transducer, but

these are only two of a wide variety of sugars metabolized by enteric organisms. In *E. coli* , the phosphotransferase system (PTS) is used to transport glucose, fructose, mannose, N-acetylglucosamine, mannitol, sorbitol and galactitol; some of these PTS sugars elicit a strong chemotactic response. It is believed that an element of the phosphotransferase machinery somehow interacts with the Che system, downstream of the MCP, integrating transport information into the taxis signalling pathway to produce a response. Taxis to PTS sugars, including glucose, has been demonstrated with mutant strains in which the MCP, Che B and Che R proteins have been deleted.

The phosphotransferase system is a group translocation system in which the transported solute is concomitantly phosphorylated (eg: glucose is converted into glucose-6-phosphate) using phosphoenol-pyruvate as the phosphoryl group donor (fig.05). The sugar is translocated into the cytoplasm via the membrane-spanning protein EII and the associated enzyme, EIII; it is at this point that the sugar is phosphorylated. A phosphoryl group is passed from phosphoenol-pyruvate to the cytoplasmic enzyme EI, thence to the histidine residue of a small heat-stable protein, HPr, thence to EII and finally to the sugar. EIII catalyses the phosphorylation of the sugar. The magnitude of the chemotactic response is related to the rate of translocation, but transport alone is not sufficient to produce a response; neither is the metabolism of the transported sugar a requirement for chemotaxis. Pre-phosphorylated sugars can be transported, but produce no taxis, whereas non-metabolizable analogues, which are transported and

concomitantly phosphorylated, act as strong attractants.

Fructose also enters the cell via a PTS pathway, but elicits no tactic response. Only one element of fructose transport appears to be different from other PTS transport pathways, the phosphotransfer role of HPr is fulfilled by a related protein, FPr. Although FPr can substitute for HPr in terms of phosphorylation, it cannot fulfil its role interfacing with the signal transduction system. (However, if fructose is transported by a previously induced mannose [HPr] pathway, chemotaxis *will* occur) Mutants with no HPr phosphotransferase activity cannot phosphorylate glucose, transport glucose or respond tactically to glucose. Mutants possessing variable HPr phosphotransferase activity demonstrate a clear relationship between phosphotransferase activity of HPr and chemotaxis. From this evidence it is proposed that the ratio of phosphorylated to unphosphorylated HPr acts as an indicator of environmental PST sugar concentration and that an unidentified cytoplasmic sensor molecule (the phosphoryl-chemotaxis protein, PCP) monitors this ratio, interfacing with Che system, transducing a sensory input to facilitate a chemotactic response. Both the nature of the putative sensor and the exact signal input point remain obscure. Che A and/or Che Y could both reasonably function as interface molecules, and a number of mechanistic theories have been produced to explain signal integration involving these components, but none of them have been proven; it seems almost certain, however, that Che Y is necessary, indicating that the PCP

sensor probably does not interact directly with the switch/motor complex.

Interestingly, an abundance of any of the PTS sugars strongly inhibits the active transport of sugars by other systems by expressional control of genes coding for transport enzymes. High glucose concentrations inhibit the production of cyclic AMP, which is required (in the formation of the CAP/cAMP complex) for transcriptional initiation of the relevant inducible catabolic operons. Transcription of the master operon of flagellum and chemotaxis gene expression is also controlled by the CAP/cAMP system, and therefore is catabolite repressed by glucose; so in an environment where glucose is plentiful, and chemotaxis confers no selective advantage, *E. coli* will produce neither Che proteins nor a flagellum, economizing on the production of these energetically expensive systems.

Some organisms respond to certain chemoeffectors in a way that is neither MCP/Che dependent nor PTS dependent. *R. sphaeroides*, for instance, is strongly chemotactic to mannose, sorbitol and glucose, none of which are transported via a PTS system in this organism; indicating yet another signalling pathway.

1.6.3

Electron Transport Dependent Taxis

Electron transport is intimately coupled with the production of the transmembrane proton gradient, a fundamental energy currency in living systems powering diverse biochemical reactions from ATP production to flagellar rotation. Although most of the research into tactic signal reception and transduction has been concentrated upon dedicated chemosensory systems, there is considerable evidence suggesting that bacteria have evolved a sensory system that monitors electron transport, and therefore the general energy state of the cell. An intrinsic problem in the investigation of electron transport dependent taxis is that since pmf and motor function are inseparably linked, it is a practical impossibility to distinguish between effects caused by perturbation of electron transport acting solely on sensory transduction apparatus from those acting on the motor alone. Aerotaxis and eubacterial phototaxis are both mediated independently of MCP's (and possibly Che proteins). Whether they possess dedicated signal transduction components of their own is unknown, but both forms of behaviour are electron transport dependent.

Aerotaxis is the movement of bacteria towards oxygen, and may be thought of as a specific form of terminal electron acceptor taxis. At the proximal end, electron transport is dependent on the availability of electron donors with high electronegativities, whose electrons are made available (via, for instance, glycolysis and the Krebs cycle) and at the

distal end, on the availability of electron acceptors with low electronegativities. Bacteria will induce different terminal reductase enzymes depending upon the availability of different terminal electron acceptors. Under aerobic conditions, production of the nitrate reductase of *E. coli* will be inhibited and taxis will occur to oxygen, but not to nitrate. If oxygen becomes limited, nitrate reductase expression becomes derepressed and *E. coli* shows chemotaxis towards nitrate. This taxis has been shown to be MCP independent; involvement of Che proteins has not been shown (Armitage *et al*, 1990). Electron transport has been shown to be essential for this type of taxis and binding of a terminal electron acceptor to the terminal reductase is not in itself sufficient to cause a response. It seems probable therefore, that some intermediate of the electron transport chain acts as an indicator. (It is also possible that a change in pmf, induced by electron transport, is sensed directly). The redox balance between the oxidized and the reduced forms of such an indicator molecule may be monitored by a sensor molecule which then transmits information either directly, or via intermediate species (which could amplify the signal) to the motor, to cause switching. Many organisms possess branched electron transport pathways. This allows them to flexibly exploit a changeable and nutrient-poor environment containing a range of different electron donors and acceptors in variable concentrations. *Rhodobacter sp*, for instance, have evolved to survive in just such an environment and possess, in addition to O_2 dependent and cyclic photosynthetic systems, three different oxidative enzymes which can funnel electrons into the electron transport chain, and five

different terminal reductases (fig.12). In such a case, it would seem most metabolically efficient to use, as an indicator, a component that was common to all possible electron transport pathways, for example, ubiquinone.

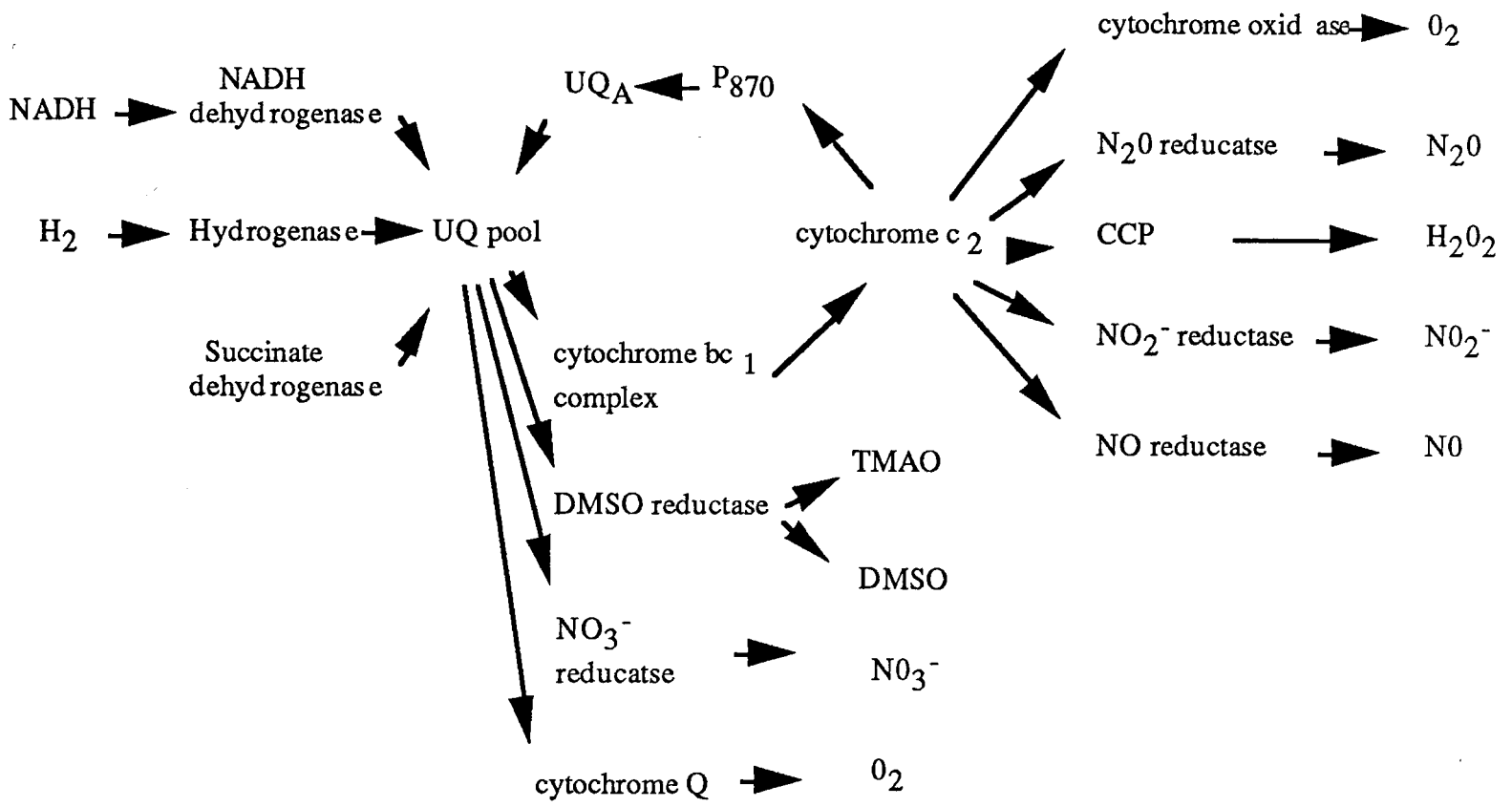


Fig.12: The electron transport pathways of *Rhodobacter* species. The fact that this organism can use three different electron donors and several different terminal electron acceptors gives it great flexibility in metabolically exploiting a wide range of environments. UQ = ubiquinone; DMSO = dimethyl sulphoxide; TMAO = trimethylamine N oxide; CCP = cytochrome c peroxidase.

Phototaxis in eubacteria, such as *R. rubrum*, causes the organism to reverse direction when it crosses a boundary from light to dark (but not from dark to light). Because the motor does not just stop, but actively reverses, this cannot simply be the result of a reduction in photo-synthetically derived pmf. *R. sphaeroides*, when grown photo-

synthetically, does not show such a dramatic response, but an increase in stopping frequency is caused by a reduction in light intensity. This is the equivalent of a negative chemotactic response, comparable to tumbling in enteric bacteria.

No evidence of MCP or Che involvement has been found in phototaxis, however, a close correlation has been demonstrated between the rate of photosynthetic electron transport and phototaxis (Clayton, 1953a; Clayton, 1953b). This evidence suggests phototaxis is mediated via an indicator molecule (or system of molecules) that monitors the redox poise of an intermediate of photosynthetic electron transport (Armitage *et al*, 1992). Inhibitors of electron transport inhibit phototaxis and reaction centre mutants with full pigment complement do not show phototaxis (Harayama, 1977; Armitage and Evans, 1981; Armitage, 1993). Phototaxis and aerotaxis may share much of the same signal transduction machinery, differing only at the input end, both may work by sensing a change in the rate of electron transport.

1.7

The Signal Transduction System of Enteric Organisms is Not Universally Representative

Although *E. coli* and *S. typhimurium* provide the best defined chemotactic systems to date, they were not chosen on the basis of being the most representative organisms and indeed it must be remembered that enteric bacteria have evolved to exploit relatively limited

environments which are biochemically and physiologically quite stable, and will have concomitantly evolved to become less adaptable as they have become more specialized. The mammalian large intestine is a nutrient-rich environment in which *E. coli* has no difficulty obtaining carbon and energy sources, consequently enterics will have developed a sensory system which "assumes" an adequate supply of carbon, nitrogen, phosphorus and sulphur-containing compounds. Aspects of the progenitory sensory system which are crucial for survival in nutrient-poor environments will have become de-accentuated over evolutionary time and may have been lost entirely, whereas components which confer the greatest advantage in a nutrient-rich environment will have become dominant. Accordingly, the MCP/Che system has become the dominant system in enteric bacteria.

Most bacteria, including *R. sphaeroides*, inhabit less well-defined niches and require a highly flexible and sensitive sensory system to allow exploitation of nutrients which would vary greatly in type, availability and concentration. *R. sphaeroides* is an extremely flexible microbe, able to exploit almost any environment in which it finds itself. Under certain conditions, the possession of MCP's could be seen as a disadvantage. The possession of a dedicated sensory transduction system will "compel" *E. coli* to move towards a carbon source such as serine or galactose even though carbon is not a growth-rate limiting metabolite. *E. coli* will show this response even though positive taxis to the carbon source will cause it to move away from a

source of oxygen, the concentration of which *is* growth-rate limiting; aerotactic signals being over-ridden by MCP-derived signals, at least under laboratory conditions. *R. sphaeroides*, however, will only respond chemotactically to useful metabolites and will give preference to groups of substances that are growth-rate limiting (Poole and Armitage, 1989). This type of flexible response is more energetically efficient and better suited to an environment where nutrients are variable and may be in short supply. Such a response suggests a metabolically integrated signal transduction system.

1.8

Differences Between Enteric Organisms and *R. sphaeroides*

R. sphaeroides is a Gram negative, purple, non-sulphur bacterium which can grow both photoheterotrophically under anaerobic conditions or chemoheterotrophically under aerobic conditions, displaying dramatically different morphological and biochemical features in these different environments (Pellerin and Gest, 1983; Kiley *et al*, 1988). *R. sphaeroides* is significantly different from enteric bacteria in a number of ways that pertain to chemotaxis (fig.13).

R. sphaeroides is a mono-flagellate possessing a sub-polar flagellum with a straight hook. Despite possessing only one flagellum (in contrast to about six possessed by enterics) *R. sphaeroides* displays relatively fast run speeds of up to 100 micrometers per second, compared with about 40 micrometers per second for *E. coli*.

Fig.13: Differences between *Rhodobacter sphaeroides* and *Escherichia coli* that pertain directly to chemotaxis.

Rhodobacter sphaeroides	*Escherichia coli*
Single sub-polar flagellum	Peritrichous
Straight hook	Curved hook
Unidirectional flagellum rotation	Bidirectional rotation
Reorientation by Brownian motion	Reorients by tumbling
Demonstrates kinesis	No kinesis
Only a fraction of the population will respond to a stimulus	"All-or -none response"
Heterogeneous behaviour - motility characteristics very variable throughout same population	Homogeneous behaviour - motility characteristics similar throughout same population
No membrane spanning MCP's known.	MCP's span membrane
Second Che Y protein present, dual sensory pathway proposed [This study]	Only one Che Y, single chemosensory pathway.
Chemoattractants require transport to produce response	Transport not required to produce response
All chemoattractants are metabolites or ions such as K^+ or Rb^+	Attractants include non-metabolizable analogues
Some metabolism required for taxis	No metabolism required for taxis
Strongly attracted by organic acids	Strongly repelled by organic acids
Only fructose known to be transported by PTS (HPr)	Many sugars transported by PTS

Unlike the bidirectional flagellum of the peritrichous enterics, the flagellum of *R. sphaeroides* rotates unidirectionally, either clockwise or counterclockwise, but never shows reversal (Packer and Armitage, 1993). Unlike enterics, reorientation of *R. sphaeroides* is achieved without the aid of flagellar reversal, but Brownian motion causes the cell to be turned randomly during brief stops caused by a complete cessation of motor rotation (Armitage and Macnab, 1987). It also seems likely that reorientation could occur due to the recoiling of the filament when torque is released. The relatively high viscosity of the environment would provide sufficient anchorage to allow the recoiling filament to subject the head to force sufficient to cause movement. In the above respects, the *R. sphaeroides* motor is more similar to the unidirectional motor of *R. meliloti* (Gotz and Schmitt, 1987). Unlike *E. coli*, in which flagellin synthesis is tightly controlled by a de-repression system involving the cap (Fli D) protein, *R. sphaeroides* appears to produce flagellin throughout the cell cycle (N. Takahashi, D. Phil thesis, 1995, Oxford).

When investigating interactions involving the motor complex, *R. sphaeroides* provides the advantage of possessing only one motor, and so has only one holoenzyme unit per cell. This means that any biochemical reaction of a sufficient magnitude to affect the motor will affect whole cell behaviour. This provides a rare opportunity to observe and investigate the function of a single enzyme, impossible with peritrichous species.

Unlike *E.coli*, *R. sphaeroides* shows great behavioural heterogeneity (in run velocity, stopping frequency, duration of stops and duration of runs). This heterogeneity is observed even under conditions where the pmf is well above that required for the saturation of motor rotation (Poole and Armitage, 1988). A population of *R. sphaeroides*, unlike a population of *E. coli* , does not respond to a chemoeffector *en masse* (an "all-or-nothing" response), but only a fraction of the cells in the population will show taxis. Such heterogeneity makes measurements based on the behavioural changes of cell populations of *R. sphaeroides* less clear cut than with *E. coli*.

In response to an increase concentration of some chemoattractants, *R. sphaeroides* demonstrates chemokinesis , which is a sustained increase in run speed independent of any change in stopping frequency and is therefore independent of chemotaxis (Brown *et al*, 1993). Kinesis increases mean swimming speed by up to 25%, depending on the extent of starvation for the metabolite causing the response, and is maintained as long as the effector concentration remains elevated. The increase in speed is caused by an increase in the rate of flagellar rotation, but is not associated with any increase in membrane potential (Poole *et al*, 1991). It can be induced by a limited number of metabolites as well as non-metabolites, such as betaine (Packer *et al*, 1994). Chemokinesis is not caused equally by all chemoattractants. Fructose, which produces strong chemotactic accumulation, causes only a weak kinetic

response, whereas organic acids provoke a strong chemokinetic response; with the exception of aspartate, amino acids cause no kinetic response (Armitage, 1993). Chemokinesis is invariably accompanied by cell swelling and the increase in speed may be a by-product of this, possibly due to a rearrangement of membrane-bound components around the motor. As chemokinesis is not a directional response, it is thought that the function of it may be to spread the population throughout a favourable environment. Chemokinesis has also been observed in *Rhizobium meliloti* and *Azospirillum brasilense*, two other species in which membrane spanning transducers have not been identified and which are both members or the α-subgroup of bacteria, as is *R. sphaeroides* (Zhulin and Armitage, 1993; Greck *et al*, in press).

R. sphaeroides responds positively to a variety of chemicals including monosaccharides, disaccharides, amino acids, ammonia, potassium, rubidium and shows particularly strong taxis towards organic acids including acetate, propionate and butyrate, which repel enterics. Fructose, which provokes no taxis in enterics, produces a strong accumulation response in *R. sphaeroides*. Chemicals which repel enterics do not seem to have the same effect on *R. sphaeroides* and only oxygen has been shown to provoke a repellent response, and then only if *R. sphaeroides* has been grown under strictly anaerobic, photoheterotrophic conditions.

Adaptation to a step-down response (those involving a temporal

decrease in attractant concentration) has recently been demonstrated (Packer *et al*, in press), but no adaptation to a step-up response has been shown and it may be that *R. sphaeroides* responds only to a decrease in chemoattractant concentration and not to an increase (Poole and Armitage, 1988). Computer modelling has demonstrated that such a response *would* result in accumulation, although at a slightly slower rate than accumulation mediated by an organism that responds to both increases and decreases in effector concentration (Packer and Armitage, in press).

In contrast to the situation in enterics, which transduce a signal from the environment to the cell interior via a transmembrane receptor and hence do not require a chemical to enter the cell in order to cause a response, non-transportable analogues of attractants do not produce a response in *R. sphaeroides* (Poole *et al*, 1993). Analogues of chemoattractants that *are* transported, but not metabolized, such as 2-aminoisobuturate, a non-metabolizable analogue of alanine, also cause no chemotactic response and it has been shown that both transport and metabolism (at least to a limited extent) of effectors is required to cause chemotaxis in *R. sphaeroides* (Ingham and Armitage, 1987; Poole *et al*, 1993).

In addition, *R. sphaeroides* does not appear to possess membrane-spanning MCP's (Armitage *et al*, 1985; Ingham and Armitage, 1987; Poole *et al*, 1992). Western blotting using antibodies raised against

enteric Tar, Trg and Tsr MCP's produced no hybridization signal with *R. sphaeroides* protein extracts (Morgan *et al*, 1993; Sockett *et al*, 1987). Protein methylation assays and methanol production assays have also failed to demonstrate the presence of MCP-like trans-membrane transducers (Armitage, 1992), this fits well with the requirement for transport.

Previous studies have failed to identify any homology on the DNA or protein level between *R. sphaeroides* and *E. coli* Mot or Che systems (Sockett *et al*, 1987). Western blotting using antibodies raised against *E. coli* Che A and Che Y revealed no protein homology for *R. sphaeroides* (W. Havelka, D.Phil thesis, Oxford, 1991).

Southern blotting using probes made from fragments of the *E. coli* Mocha operon (including *che* A) was done, but no hybridization signal was seen (W. Havelka, D.Phil. thesis, Oxford, 1991). [It should be noted that *R. sphaeroides* has a high G:C content (68%) which would inhibit hybridization of *E. coli* probes, even under low stringency].

Although *R. sphaeroides* responds to a number of sugars which in enterics are transported (and responded to) via the PTS system, only one sugar, fructose, seems to be taken up via a PTS pathway in *R. sphaeroides* (Sockett *et al*, 1987; Hazelbauer *et al*, 1990; Armitage, 1993). In the fructose transport system FPr, not HPr, is the phosphate donor and in enterics this system elicits no chemotactic response (see previous section).

1.9

Background of This Study

Prior to the start of this investigation none of the elements of the chemotactic signal transduction system of *R. sphaeroides* had been identified. The behaviour of the organism (as discussed above) had been shown to be strikingly different from that of the enteric bacteria, and this provided tantalizing evidence of functional and therefore of structural differences in the chemosensory and signal transduction systems between the organisms.

A previous investigator had used transposon mutagenesis in an attempt to produce chemotactic mutants of *R. sphaeroides*. This had led to the identification of the Tn*pho* A mutant Q12 which had been characterized as being non-chemotactic to organic acids when grown on propionate and non-chemotactic to acetate and to propionate when grown on succinate; when grown on butyrate however, it showed taxis to all organic acids (W. Havelka, D. Phil thesis, 1991). The Q12 genome was used to generate restriction fragments which were cloned into pUC 18 (pWH1, pWH6, pWH8 and pEKn12-18; see Materials and Methods). A fragment flanking the Tn*pho* A insertion site was used to produce a probe which hybridized with cosmid 324 (c324) from the *R. sphaeroides* cosmid library and this cosmid was used to generate sequencing clones. This work had proceeded to a stage at which an open reading frame ("ORF W") of the Tn*pho*A-interrupted gene had

been identified and partially sequenced. It had been determined that ORF W "contains strong identity to a σ54 consensus sequence and possesses limited homology on the protein level to the response regulator subgroup of the two component sensory system family." The sequencing clone pWH8 was believed to contain over 70% of the putative gene's open reading frame.

The association of a response regulator-like protein with an aberrant behavioural phenotype suggested that a central component of the chemotactic signalling system had been discovered. It was therefore important to investigate the gene, to precisely define its function and to complete the sequence of ORF W. During this project, serious flaws were revealed in the previous work.

1.10

Aim and Approach of This Study

The aim of the investigation presented here has been to explore and to characterize the chemotactic signal transduction system of *R. sphaeroides* on a molecular and on a genetic level. Three major approaches were used in the investigation. The first was to build on the previous investigation of Q12. This would initially involve reproduction of experimental results and confirmation of phenotype and then would progress to characterization of the ORF W gene, protein purification and determination of the gene function. As will be discussed later, problems were revealed with the Q12 mutant which

made it an unsuitable subject for further investigation.

The second approach was to produce mutants in the hope of isolating a true non-chemotactic phenotype, to characterize them behaviorally and then genetically. Transposon mutagenesis was used initially and the transposon Tn5 was chosen both for ease of delivery into the target genome and for it's convenient kanamycin resistance gene which served as a selection marker. Transposon mutagenesis is a coarse but relatively simple and effective means of producing mutations, with Tn5 insertion likely to inactivate entire gene products and to have down-stream effects. This method had been used in previous investigations (Duggleby *et al*, 1990; Stock *et al*, 1989; Hunter, 1988) to successfully identify elements of the chemotaxis signal transduction system of *E. coli* and *S. typhimurium*. It was reasoned that if the chemotaxis system of *R. sphaeroides* consisted of functionally similar cytoplasmic elements, these could be eliminated by transposon insertion, producing aberrant chemotactic phenotypes, without affecting motor assembly or anchorage.

If, however, the common components of the *R. sphaeroides* sensory transduction system were not cytoplasmic, but formed an integral part of the motor/switch complex, or if they were transcribed polycistronically on an operon that also contained a sigma (or other regulatory) factor essential for the expression of subsequent flagellar/motor components, then transposon mutagenesis would be too crude to produce non-chemotactic mutants that could assemble a normal flagellum, and no flagellated, non-chemotactic mutants would be produced. If transposon

mutagenesis was not successful in producing non-chemotactic phenotypes, chemical mutagenesis using ethane methane sulphonate (EMS) could theoretically be used to produce point base-substitutions in *R. sphaeroides* DNA. Assuming that individual residues involved in switching or signal transduction were not also essential for assembly, anchorage and/or rotation, then point mutations of these functionally critical bases would produce non-chemotactic mutants. These mutants would then be characterized by motion analysis and then investigated genetically. However, if *R. sphaeroides* contained proteins which were structural but not exact functional equivalents of the enteric proteins, then the product of transposon or of chemical mutagenesis would not necessarily be apparent using screening procedures developed for the identification of chemotaxis mutants. As became apparent from this and other studies, although successful in enterics, this method is unlikely to work in *R.sphaeroides.*

The third approach only became apparent as a possibility relatively late into the investigation due to the fortuitously timely identification of *R. meliloti che* gene homologues by R. Schmitt in Regensburg, Germany. This approach, which was ultimately to prove the most productive, entailed investigation of genetic homology between the genome of *R. sphaeroides* and the *che* homologues of *R. meliloti,* which is a bacterium considerably more closely evolutionarily related to *Rhodobacter* than are *Escherichia coli* or *Salmonella typhimurium* (Woese, 1987).

DNA probes, produced from *R. meliloti* gene fragments, displayed homology with a cosmid from the *R. sphaeroides* cosmid bank, c292. Subsequent investigation, including sequencing, revealed three genes with considerable homology to *R. meliloti* and *E. coli* genes involved in chemotaxis signal transduction. These *R. sphaeroides* genes appear to be clustered together in a functionally related operon which is now proposed to include homologues for four out of the six chemotaxis genes of enterics (*che* A, *che* Y, *che* W, *che* R) as well as a second *che* Y gene, "*che* Y2", homologous to the *che* Y2 gene recently discovered in *R. meliloti* (Ward *et al*, [in press] 1995; Greck *et al* [in press].1995) This information was used in deletion and complementation experiments with an aim to determine the function of these genes in *R. sphaeroides*.

2. MATERIALS AND METHODS

2.1

Growth Media and Growth Conditions

Rhodobacter sphaeroides WS8N (a spontaneous naladixic acid resistant strain of WS8 which was a gift from W. R. Sistrom) was grown both aerobically in the dark and anaerobically in the light in either 7 mM succinate medium or in M22 minimal medium supplemented with a single 7 mM carbon source (Fig. 14). Sodium salts of carbon sources and of buffers were used in preference to potassium salts as K^+ is known to be a strong chemoattractant for *R. sphaeroides* (Poole *et al.*, 1989).

Growth conditions were as follows: Photo-heterotrophically, cells were grown anaerobically in liquid medium, at 28°C (in a filled 200 ml medical flat bottle), with tungsten illumination of c.100 microeinsteins or on medium solidified with 2% agar at 28°C in an environment of 3% carbon dioxide and 97% nitrogen (Don Whitely Anaerobic Cabinet Mk. III). Heterotrophically cells were grown in liquid medium at 30°C in the dark, thoroughly aerated on a shaker (10 ml in a 250 ml conical flask) or on medium solidified with 2% agar at 28°C in the dark.

Escherichia coli strains were grown heterotrophically in Luria broth (LB) at 37°C with shaking (Sambrook *et al*, 1989) or on medium solidified with 2% agar at 37°C in the dark. Antibiotics, when

appropriate, were added to media in the following concentrations: ampicillin: 50 μg/ml; chloramphenicol: 25 μg/ml; kanamycin: 25 μg/ml; nalidixic acid: 20 μg/ml; streptomycin: 50 μg/ml; tetracycline: 15 μg/ml for *E. coli* or 1 μg/ml for *R. sphaeroides*. Bacterial strains and plasmids are listed in fig.15.

Bacterial growth was monitored spectrophotometrically at 700 nm using a Shimatsu UV-1201 spectrophotometer and also directly by Coulter Counter (Coulter Electronics Inc).

2.2

Growth Characteristics

Growth curves were produced for both aerobically and anaerobically grown *R. sphaeroides*. 1 ml of a log-phase culture was used to inoculate 200 ml of 7 mM succinate medium containing 25 μg/ml naladixic acid. These cultures were incubated aerobically and anaerobically (as above) over a period of three days. Small samples (0.5 ml) were taken at regular intervals and cell density was quantified both spectrophotometrically and directly, using the Coulter Counter. Graphs were produced of spectra-photometric absorption (Abs_{700}), and of cell number against incubation time.

Figure 14. Bacterial Growth Media

Succinate Medium

Concentrated Base solution	20 ml
Potassium Phosphate Buffer (1 M)	20 ml
Ammonium Sulphate	0.5 g
Sodium Succinate	2.0 g
Cassamino Acids	1.0 g
Growth Factor solution	2 ml
ddH_20	to 1 L

pH 7.2 with 5 M Potassium Hydroxide solution

M22 Minimal Medium

Concentrated Base solution	20 ml
Potassium Phosphate Buffer (1 M)	20 ml
Ammonium Sulphate	0.5 g
Growth Factor solution	2 ml
ddH_20	to 1 L

pH 7.2 with 5 M Potassium Hydroxide solution

Luria Broth

Tryptone	10 g
Sodium Chloride	10 g
Yeast Extract	5 g
ddH_20	to 1 L

pH 7.5.

Concentrated Base Solution

Nitriloacetic Acid	5.9 g
Metals 44 Solution	25 ml
Magnesium Sulphate 7 H_20	14.5 g
Calcium Chloride 6 H_20	2.5 g
Ferrous Sulphate 7 H_20	50 mg
Ammonium Molybdate 4 H_20	4.6 mg
ddH_20	to 1 L

The nitriloacetic acid was dissolved in 500 ml ddH_20. The undissolved residue was removed and the pH adjusted to 5.0 with potassium hydroxide. The magnesium sulphate was added and allowed to dissolve, followed by the calcium chloride, ammonium molybdate, ferric sulphate and Metals 44 solution. The final pH was adjusted with potassium hydroxide dropwise to 6.8. Water was added to give a final volume of one litre.

"Metals 44" Solution

Tetra Sodium Versanate	2.5 g
Zinc Sulphate 7 H_20	11 g
Ferrous Sulphate 7 H_20	5 g
3M Sulphuric Acid	1.5 ml
Magnesium Sulphate 4 H_20	2 g
Copper Sulphate 5 H_20	0.39 g
Boric Acid	0.12 g
Cobalt Chloride 6 H_20	0.2 g
ddH_20	to 1 L

Figure 15. Bacterial Strains, Plasmids and Bacteriophage

Strain or Plasmid	Characteristics and References
Strains:	
R. sphaeroides WS8-N	Wild type; spontaneous Nal^r strain of WS8. (Sockett and Armitage, 1991).
R. sphaeroides Q12	Tn*pho*A mutant of WS8-N. Nal^r. (Havelka, doctoral thesis, 1992).
R. sphaeroides NM 1 - 19	Tn *pho*A mutants of WS8 and WS8-N. Nal^r (NM 9 - 19); Kn^r (This laboratory).
R. sphaeroides TAB 1 to 95	Tn5 mutants of WS8-N. Nal^r Kn^r (This study).
R. sphaeroides CAB 1 to 9	Ethyl methane sulphonate mutants of WS8-N. Nal^r (This study).
R. sphaeroides WS8NXW	Recombinant mutants of WS8N produced by homologous recombination with the suicide construct of the pSUPW series. Kn^r Cm^s. (This study)
R. sphaeroides WS8NXR	Recombinant mutants of WS8N produced by homologous recombination with the suicide construct of the pSUPR series. Kn^rCm^r. (This study)
R. sphaeroides WS8N::pJPW15/21	Recombinant mutants of WS8N produced by homologous recombination with the suicide construct pJPW15 or pJPW21 (this study).
R. sphaeroides WS8NXW*	Recombinant mutants of WS8N produced by homologous recombination with the suicide construct of the pSUPW* series. Kn^r Tc^s. (This study)
E. coli JM83	F^- *ara* Δ(*lac-pro*)AB, alpha $comp^+$ *rsp*L. (Maniatis, 1983).
E. coli DH5a	F^-*lac*ZΔM15 *rec*A1 hsdR17 (Suwanto and Kaplan, 1991).
E. coli S17-1	Sm^r Pro^- Res^- Mod^- *rec*A integrated plasmid RP4-Tc::Mu

	Kn::Tn7. Broad host conjugation strain. (Simon *et al.*, 1983)
E. coli GM48	F⁻ *thr leu thi Lac*Y *gal*K *gal*T *ara* fhuA *dam*⁻ *dcm*⁻ supE44 (Maniatis, 1983).
E. coli RP4606	che W⁻ mutant: che W (Amber) 113. (gift from J.S.Parkinson)

Plasmids:

pUC18 and pUC19	Ap^r Multicopy clonong vectors. (Yanisch-Perron *et al.*, 1985).
pLA2917	Km^r Tc^r cosmid cloning vector, single copy number vector (Allen and Hanson,1984).
pSUP2021	Km^r Ap^r Cm^r pSUP202::Tn5. Source of Tn5 for mutagenesis. (Hunter, 1988).
pUI800	Tc^r Cm^r Km^r derivative of pSUP203 bla::Tn*pho*A (Moore and Kaplan, 1989).
pMH1701	Kn^r Vector containing Tn5 *sac* (Duggleby *et al.*, 1990).
pSUP202	A pBR325-derived mobilizable suicide vector Km^r Ap^r Cm^r. (Simon *et al*, 1983)
pJP5603	R6K-based suicide vector requiring a trans supply of the *pir* encoded π protein. lacZ'/MCS. Kn^r (Penfold and Pemberton, 1992).
pJP∂R	3.8 kb pJP5603-derived suicide plasmid containing a 694 bp *Nru* I *che* R fragment from pCH2. ΔLac Z, Kn^r. (this study).
pJP∂Y2	3.8 kb pJP5603-derived suicide plasmid containing a221 kb Kpn I / Sal I *che* Y2 fragment from pCH2. ΔLac Z, Kn^r. (this study).
pUI523A	Tc^r Δ*lac* P O , Broad host translational (lac) fusion vector derived from RSF1010. (Tsu-Ning Tai *et al.*, 1988).
pUI-ORFW 1,2 and 3	pUI523A-derived clones containing a 1.3 kb *R. sphaeroides Eco* RI - *Hind* III fragment from pWH8 (thought to contain ORFW). Tc^r. This study.
pGEX-3X	GTS gene fusion expression vector, Ap^r (Blackwood, 1991).

pTM30	Expression vector using a Ptac IPTG-inducible promoter. Ap^r (a gift from J.S.Parkinson, University of Utah).
pE1.5, pE2.3 and pE2.7	*Eco* RI pUC18 clones of *R. meliloti mcp* and *che* homologue genes (a gift from R. Schmitt).
pCH1 and pCH2	Clones containing the 3.1 kb *Eco R*I fragment from c292 cloned into the *Eco* RI site of the pUC18 polylinker; both orientations Ap^r.(This study).
pCH3 and pCH4	*Bam* HI deletion clones of pCH1 and pCH2; Ap^r (This study).
pCH5 and pCH6	*Sal* I deletion clones of pCH1 and pCH2; Ap^r (This study).
pCH7 and pCH8	Clones containing the 2.7 kb *Bam* HI fragment from c292 cloned into the *Bam* HI site of the pUC19 polylinker, both orientations; Ap^r (This study).
✳ pUC∂R	pUC18-derived clone containing an internal 695 bp *Nru* I fragment of the *R. sphaeroides che* R gene cloned into at the Sma I site of the pUC18 polylinker. Ap^r. (This study).
✳pUC∂Y2	pUC19-derived clone containing an internal 221 bp fragment from the *R. sphaeroides che* Y2 gene cloned into the *Kpn* I - *Sal* I site of the pUC19 polylinker. Ap^r. (This Study).
pCH2T series	A series of pUC18-based clones derived from pCH2, mutagenised by the insertion of mini-Tn10 into the che W gene in the insert; Ap^r Kn^r (This study).
pJPW15	Mobilizable suicide clone based on pJP5603 containing 4.9 kb (mini-Tn10 interrupted) *Eco* RI fragment derived from pCH2T15 in opposite orientation to vector; Ap^r Kn^r mob^+ (This study).
pJPW21	Mobilizable suicide clone based on pJP5603 containing 4.9 kb (mini-Tn10 interrupted) *Eco* RI fragment derived from pCH2T21 in same orientation to vector; Ap^r Kn^r mob^+.
pSUPW series	pSUP202-derived suicide vectors containing *R. sphaeroides che*W::Tn10 (3 Kb *Sal* I fragment from pJPW)

✳ Note: "δ" in this text signifies an internal fragment of the following gene.

	cloned into the *Sal* I site. Ap^r Cm^r (This study)
pSUPR series	pSUP202-derived suicide vectors containing a 694 bp Nru I fragment of *R. sphaeroides che*R, cloned into the (blunted) *Sal* I site. Ap^r Cm^r (This study)
pSUPW* series	pSUP202-derived suicide vectors containing *R. sphaeroides che* W::Tn10 (2 Kb *Eco* RI - *Pst* I fragment from pCH2T21) cloned into the *Eco* RI - *Pst* I sites of pSUP202. Km^r Tc^r Ap^S Cm^S (This study)
pTMW26	Construct containing *R. sphaeroides che* W gene in *Kpn* I site of pTM30 (This study)
c324	20kb fragment of *Mbo* I partial digest of *R. sphaeroides* WS8 chromosomal DNA inserted into the *Bgl* II site of pLA2917 (Havelka thesis, 1992).
c292	21kb fragment of *Mbo* I partial digest of *R. sphaeroides* WS8 chromosomal DNA inserted into the *Bgl* I site of pLA2917 (this study)
pWH1	800 bp *Eco* RI - *Hpa* I fragment from pEKn12-18 in the *Eco* RI - *Hinc* II site of pUC18 (Havelka thesis, 1992).
pWH6	6kb *Hin*d III fragment from c324 in the *Hin*d III site of pUC18 (Havelka thesis, 1992).
pWH8	1.3kb *Sal* I - *Nru* I fragment from pWH6 in the *Sal* I - *Sma* I site of pUC18 (Havelka thesis, 1992).
pEKn12-18	6kb *Eco*RI fragment of Q12 DNA in the *Eco*RI site of pUC18 (Havelka thesis, 1992).
pAB1	5.5kb *Hin*d III fragment of Q12 DNA from pWH6 in the *Hin*D III site of pLA2917 (This study).

Bacteriophage:

Lambda NK1316	Phage vehicle (P*am* 80 lambda hop, *Att*$^-$ cI857 repres.) for the delivery of the Kn^r mini-transposon mini-Tn10,derivative 103 of Tn903, with the *Ptac-ats1 ats2* transposase gene in *cis*.

2.2a

Pigment Analysis

Absorption spectra (ε_{772} = 75 mM^{-1} cm^{-1}) with a range of 300 - 900 nm were determined using an initial cell concentration of c. $2x10^8$ cells/ml from which photosynthetic pigments were extracted into acetone : methanol (7 : 2) (Clark and Jackson, 1981). 1 ml of photosynthetically grown cells, was pelleted and washed in sterile water, then resuspended in an equal volume of the solvent mixture, which causes cell lysis. The cell debris was pelleted at 10000g for four minutes, the supernatant was decanted and the absorption spectrum was measured using a Beckman DU-64 or a Shimatsu UV-1201 spectrophotometer with an acetone : methanol blank.

2.2b

Flagellar Staining

The presence of flagella was determined by staining using a tannic acid (5 M) and aluminium potassium sulphate (a saturated solution) mordant with a crystal violet stain. (Kodaka *et al.*, 1982). 1.0 ml of a mid-log phase culture was pelleted, washed and resuspended in 100 µl of distilled water. 12 µl of this suspension was smeared onto a slide and air dried. The slide was then flooded for 5 minutes with a mixture (10 : 1) of mordant and stain. The slide was washed in running water and air dried, then viewed, without a cover-glass, under the 100x oil immersion objective of the Nikon Optiphot microscope. The mordant causes cell aggregation as well as thickening of the flagella, and the

flagella can clearly be seen projecting from dense rafts of cells.

2.3

Motion Analysis, Chemokinesis and Chemotaxis Assays

Motility, chemokinesis and chemotaxis characteristics of wild-type and mutant *R. sphaeroides* strains was characterised by a number of methods. These methods included macroscopic assays based on swarm formation and chemotactic accumulation (swarm-plate, mini-swarm, injection-swarm and plug plate assays). Detailed analysis of motility characteristics was carried out by computerised tracking and analysis using both free-swimming and tethered cells (see below)

2.3a

Computerised Motion Analysis

Motility characteristics of *R. sphaeroides* populations were measured using the Seescan computerized tracking system (Armitage *et al*, 1988; Poole and Armitage, 1988; Packer and Armitage, 1994a; Harrison *et al*, 1994) and using the Hobson Bac-Tracker system (Packer and Armitage, 1994b). Parameters measured included mean run length, mean run speed, translational run speed (speed between, but not including stops), mean stopping frequency, mean stop duration and percentage motility. Total tracking time for the Seescan system was 3 x 5 minutes, giving about 450 cells recorded per sample. For the Hobson Bac-Tracker, total tracking time was 4 x 2 minutes, tracking

over 4000 cells per sample (greater sample number due to different magnifications used).

For analysis of free-swimming populations (as supposed to tethered bacteria) cells were grown aerobically or anaerobically to mid-log phase (about 10^9 cells/ml), harvested by gentle centrifugation (2000g for 10 minutes), washed and resuspended in ten times the original volume of 10mM Na HEPES buffer, pH 7.2 which had been warmed to 30°C to prevent temperature shock. If the cells had been grown anaerobically, the HEPES buffer was first made anaerobic by vigorous sparging for 15 minutes with oxygen-free nitrogen. The cell suspension was then drawn into a flattened glass capillary tube with a thickness of 0.05mm (Camlab, Cambridge, U.K.) which was placed on a slide, plugged at either end with petroleum jelly and viewed using phase contrast microscopy (10000X magnification for Seescan and 1000X magnification for the Hobson Bac-Tracker). The image of the moving cells was recorded onto U-Matic video tape via an electronic video camera mounted on the microscope turret and motility was analysed using Seescan or Hobson Bac-Tracker software. Chemokinesis was measured with the same apparatus immediately following the addition of an equal volume of 1 mM putative chemoeffector, pH 7.2 (Brown *et al*, 1992). A 10mM Na-HEPES solution (pH 7.2) was used as a control in all assays.

For analysis of tethered cells, 5 µl of *R. sphaeroides*, grown and

harvested as above, was incubated on a cover slip in a humidity chamber with an equal volume of crude anti-flagellar antibody for 20 minutes. The tethered cells on the cover slip were then loaded into a flow chamber and observed by phase contrast microscopy at 1000x magnification. Buffers containing 1 mM Na acetate or 10 mM HEPES, pH 7.2, were passed through the flow chamber and rotational behaviour of individual tethered cells was measured using the Hobson Bac-Tracker software (Packer and Armitage, 1994b). Graphs of stop probability against time were produced. Each graph is a composite of at least ten individual tethered cells. The rotation rate of each cell was measured every 0.006 seconds and the data displayed is the mean stopping probability over a 20 second period, computed every 2 seconds.

2.3b

Miniswarm Chemotaxis Assay

Chemotactic mutants were initially screened in 0.3% swarm agar containing a single 7mM carbon source into which mutagenised cells were diluted (Parkinson, 1987). As a colony grows, it reduces the local concentration of the carbon source, thus establishing a gradient. A normally chemotactic population will swim up the gradient, creating a wild-type swarm; a non-chemotactic population will not be able to transduce information about the gradient to its switching apparatus and will form reduced swarming colonies.

2.3c

Plug Plates

Chemotaxis to a range of putative chemoeffectors was assayed by the use of plug plates (Poole *et al*, 1993). 200 ml of mid-log phase cells, grown photoheterotrophically, were pelleted, washed and resuspended in 10 mM Na-HEPES, pH 7.2 which had been sparged for twenty minutes with oxygen-free nitrogen. This cell suspension was then mixed with an equal volume of 0.5 % Bacto agar at about 40°C containing 50 μg/ml chloramphenicol (to inhibit growth), poured into Petri dishes and allowed to set in the light. Hard agar plugs containing 50mM putative attractant were inserted into the agar. Positive taxis was manifested by a visible disc around the plug within five hours of incubation in the light (c.100 microeinsteins) at 28°C.

2.3d

Injection-Swarm Plates

Injection-swarm plates were developed as an alternative to stab-swarm plates in order to overcome the problem of different degrees of swarm formation being caused by different inoculum sizes. A single colony, having been inoculated into a liquid medium containing a 7 mM carbon source and antibiotics as appropriate, was grown anaerobically in the light to mid-log phase. The culture density was then measured by counting cell numbers per unit volume using a Coulter Counter, and adjusted to eliminate differences between strains to be compared. 5 μl of this culture was injected into a 0.3% (swarm) agar plate using a

Gilson pipette to form a stable column of cells. The plate was then incubated in a sealed box (to prevent evaporation) and incubated at 30°C for four days. After this time, swarming, if any, is readily apparent and degree of swarming can be easily and meaningfully compared between strains, knowing that the initial inoculum in each case was the same. This assay assumes growth rates of the strains to be the same.

2.3e

Swarm Plates for the Analysis of *E. coli che* Mutants

E. coli che W^- mutants (a gift from J.S.Parkinson, University of Utah) were transformed with an expression construct, pTMW26, which contained the *R. sphaeroides che* W gene inserted in frame in front of the IPTG-inducible Ptac promoter of pTM30 (Materials and Methods, 2.4h). These transformants were assayed using swarm plates in order to determine if *che* W complementation had occurred. A 5 µl sample of a stationary culture was pipetted onto the surface of an LB plate containing 0.3% agar, 25 µg/ml of ampicillin and 40 µg/ml of IPTG, dried at room temperature overnight. The plates were incubated at 30°C to allow swarming and photographs were taken at intervals over three days.

2.4

Genetic and Molecular Biology Techniques

2.4a

DNA Preparation, Purification, Electrophoresis and Restriction

Small scale plasmid preparations of *E. coli* were performed by the alkaline lysis method "mini-prep" method of Birnboim and Doly (Sambrook *et al*, 1989) . When particularly high quality plasmid DNA was required, for use in sequencing for instance, Promega "Magic miniprep" columns were used. Alkaline lysis followed by CsCl-ethidium bromide purification (Sambrook *et al*, 1989) was used for large scale plasmid preparations. Plasmid preparations of *R. sphaeroides* required two additional phenol extraction steps to remove photosynthetic membrane proteins. Chromosomal DNA preparations were done by the method of Maquat and Reznikoff (Sambrook *et al*, 1989).

All DNA manipulation was carried out using Gibco BRL or Promega enzymes and buffers according to manufacturers' instructions. Restriction fragments were separated by electrophoresis on 1xTBE or 1xTAE, 0.8% to 1.5% agarose gels (Sambrook *et al*, 1989) . When required for probe production or cloning, DNA was recovered and salts and proteins were removed using GeneClean® (Bio.101 Inc.).

2.4b

DNA Hybridization

DNA hybridization of chromosomal and cosmid restriction fragments was performed from a 19 cm agarose gel, run overnight at 40 volts. The DNA was blotted onto nylon membranes (Amersham International, Hybond-N) using the capillary method of Southern and using a Promega vacuum blotting apparatus (Sambrook *et al*, 1989) . DNA was chemically denatured, depurinated and neutralized prior to blotting (Southern, 1979). Hybridized DNA was fixed to the membrane by UV crosslinking on a transilluminator for 3 minutes (Maniatis *et al*, 1982).

2.4c

Chemical Mutagenesis

Chemical mutagenesis of heterotrophically grown, log phase *R. sphaeroides* was performed using 3% (v/v) ethyl methane sulphonate (EMS) with an exposure time of 30 minutes in growth medium in a 250 ml conical flask, with shaking. This was followed by three washes in 7 mM succinate medium and a five hour recovery period in 7 mM succinate medium. The cell suspension was then serially diluted and plated into 0.3% swarm agar containing 5 mM succinate and 25 µg/ml nalidixic acid and incubated at 30°C for five days in the dark.

2.4d

Tn5 and Tn10 Transposon Mutagenesis

Transposon mutagenesis using the transposon Tn5 (Duggleby *et al*, 1990) was performed upon the *R. sphaeroides* genome with the aim of producing behavioural mutants by interrupting genes whose (putative) products are central to chemotactic signal transduction or rotational switching. This mutagenesis was achieved by the conjugation of WS8-N with donor *E. coli* S17-1 containing the broad host-range, mobilizable suicide vector pSUP2021 (pBR325-*Mob*::Tn5) which contains Tn5 (Hunter, 1988). The resulting transconjugants were Nal^r by virtue of WS8-N and Kn^r due to Tn5. *E. coli* was selected against by both the presence of naladixic acid and the absence of proline, for which *E. coli* S17-1 is auxotrophic. Transconjugants were serially diluted into 0.3% agar swarm plates containing M22, 7mM succinate, 20 μg/ml naladixic acid and 25 μg/ml kanamycin. Reduced swarming colonies were picked and colony purified three times, then stabbed into soft agar to check for consistency of swarming characteristics. Controls were run to determine initial and final *R. sphaeroides* and *E. coli* cell concentrations; this information was used to calculate survival and mating ratios.

Transposon mutagenesis was also used for site-specific mutagenesis of *R. sphaeroides che* R and *che* W homologues cloned into pUC18. In these cases bacteriophage λNK1316 (Kleckner *et al*, 1991) was used to introduce mini-Tn10 into an *E. coli* strain carrying the appropriate construct (see below).

2.4e

Transduction with Bacteriophage λNK1316

Transductions were performed in order to produce mini-Tn10 insertions in cloned *R.sphaeroides* genes. A clone, pCH2 (fig.16) containing the *che* W and *che* R gene homologues was constructed using pUC18, and this construct was transformed into *E. coli* C3000, which is non-suppressing for amber mutations. Bacteriophage λNK1316 acts as a phage vehicle for a mini-transposon derivative (#103) of Tn10 (fig.17),which has been constructed to maximize randomness of insertion and to abolish excision and repeated transposition events by placing the transposase gene outside from the insertion sequences (Kleckner *et al*, 1991).

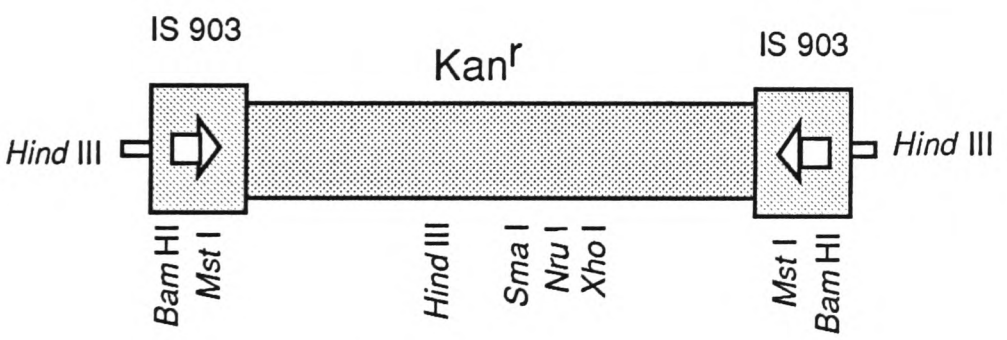


Fig.17: Schematic diagram of mini-transposon Tn10 (derivative 103), delivered from phage vehicle λNK1316 (Terminal *Bam* HI sites are not transposed).

λNK1316 possesses an amber nonsense mutation and can only replicate in an amber suppressor strain, so when it is transduced into *E. coli* C3000 the phage is excluded and the transposon is "forced" to insert into other DNA.

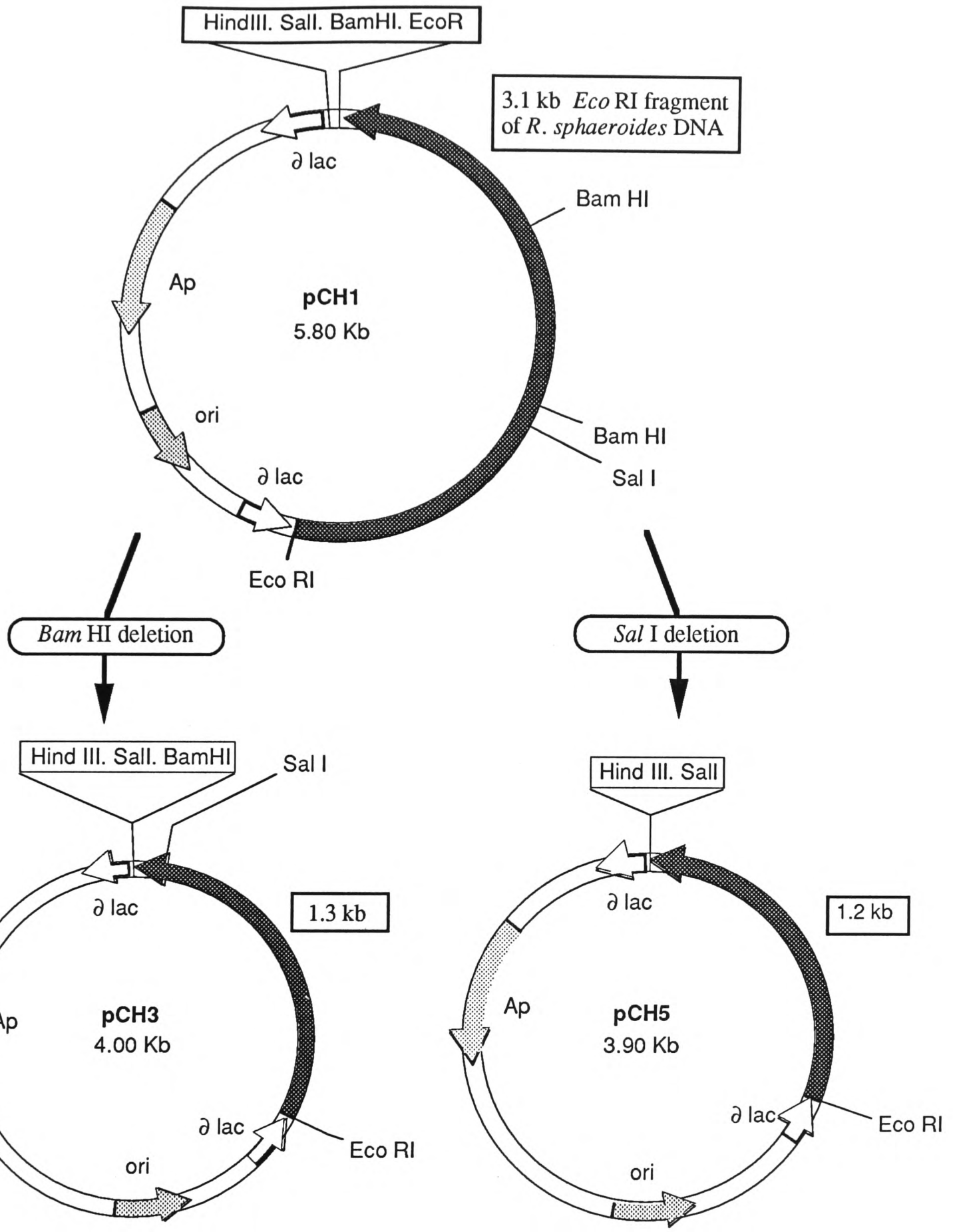


Fig.16a: Parent clone pCH1 which was constructed from a 3.1 kb *Eco* RI fragment of *R. sphaeroides* DNA cloned into pUC18. This insert was found to contain the *che* W, *che* R and *che* Y2 homologues as well as at least two other open reading frames with no similarity to any known genes. The sub-clones pCH3 and pCH5 were produced from pCH1 by making *Bam* HI and *Sal* I deletions of the insert. Arrows indicate gene orientation.

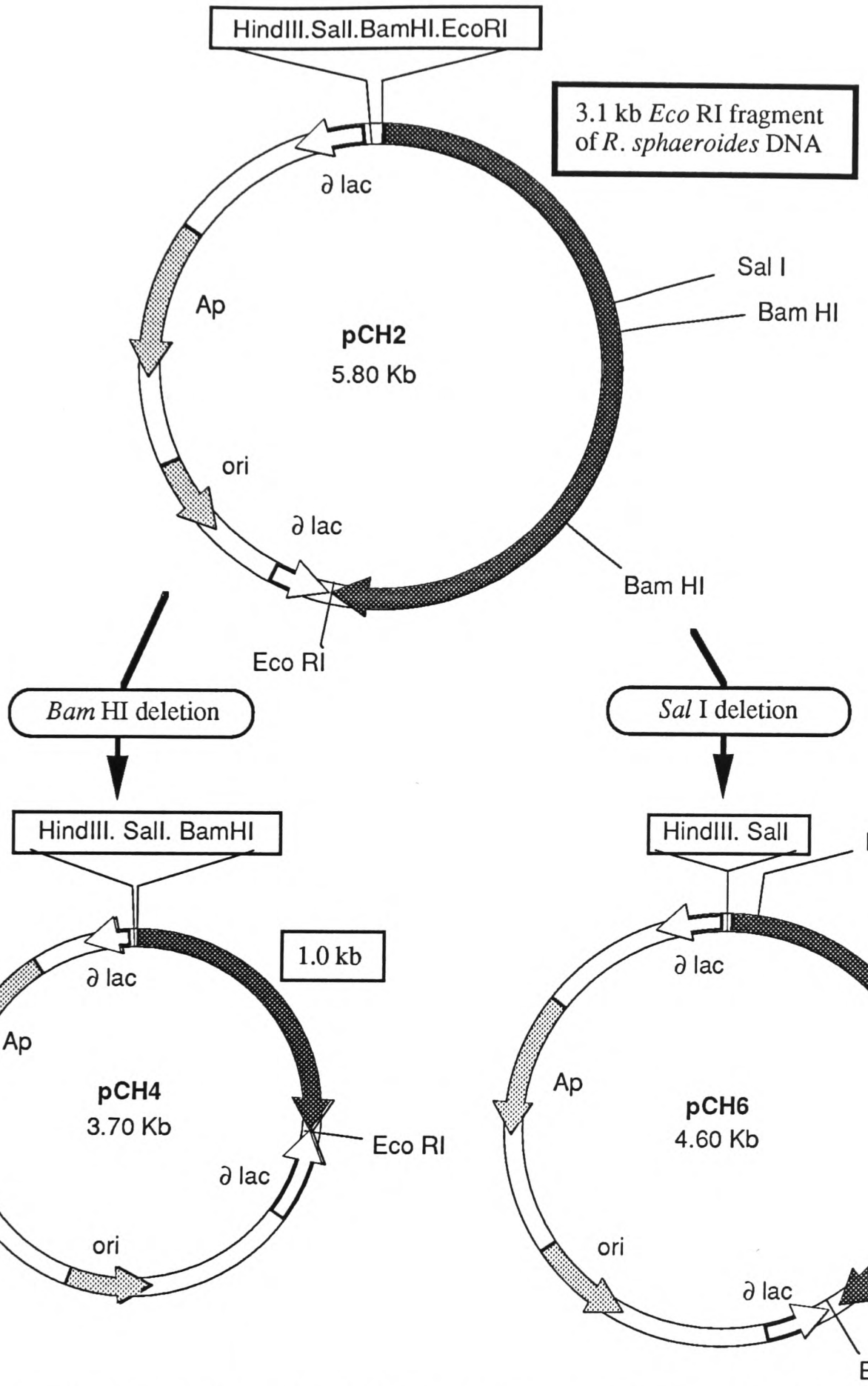


Fig.16b: Parent clone pCH2 which was constructed from a 3.1 kb *Eco* RI fragment of *R. sphaeroides* DNA cloned into pUC18. This insert was found to contain the *che* W, *che* R and *che* Y2 homologues as well as at least two other open reading frames with no similarity to any known genes. The sub-clones pCH4 and pCH6 were produced from pCH2 by making *Bam* HI and *Sal* I deletions of the insert. Arrows indicate gene orientation.

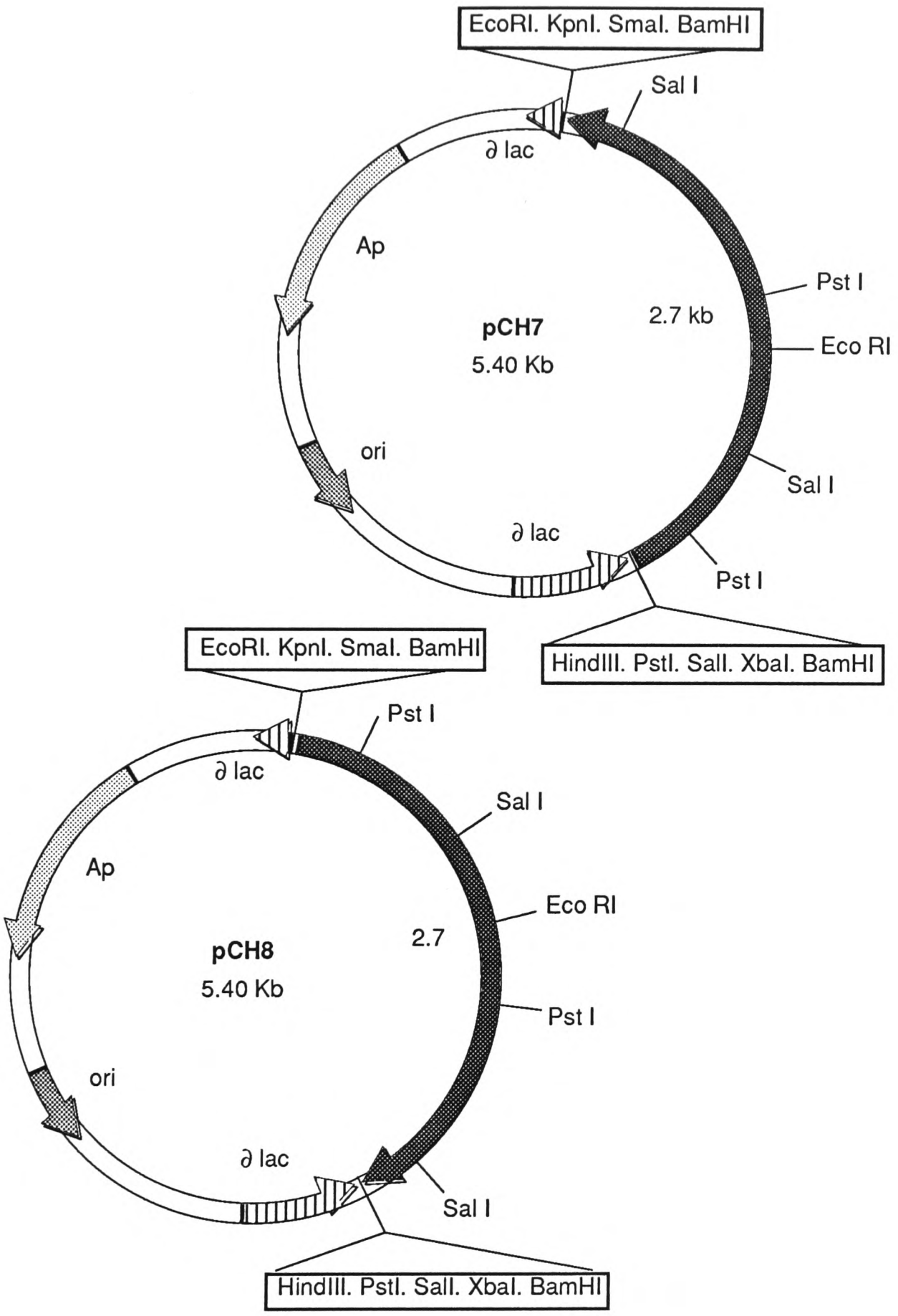


Fig.16c: clones pCH7 and pCH8. Constructed by cloning a 2.7 kb *Bam* HI fragment of *R. sphaeroides* DNA into to pUC 19. This insert was found to contain homologues for half of che A gene and the entire *che* W, *che* R and *che* Y2 genes. Arrows indicate gene orientations.

For a genome of approximately $3x10^6$ bp and a vector of 4200 bp the probability of insertion into the plasmid is 1:714 , assuming random transposition, and considerably less for targeting of the insert alone. Selection of strains in which mini-Tn10 has become inserted in the plasmid, rather than the chromosome, is accomplished by using a high concentration (300 μg/ml) of kanamycin in broth and solid media. The transduction protocol is as described below.

a. *E. coli* C3000 was transformed with the chosen pUC-based clone and cultured overnight at 37°C in LB containing 25 μg/ml ampicillin.

b. 1.0 ml of the overnight culture was pelleted and resuspended in 100 μl of LB without antibiotics. This suspension was infected with λNK1316 to give a multiplicity of infection of 0.1 (in this case the phage concentration was 10^9 plaque forming units per ml.) and the mixture was left for one hour at 37°C to allow infection and the expression of antibiotic resistance genes.

c. 100 μl of the infected culture was spread onto LB plates containing 25 μg/ml ampicillin and 300μg/ml (high concentration) kanamycin and incubated at 37°C overnight.

d. Colonies were picked, cultured, plasmid-prepped and restricted to determine the site of transposon insertion.

The products of this mutagenesis were then cloned out of the pUC vector into the suicide vector pJP5603 (fig.18) and transformed into

E. coli S17-1 to facilitate conjugation with *R. sphaeroides* WS8N. The aim of this conjugation was to produce homologous double crossover recombinants with the *che* R or *che* W homologue interrupted by a transposon insertion. The resultant phenotypes would elucidate the function of the gene product.

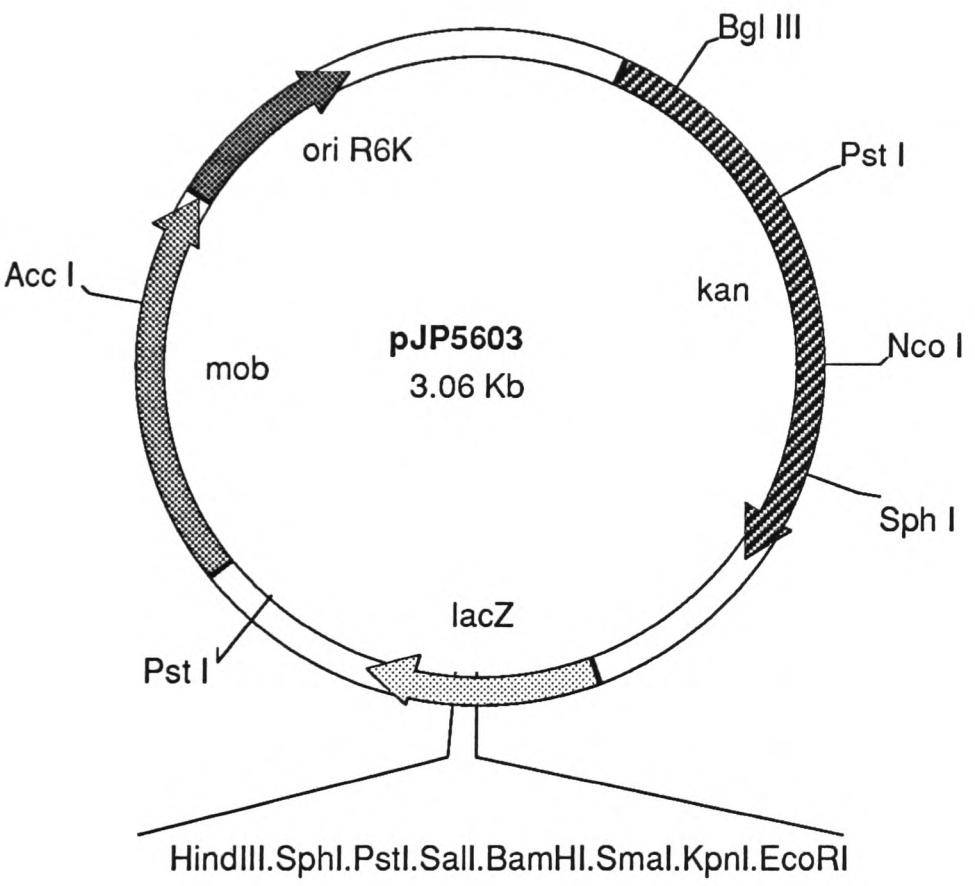


Fig 18: pJP5603, an R6K-based suicide vector requiring a trans supply of the *pir* encoded π protein. lacZ'/MCS. Kn^r (Penfold and Pemberton, 1992).

2.4f

Diparental Conjugation

The transfer of various mobilizable (*mob*$^+$) constructs was performed by diparental conjugation between *R. sphaeroides* and the mobilizing (*tra*$^+$) donor strain *E. coli* S17-1 (Simon *et al*, 1983) as detailed below:

a. A single colony of *R. sphaeroides* was inoculated into 30 ml. of succinate medium containing appropriate antibiotics and incubated at 30°C overnight with vigorous shaking to inhibit the induction of photosynthetic pigments. A single colony of *E. coli* S17-1 transformed with the construct was inoculated into 60 ml. of LB containing appropriate antibiotics and incubated at 37°C overnight.

b. 0.5 ml. of the *E. coli* culture was inoculated into 100 ml. of fresh medium and allowed to grow to mid-log phase (6 hrs.). 1 ml. of mid-log phase *E. coli* culture was gently mixed in an Eppendorf tube with 1.0 ml of *R. sphaeroides* and spun for 2 mins. at 3000g. The pellet was gently resuspended in 100 µl of M22 medium and spotted onto a Millipore nitrocellulose 0.2 µm pore-size filter on a dry succinate agar plate and left to conjugate overnight at 30°C in the dark.

c. Transconjugants were washed off the filter into 1 ml. of M22 medium and dilution plated into 0.3% swarm agar containing appropriate antibiotics and a carbon source but lacking proline (for which *E. coli* S17-1 is auxotrophic). Colonies become visible within three days.

2.4g

Homologous Recombination

Gene recombination was achieved by cloning *R. sphaeroides* genes into a suicide vector and then conjugating that construct into wild-type *R. sphaeroides* WS8N. Two strategies were used to produce the suicide clones. One strategy used a transposon-interrupted copy of the target gene as an insert. When recombined into the genome via a double crossover, the transposon-interrupted copy would displace the native gene, preventing functional protein expression. The second strategy employed an internal fragment of the target gene as the clone insert. When recombined via a single crossover event, the entire construct would be inserted, preventing normal protein expression.

Two suicide vectors were used for each approach, pSUP202 (fig.19) and pJP5603 (fig. 18). For the experiment in which pJP5603 was used to delete Che W function, the 4.9 kb *Eco* RI fragment from pCH2T21 (or pCH2T15), which contained the transposon-interrupted *R. sphaeroides che* W gene (Materials and Methods, 2.4d and 2.4e), was cloned into the polycloning site of pJP5603 (Results, 3.8.4 and 3.8.4b). The resultant 8.0 kb, kanamycin resistant suicide constructs, pJPW21 and pJPW15, were transformed into permissive strains of *E. coli*. The *pir*-encoded π protein is required to maintain the suicide vector and is supplied in *trans* by the strain in which it is maintained. The permissive strains used were *E. coli* JM109 λ *pir,* and the

specially modified, mobilizing strain, *E. coli* S17-1 λ *pir* (Penfold and Pemberton, 1992). Diparental conjugation was performed between *E. coli* S17-1 λ *pir* containing the suicide construct and *R. sphaeroides* WS8N (Materials and Methods, 2.4f). Resultant transconjugants were dilution plated into 0.3% swarm agar containing 25 µg/ml kanamycin, 20 µg/ml naladixic acid and a 7 mM carbon source. Resultant colonies were examined for phenotype by swarm assays, computerised motion analysis and by flagellar staining (Materials and Methods, 2.3b, 2.3a and 2.2b).

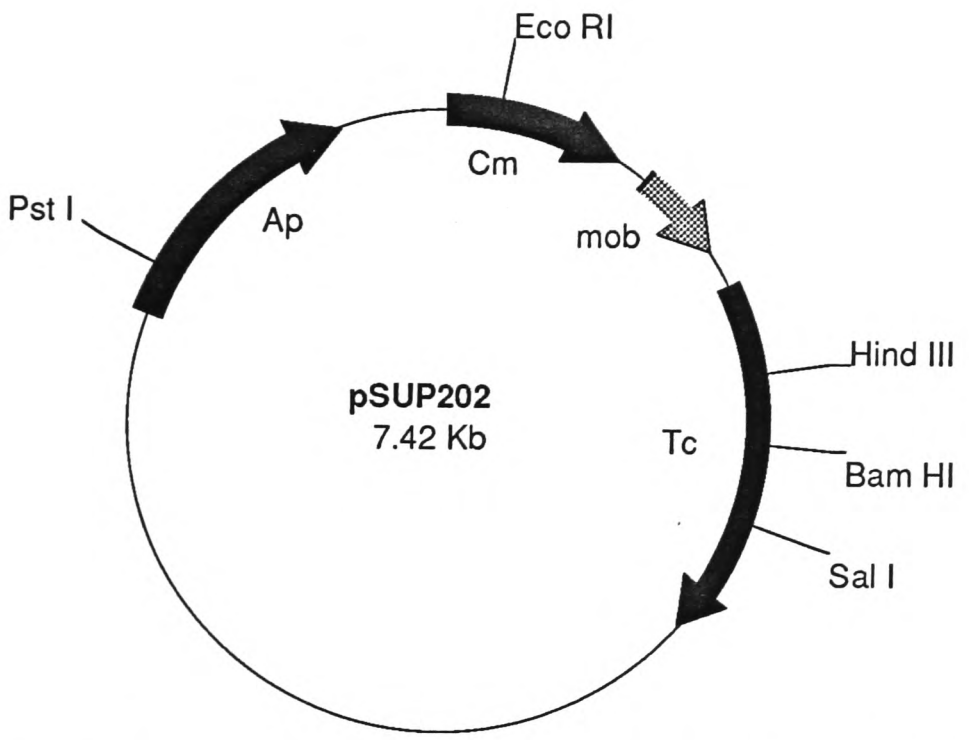


Fig.19: pSUP202, a pBR325-derived mobilizable suicide vector Km^r Ap^r Cm^r (Simon *et al*, 1983).

The method used to produce the pJP5603-derived *che* R suicide clone (pJP∂R) was similar to that used above, except that the insert used was a 694 bp *Nru* I internal fragment of the *R. sphaeroides che* R gene. (Results, 3.8.4 and 3.8.4a). The reason for the difference in strategies was the unexpected specificity of transposon insertion ("hotspotting"),

which meant that no transposon-interrupted clones of *che* R were produced (see Results, 3.8.2a). Since there was no transposon marker available in this experiment, the suicide constructs were transformed into *E. coli* JM109 λ *pir* and screened by α-complementation on 2% LB agar plates containing 40 μg/ml isopropyl-ß-thiogalactoside (IPTG) and 30 μg/ml 5-bromo-4-chloro-3-indole-ß-D-galactoside (X-gal). Suicide clones were transformed into *E. coli* S17-1 λ *pir* and then conjugated into *R. sphaeroides* WS8N. Transconjugants in which a single crossover had occurred would be kanamycin resistant, by virtue of the integrated construct. These transconjugants would be screened for phenotype by dilution plating into 0.3% swarm agar containing 25 μg/ml kanamycin, 20 μg/ml naladixic acid and a 7 mM carbon source.

The procedure for experiments whose aim was deletion of Che Y2 function was the same as that used with *che* R, except that A 221 bp internal *Kpn* I / *Sal* I fragment of the *R. sphaeroides che* Y2 gene was cloned into the polycloning site of the pJP5603 (Results, 3.8.5a).

The narrow host range, 7.5 kb replicon, pUSP202 (Simon *et al*, 1983a and 1983b) was also used to construct suicide clones with the aim of deleting the gene function of Che W and of Che R in *R. sphaeroides*. The vector pSUP202 is based on pBR325, and cannot survive outside *E. coli* (or closely related organisms), but because of the RP4-derived broad host range transfer functions, it can be conjugated into a wide range of gram negative bacteria, including *R. sphaeroides*. Suicide

constructs made using pSUP202 employed the same inserts as used with pJP5603 (above). The Tn10-interrupted *R. sphaeroides che* W gene was cloned into pSPU202 at the *Sal* I site to produce the 10.5 kb plasmid pSUPW (fig.20). The 694 bp internal fragment of *R. sphaeroides che* R was cloned into pSPU202 at the *Sal* I site to produce the 8.2 kb plasmid pSUPR (fig.21). The suicide clones produced were tetracycline sensitive, but ampicillin and chloramphenicol resistant (Results, 3.8.2a and 3.8.3a). The resultant suicide constructs were transformed into competent *E. coli* S17-1 and then conjugated into *R. sphaeroides* WS8N (Materials and Methods, 2.4f). The transconjugant recombinants (WS8NXW and WS8NXR) were dilution plated into 0.3% swarm agar containing appropriate antibiotics: WS8NXW was screened for 25 μg/ml kanamycin 1 μg/ml tetracycline sensitivity (Results 3.8.2a); WS8NXR was screened for 25 μg/ml chloramphenicol resistance (Results 3.8.3a).

2.4h

Complementation of the *E. coli che* W Mutant RP4606

The *R. sphaeroides che* W gene was cloned into the expression vector pTM30 , downstream of the IPTG-inducible P*tac* promoter . This construct, "PTMW26" (fig.57, Results) was then transformed into a *che* W^- mutant of *E. coli*, RP4606, which has a non-swarming phenotype on LB agar swarm plates. The transformants were assayed for swarming activity on 0.3% agar swarm plates containing 40 μg/ml IPTG (section 2.3e).

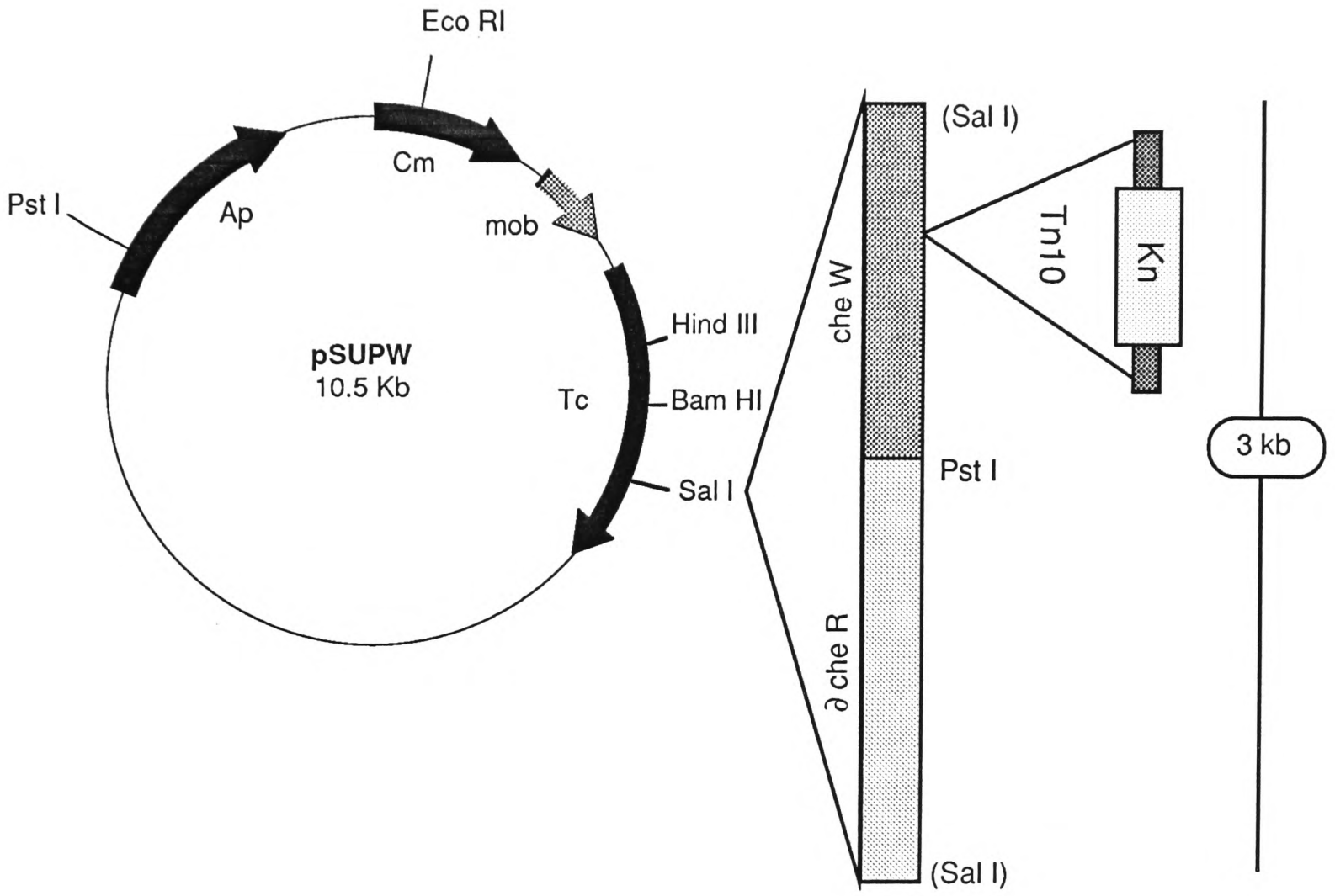


Fig.20: pSUPW: A mobilizable suicide clone based on pSUP202, containing the *Sal* I fragment from pCH2T12 which carries the Tn 10 interrupted *R. sphaeroides che* W gene.

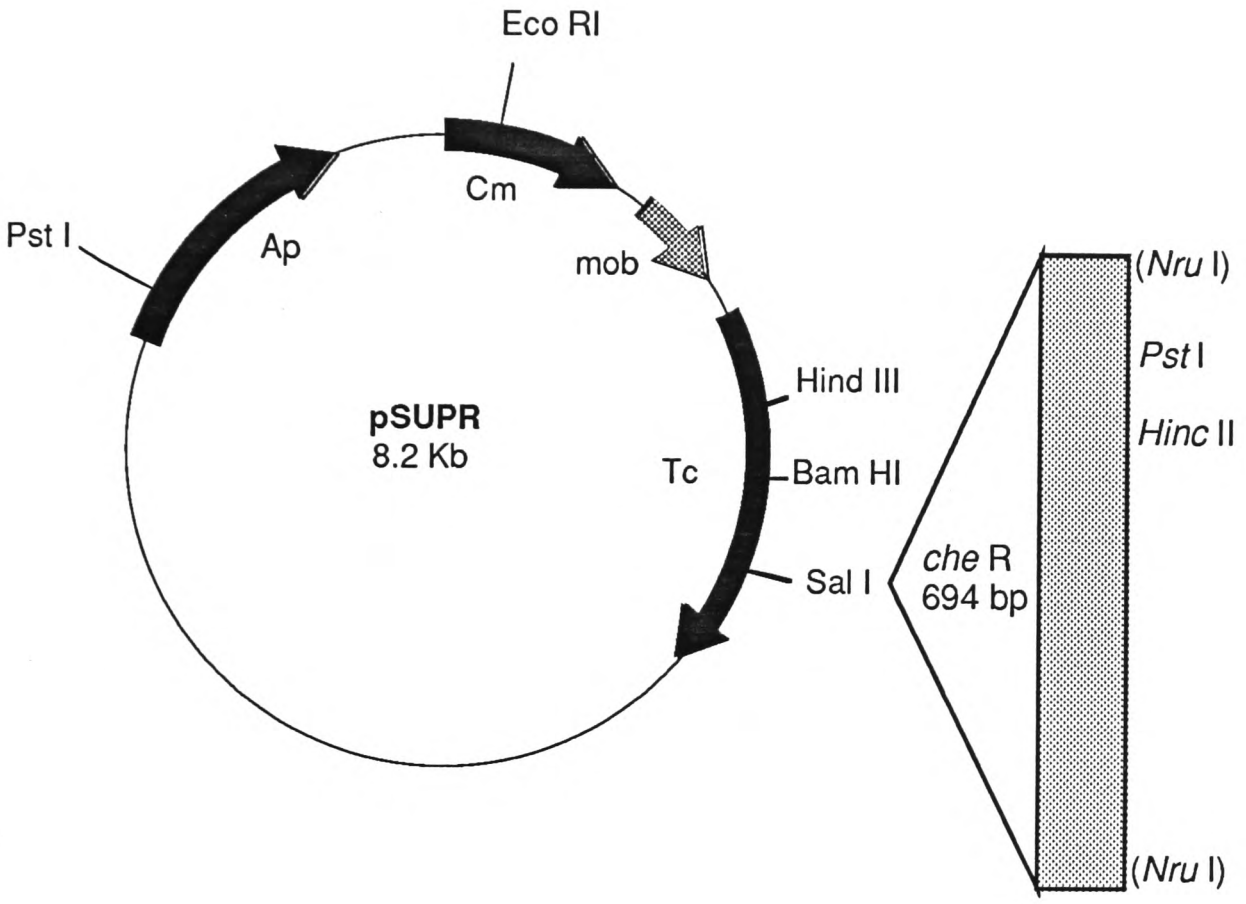


Fig.21: The 8.2 kb suicide clone pSUPR constructed by ligating the 694 bp *Nru* I fragment of *R. sphaeroides che* R into the blunted *Sal* I site of pSUP202. The insert interrupts the tetracycline resistance gene and this feature was used for sensitivity screening of the clones.

The restriction site used in the vector was *Kpn* I (to obtain the correct reading frame) which was blunted using the 3' exonuclease activity of T4 polymerase. The insert *(R. sphaeroides che* W) was prepared by PCR, phosphorylated using T4 kinase and purified using GeneClean. The forward primer for the PCR reaction was:.5'-ATG GCG GCC GTG AGC CTG-3' and the reverse primer was: 5'-GCA GTC GGG GGT TGT CGC-3'. The cosmid c292, diluted 1:100 in water, was used as a template. The PCR reaction mixture below was determined to be optimal for the template and primers used:

66 µl distilled water
10 µl DIG-dNTP's (2.5 mM of dATP, dCTP, dGTP and dTTP)
6 µl forward primer
6 µl reverse primer
10 µl NEB buffer
1µl c292 template
1 µl *Thermococcus litoralis* (exo$^-$) DNA polymerase.

The following PCR cycle was used:

a. denature at 95°C for 60 s
b. anneal at 60° C for 60 s
c. extend at 75°C for 45 s
d. repeat steps "a" through "d" 30 times.
e. 75°C 7 min. then stop

The ligation mixture contained: 0.5 µl (c. 20 ng) of pTM30 (*Kpn* I cut and blunted), 1.5 µl of insert (c. 40 ng), 10 µl of water, 1.5 µl of T4 buffer and 1 µl of T4 DNA polymerase. Ligation was carried out at

4°C overnight and the products of this were transformed into *E. coli* DH5α. Colonies were screened by restriction to determine presence and orientation of the insert .

The resultant clone, pTMW26, was isolated and transformed into the non-swarming mutant *E. coli* RP4606. Chemotactic screening of the successfully complemented strain was carried out on LB agar swarm plates containing 40 μg/ml IPTG (see section 2.3e).

2.4i

Translational Fusion Experiments

Translational (*lac* Z) fusion experiments were done in an attempt to identify a promoter region thought to exist in the previously identified ORFW region of *R. sphaeroides* Q12. The broad host-range, mobilizable vector pUI523A was used (Tai *et al.*, 1988). pUI523A was grown in the dam$^-$ dcm$^-$ *E. coli* strain GM48 to allow restriction by *Sma* I and *Stu* I. The insert, a 1.3 kb fragment of *R. sphaeroides* Q12 DNA,was produced by restricting pWH8 with *Eco* RI and *Hin*d III, separating the 1.3 kb fragment by electrophoresis, recovering it by using GeneClean®, then blunting the 5' overhanging ends by using the Klenow fragment. pUI523A was restricted with the blunt-cutting enzymes *Dra* I, *Sma* I and *Stu* I to provide insertions in all three reading frames. Ligations were carried out using T4 DNA ligase at 16°C overnight.

The constructs "pUI-ORFW 1, 2 and 3" was transformed into *E. coli* S17-1 which had previously been made competent by the $CaCl_2$/heat shock method (Davies *et al*, 1980). pUI-ORFW was then conjugated from *E. coli* S17-1 into *R. sphaeroides* WS8N (Materials and Methods, 2.4f). Transconjugant colonies were screened for ß-galactosidase production on agar plates containing 30 µg/ml 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal) 15 µg/ml tetracycline, 25 µg/ml naladixic acid and carbon sources of varying concentrations (propionate, succinate or acetate, 5 mM, 10mM, 15mM and 20mM).

2.4j

Enrichment of Behavioural Mutants

A tube enrichment technique (fig.23) was developed in an attempt to enrich mutagenised populations for smooth swimming mutants (the functional equivalent of *che*$^-$ mutants in *E. coli*). This method involved placing an inoculum of mutagenised bacteria at the interface between a layer of chemoattractant and a layer of minimal medium,

The methodological premise behind the design of enrichment tubes was that while normally chemotactic cells would accumulate in a region of optimum chemoattractant concentration, smooth swimmers would become become oriented "head downwards", due to the greater density of the body compared with the tail, and would accumulate near the bottom of the tube regardless of local attractant concentration. Non-motile mutants, behavioural equivalents of enteric *Mot*$^-$ and *Fla*$^-$

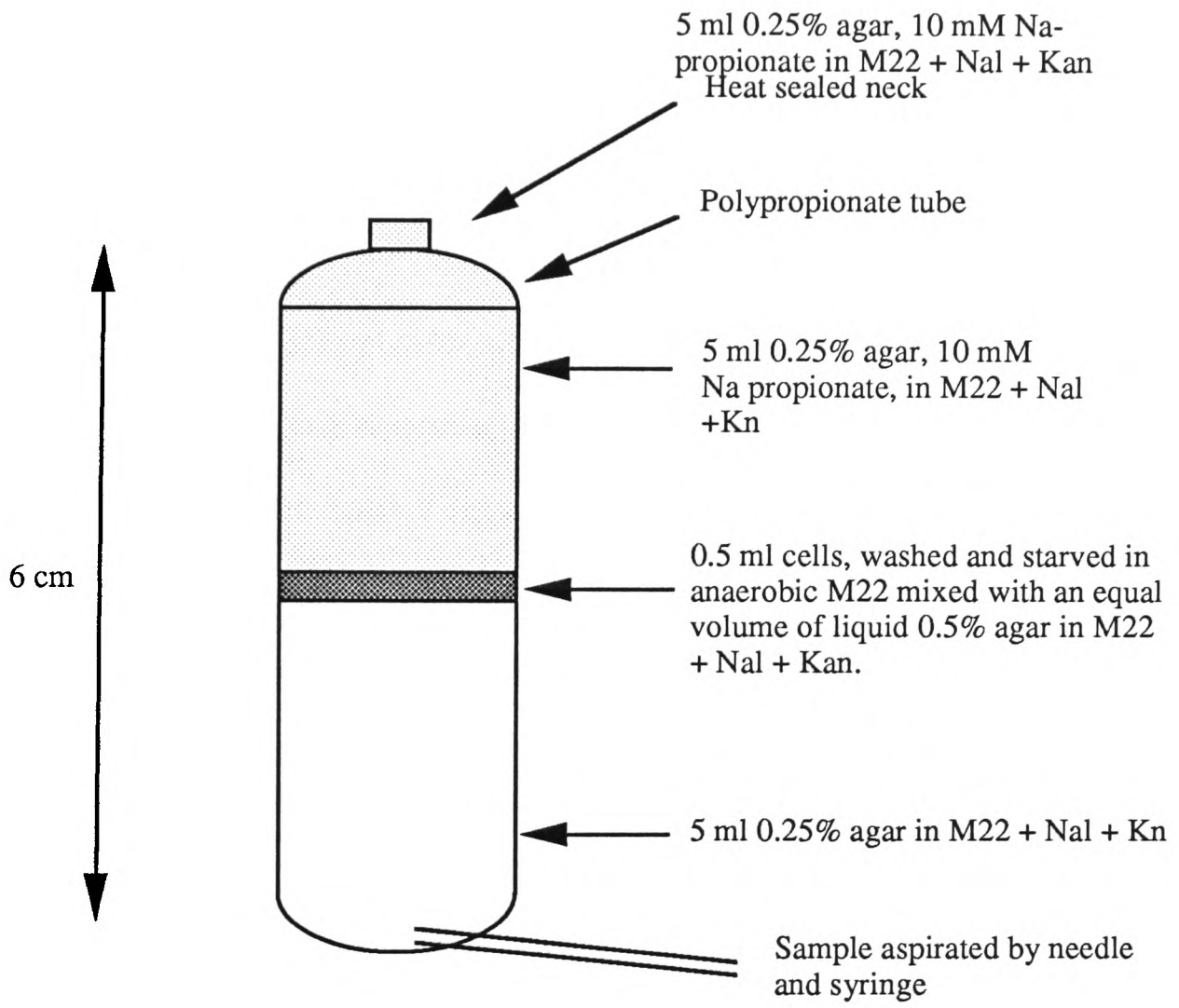


Fig.23 : Chemotaxis mutant enrichment tube. Incubation from 1 hr to 12 hrs in even illumination (c.100 microeinsteins) at 28°C. Samples aspirated by syringe.

mutants, would remain at the inoculation site. Sampling from the bottom of the tube after population redistribution from the inoculation point should therefore provide a sub-population likely to be rich in smooth swimmers. Assuming a net rate of movement of 10 μm/second (3.6 cm/hr), a bacterium should take slightly less than an hour to reach the bottom of the tube.

The tube used was a 10 ml polypropionate ultracentrifuge tube. The three layers were placed sequentially into the tube using a syringe with a wide bore needle. The lower layer contained no attractant and consisted of 0.25% agar in M22 with 20 μg/ml naladixic acid and 20 μg/ml kanamycin. The middle layer consisted of a 0.5 ml inoculum of cells mutagenised by recombination or by chemical mutagenesis. If recombination had been used, the cells were vortexed directly from the filter into 2.5 ml of anaerobic M22, which was mixed with and equal volume of liquid 0.5% agar in M22. 0.5 ml of this solution was then layered into the tube (the inoculum also contained 20 μg/ml naladixic acid and 20 μg/ml kanamycin). If chemical mutagenesis had been used, 50 ml of cells was washed three times in 7 mM succinate, resuspended in 10 ml 7 mM succinate. This suspension was mixed with an equal volume of liquid 0.5% agar in M22. 0.5 ml of this solution was then layered into the tube (with antibiotics, as above). The upper layer contained the attractant, 10 mM sodium propionate in M22 with 0.25% agarose and 20 μg/ml naladixic acid and 20 μg/ml kanamycin. Layering had to be done very carefully to prevent mixing of the layers. The tube was heat sealed

and incubated with even illumination of about 100 microeinsteins. Various incubation times were used from one to 12 hours, with longer incubation times producing more distinct layers (Results, fig.39).

Following incubation, cells were drawn from individual layers by syringe and resuspended in a small volume of 7mM succinate medium. These cell suspensions were subjected to computerised motion analysis and seeded into swarm agar plates to check for phenotypic variation.

2.4k

Production of Probes for Hybridization Experiments

Non-radioactive DNA probes were made using random hexanucleotide-primed incorporation of digoxigenin-labeled deoxyuridine triphosphate (DIG-dUTP [from Boehringer Mannheim]) into a growing polynucloetide strand catalyzed by the large fragment of DNA polymerase I. The probe templates (see below) consisted of purified DNA fragments to which homology was desired. In every case the labeling reaction proceeded for at least 16 hours at 37°C, and the reaction mixture was as follows:

500 ng to 1000 ng DNA template (denatured by boiling for 10 mins.)

5 µl sterile distilled water

2 µl dNTP mixture containing 0.35 mM DIG-dUTP, 0.65 mM dTTP and 1.0 mM for dATP, dCTP and dGTP

2 µl hexanucleotide mixture (10x)

1 µl Klenow enzyme.

The polymerase chain reaction (PCR) was also used to produce DIG labeled probes when suitable oligonucleotide primers were available (see for individual probes below).

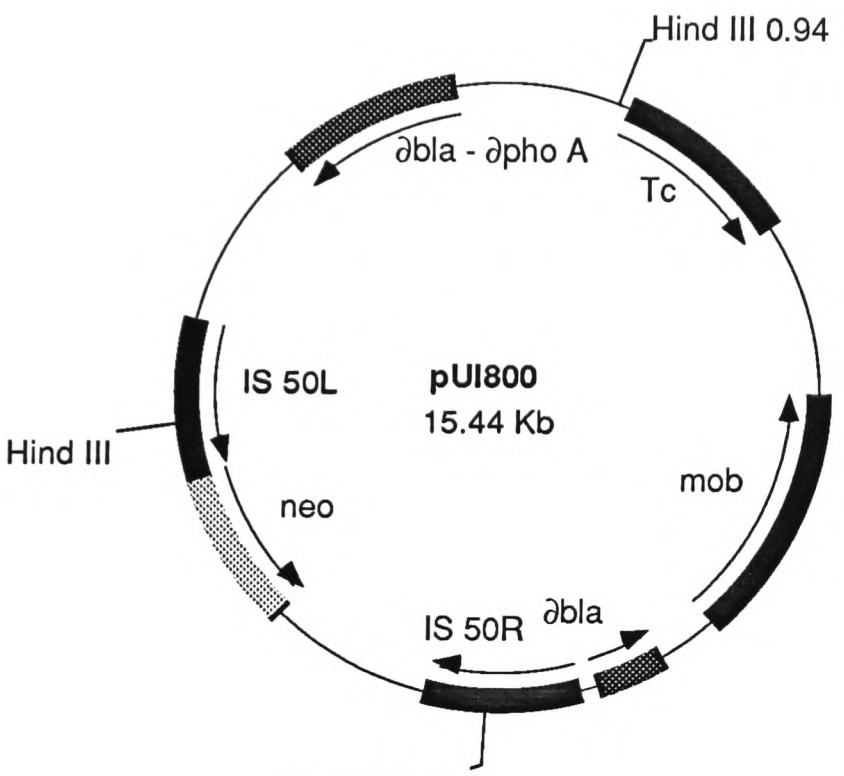


Fig.24: pUI800, a derivative of pSUP203 bla::Tn*pho*A, Tc^r Cm^r Km^r (Moore and Kaplan, 1989). The 3.2 kb *Hind* III fragment was used to produce a probe for hybridization experiments the aim of which was to locate the Tn*pho*A insertion in the mutant Q12.

2.4k (i)

<u>Probes Used in the Investigation of the Mutant Q12</u>

DIG labeled probes made for hybridization experiments involving mutant Q12 chromosomal digests were made using a 3.2 kb *Hin*d III fragment of pUI800 (fig.24) and a 6 kb *Eco* RI fragment of pEKn12-18 as templates. Additionally, PCR was used to produce 200 bp digoxigenin-labeled probes using as a template an internal Tn5

sequence within the plasmid pMH1701. The primers, derived from IS50 sequences flanking the transposon were a gift from M.J.Ward. Forward primer sequence was: 5'-TTA TAC ACA CAA GTA GCG TCC T-3'. The reverse primer sequence was: 5'-AGA ATA TTT TGC CAA TTG GG-3'. The reaction mixture below was determined to be optimal for the template and primers used:

54.5 µl distilled water
6 µl $MgCL_2$ (50 mM)
16 µl DIG-dNTP's (2.5 mM of dATP, dCTP and dGTP; 1.25 mM dTTP and of DIG-dUTP)
6 µl forward primer
6 µl reverse primer
10 µl NEB buffer
1µl pMH1701 diluted 1:100 in water (template)
0.5 µl *Thermococcus litoralis* (exo^-) DNA polymerase.

The following PCR cycle was used:

a. denature at 95°C for 60 s
b. anneal at 44° C for 60 s
c. extend at 69°C for 20 s
d. repeat steps "a" through "d" 30 times.
e. 69°C 7 min. then stop

2.4.k (ii)

Probes Used to Investigate Homology with *R. meliloti* Genes

DIG labeled probes were made for hybridization studies with the aim of determining if any homology existed between the *mcp* and *che*

homologues of *Rhizobium meliloti* (R. Schmitt, Regensburg, personal communication) and any genes in *R. sphaeroides*. The probes were made using *R. meliloti, Eco* RI fragments as probe templates (fig.25). The clone pE2.7 contained a fragment of *mcp*-like sequence; the clone pE2.3 contained a partial *mcp*-like fragment, a second, complete *mcp*-like fragment, a *che* Y homologue and part of a *che* A homologue; the clone pE1.5 contains most of a *che* W homologue, and all of the *che* R and *che* B homologues.

2.4.k (iii)

Probes Made for Identification of Tn10 Insertion Site

DIG labeled probes were made to identify the insertion site of the mini-Tn10 transposon in recombinant mutants of *R. sphaeroides*. The template used to make these probes was the 1.6 kb *Bam* HI fragment from mini-Tn10 containing the kanamycin resistance gene (fig.17)

2.4k (iv)

Probes Made for Identification of *che* W Location.

492 bp DIG labeled probes made for hybridization experiments involving the localization of the *che* W gene in homologous recombinant mutants of *R. sphaeroides* were made using PCR. The primers used flanked the *R. sphaeroides che* W sequence with the forward primer sequence being: 5'-ATG GCG GCC GTG AGC CTG-3' and the reverse primer sequence being: 5'-GCA GTC GGG GGT TGT CGC-3'. The template used was c292. The reaction mixture below

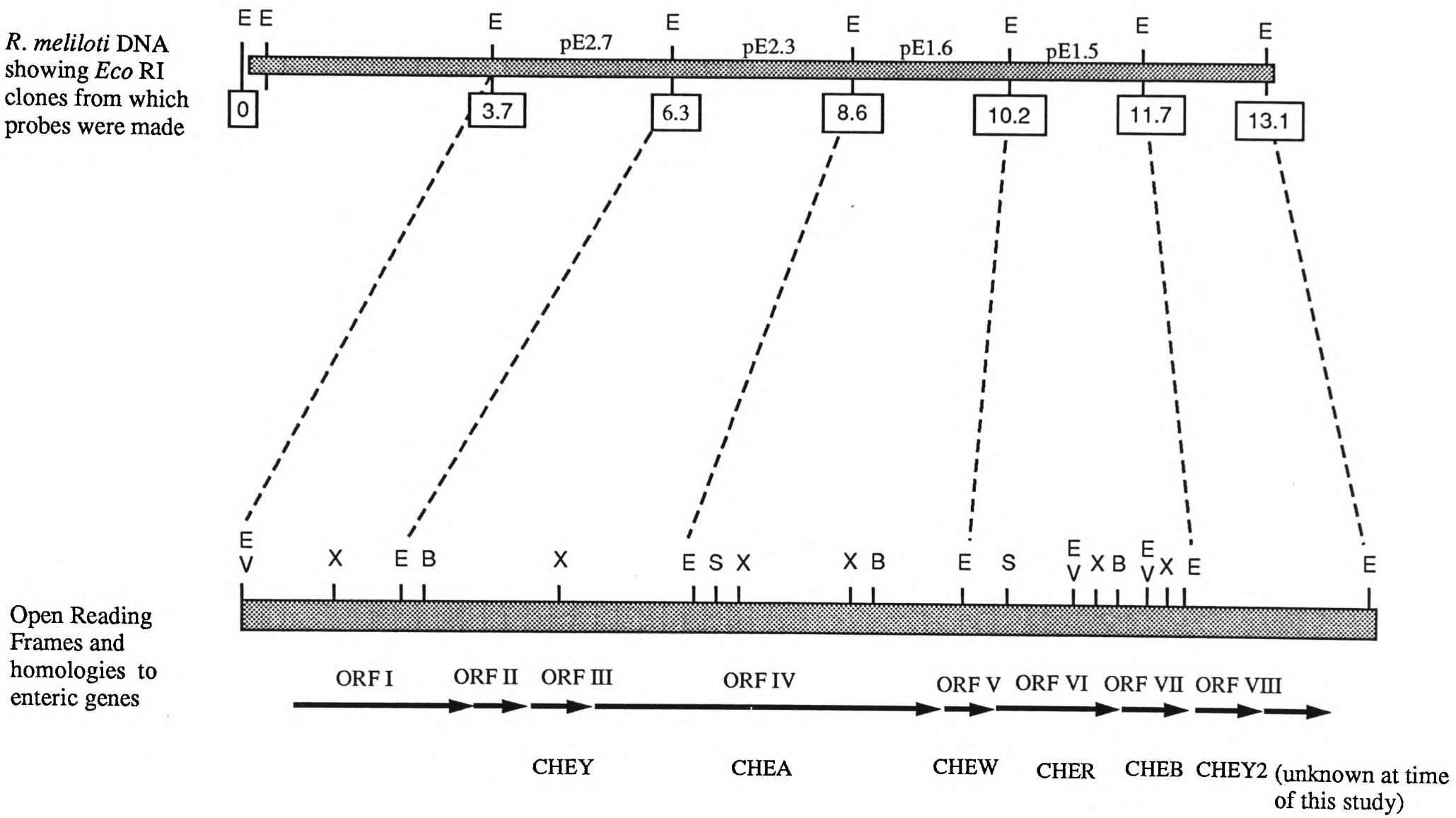


fig.25: MCP and *che* gene homologies of *Rhizobium meliloti* as determined by R. Schmitt (personal communication). Plasmids cloned from this sequence contain the following homologies and were used for the production of the digoxigenin-labeled probes used in hybridization studies with R. sphaeroides cosmid digests. pE1.5 contains Δ*che* W, che R and *che* B homologies. pE1.6 contains Δ*che* A and Δ*che*W homologies. pE2.3 contains ΔMCP ORF1, MCP ORF2, *che* Y and Δ*che* A homologies. pE2.7 contains a ΔMCP ORF1 homologue. E = Eco RI; V = Eco RV; X = Xho I; S = Sal I.

was determined to be optimal for the template and primers used:

66 µl distilled water
10 µl DIG-dNTP's (2.5 mM of dATP, dCTP and dGTP; 1.25 mM dTTP and of DIG-dUTP)
6 µl forward primer
6 µl reverse primer
10 µl NEB buffer
1µl c292 template
1 µl *Thermococcus litoralis* (exo$^-$) DNA polymerase.

The following PCR cycle was used:

a. denature at 95°C for 60 s
b. anneal at 60° C for 60 s
c. extend at 75°C for 45 s
d. repeat steps "a" through "d" 30 times.
e. 75°C for 7 min.
f. stop

2.4k (v)

Probes Made for Identification of *che* R Location

A 1332 bp DIG labeled probe, made by PCR, was to localize the *che* R gene in homologous recombinant mutants of *R. sphaeroides*. The primers used flanked the *R. sphaeroides che* R sequence with the forward primer sequence (Zeta) being: 5'-GTG AAA TAC TGG TCG CGA-3' and the reverse primer sequence (Lambda) being: 5'-CTG TCG AGC TGC ACC GCC-3'. The template used was c292. The reaction mixture below was determined to be optimal for the template and primers used:

66 µl distilled water

10 µl DIG-dNTP's (2.5 mM of dATP, dCTP and dGTP; 1.25 mM dTTP and of DIG-dUTP)

6 µl forward primer

6 µl reverse primer

10 µl NEB buffer

1µl c292 template

1 µl *Thermococcus litoralis* (exo$^-$) DNA polymerase.

The following PCR cycle was used:

a. denature at 95°C for 60 s

b. anneal at 58° C for 60 s

c. extend at 75°C for 100 s

d. repeat steps "a" through "d" 30 times.

e. 75°C 7 min. then stop

2.4k (vi)

Probes Used for Investigation of Homology with the *fli* genes of *Salmonella typhimurium*

Clones of the *fli* genes of *S. typhimurium* (a gift from R. Macnab at Yale) were used to produce DIG labeled probes. pAMH3 is a pBR322 based clone containing a 7.0 kb *Hind* III insert coding for the *S. typhimurium* genes *fli* F, *fli* G, *fli* H, *fli* I and *fli* J. pAMH6 is a pBR322 based clone containing a 3.2 kb insert coding for the *S. typhimurium* genes *fli* M, *fli* N and *fli* O. The 7.0 kb and 3.2 kb *Hind* III fragments were used as templates to produce DIG labeled probes (as above) which were used to probe *Bam* HI chromosomal

digests of *R. sphaeroides* WS8N.

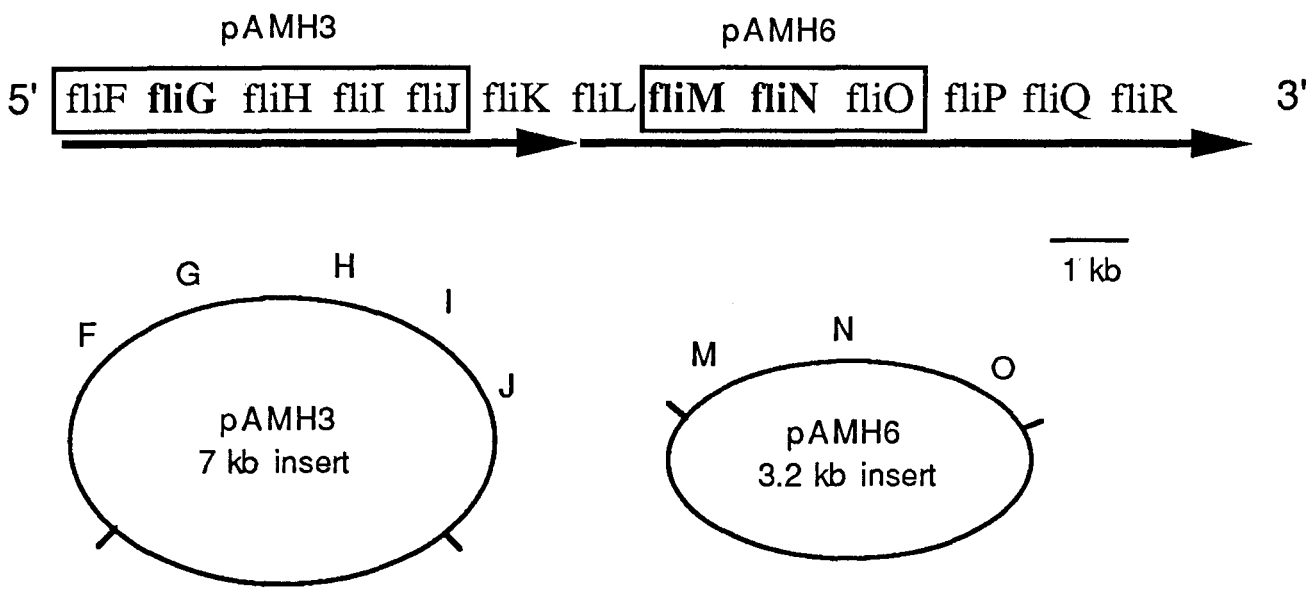


fig.26: Arrangement of the *fli* genes on the *S. typhimurium* chromosome and schematic constructs. Operon polarity is indicated by the arrows. The switch protein genes are shown in bold type. The regions contained in the clones pAMH3 and pAMH6 are boxed.

2.5

DNA Sequence Determination

Double stranded DNA was sequenced using two variations of the Sanger chain termination method (Sanger *et al*, 1977). All initial sequencing of both the 5' - 3' strand and of the 3' - 5' strand was done using the United States Biochemical Sequenase® kit, versions 1.0 and 2.0 (Tabour *et al*, 1987). The DNA dependent DNA polymerase used was a derivative of the bacteriophage T7 enzyme which possesses low 3' - 5' exonuclease activity. Due to the high GC content of *R. sphaeroides* DNA, strong symmetrical dyad structures easily develop which are not fully denatured during electrophoresis, this produces "compressions". To counter this effect DNA nucleotide analogues

(inosine and deaza purine nucleotide triphosphates) were substituted for native nucleotides during some of the sequencing reactions. These nucleotide analogues do not participate in inter-base hydrogen bonding to the same extent as the native nucleotides and therefore do not form the same secondary structures.

Thermal cycle sequencing ("PCR sequencing") methods were also used to relieve compressions. The action of repeated heating during cycling disrupts the formation of secondary structures and relieves compressions. For this method, New England Biolabs (Circumvent®) reagents were used. The exonuclease deficient DNA polymerase of *Thermococcus litoralis* used here is thermostable under the high temperatures employed during thermocycling, important with GC rich DNA for the denaturation of recalcitrant secondary structures (Murray *et al*, 1989).

The reaction mixture below was determined to be optimal for thermal cycle sequencing using the sequencing template clone pCH2.

7 µl template DNA
2 µl sterile distilled water
1.5 µl 30X triton surfactant
1.5 µl NEB 10X buffer
1 µl ^{35}S dATP (1 µCi/µl)
1 µl *Thermococcus litoralis* (exo$^-$) DNA polymerase.
3.2 µl of the above mixture was mixed with 3.0 µl of termination mixes and covered with 7 µl of mineral oil to prevent refluxing during

thermocycling. The sequence reaction was carried out in a peltier thermocycler using the cycle conditions below.

a. denature at 95°C for 60 s
b. anneal at 50° C for 60 s
c. extend at 70°C for 80 s
d. repeat steps "a" through "d" 20 times then stop.

After the addition of a stop solution and a loading dye, the samples were ready to be loaded. A 40 cm long, 6% polyacrylamide gel was used and run at 50 Watts, at approximately 1 KV for periods of 2.5 hours and 5 hours. Sequencing gels were fixed using two 15 minute immersions in 2 litres of 10% acetic acid, 12% methanol and autoradiographs were then produced using Kodak X-ray film.

Template DNA for the Sequenase® sequencing reactions was provided by the sequencing constructs pCH2, pCH3, pCH5 and pCH6 (fig.16a and 16b). The template used for the PCR sequencing reactions was the pCH2 clone only. In each case the template DNA had been prepared by the use of a single Promega Magic Miniprep® resin plasmid purification column and redissolved in a final volume of 50 µl of water. Universal forward and reverse primers of pUC18 were used for initial sequencing. Subsequent primers (12mers to 18mers) were produced by Valerie Cooper in the Dyson Perrins Laboratory, Oxford. Oligonucleotides were deprotected at 55°C overnight, precipitated with ethanol at -20°C and diluted in water to provide an Abs_{260} of 0.5, corresponding to approximately 3 ng of DNA per reaction. These

primers were designed to avoid non-specific binding and primer dimerization and and were chosen to provide high GC content especially at the termini to produce "end clamping"; the GCG programme "Primer" was used to check primer characteristics.

2.6

DNA Sequence Analysis

Computer analysis of DNA sequence was performed using the Stryder software for Macintosh and the Genetics Computer Group (GCG) software (Genetics Computer Group, Madison Wisconsin, 1991 version 7). Default parameters were selected when using GCG "pileup", "bestfit", "map", "gap" and "prettybox" programmes. Database searches for homologies and conserved motifs were done using the non-redundant EMBL, EMBLupdate, GenBank, PDP, GBupdate databases and the BLASTN 1.3.13MP search system. Default settings were used during these searches.

3. RESULTS

The major areas of investigation in this study have been:

1. The behavioural and genetic analysis of the Q12 chemotactic mutant of *R. sphaeroides*,

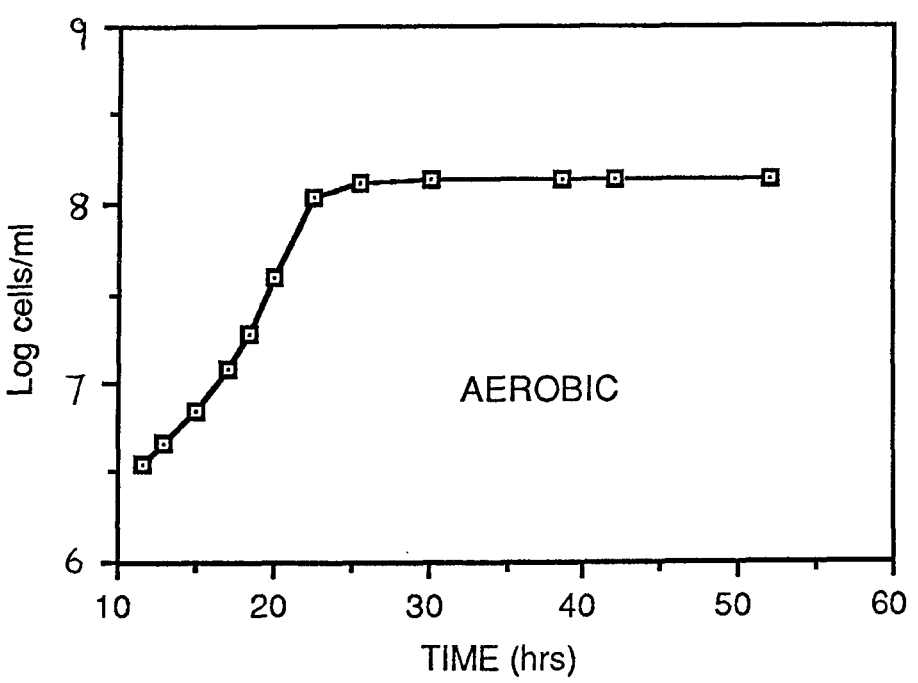
2. The transposon and chemical mutagenesis to isolate chemotactic mutants of *R. sphaeroides*,

3. The identification of chemotaxis (*che*) gene homologues in *R. sphaeroides*.

4. The production of non-chemotactic phenotypes of *R. sphaeroides* by gene disruption and homologous recombination.

For the sake of clarity, the results of these investigations are presented separately.

3.1 Anaerobic Photoheterotrophic Growth and Aerobic Chemoheterotrophic Growth of *R. sphaeroides* WS8N



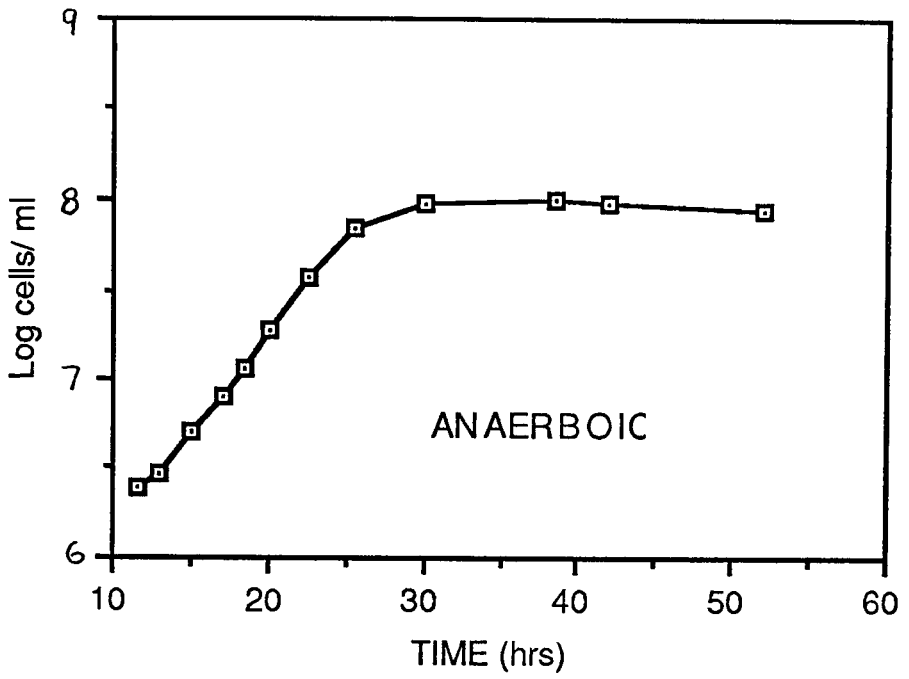


Fig.27: Log_{10} cells per ml vs time for *R. sphaeroides* grown chemo-heterotrophically (aerobically) and *R. sphaeroides* grown photo-heterotrophically (anaerobically) at a light intensity of c. 100 micro-einsteins. In both cases succinate was used as the major carbon source (see Materials and Methods, fig.14).

The above graphs (fig.27) represent the growth characteristics of aerobically and anaerobically grown wild type *R. sphaeroides* WS8-N. The generation time for aerobically grown cells is 2.7 hours and for anaerobically grown cells it is 2.9 hours under these conditions. Lag time is short as the inoculum used was taken from a log-phase culture. The aerobic cells produced no photosynthetic pigment, expression being repressed by vigorous aeration. Identical growth conditions were used throughout the study.

3.2 Analysis of the Tn*pho*A mutant Q12

3.2.1

DNA Probe Hybridization With Q12 Chromosomal Digests and With the *R. sphaeroides* Cosmid Library

In order to verify the results of the previous investigation (W. Havelka, D.Phil. thesis, Oxford, 1991) which had focussed on the *R. sphaeroides* chemotaxis mutant Q12, it was necessary to check the molecular characteristics of this mutant. Q12 chromosomal digests were probed to check the transposon insertion site and the sizes of restriction fragments identified during the previous investigation. pEKn12-18 ("a 6 kb *Eco* RI Tn fragment from Q12 in the *Eco* RI site of pUC18" -W. Havelka, D.Phil thesis, Oxford, 1991) was used to produce a probe. The pEKn12-18 construct was restricted with *Eco* RI and the resulting 6 kb fragment separated by electrophoresis, purified using GeneClean® and used as the template to produce a digoxygenin-labelled probe. A chromosomal digest of Q12 was restricted with *Eco* RI, run on an agarose gel, Southern blotted onto a nylon membrane and probed. The pEKn12-18-derived probe consistently hybridized to a 16 kb fragment, and *not* to the 6 kb fragment from which it was thought to have been made. To make sure that the probe was not hybridizing to the "wrong" fragment by virtue of having lost the transposon, probing was repeated with a digoxygenin-labelled probe homologous only to the central 3.2 kb *Hind* III fragment of Tn5 (derived from pUI800) (fig.24). This probe also hybridized with a 16 kb *Eco* RI fragment of Q12 chromosomal DNA (fig.28). Positive controls all produced a strong signal and non-specific background hybridization was minimal, making these results unequivocal.

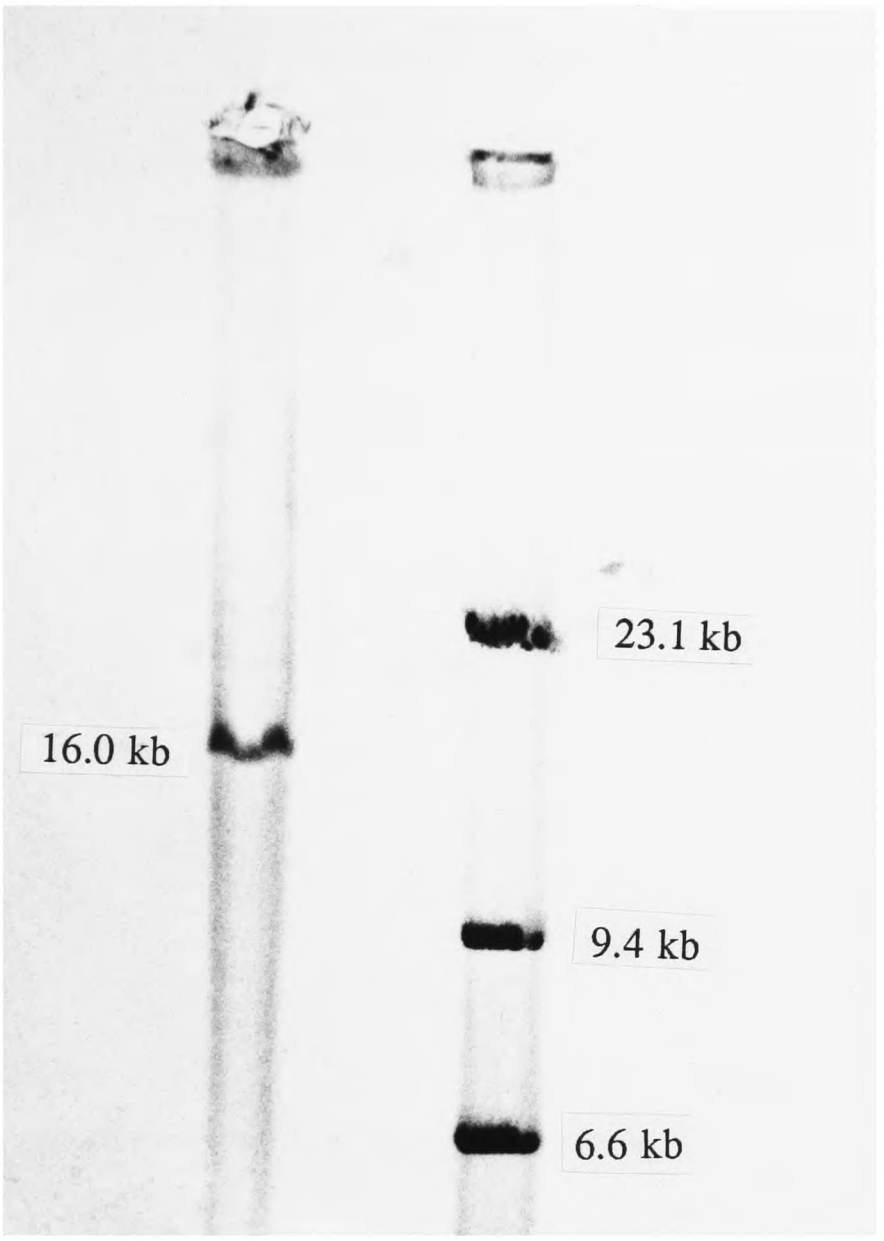


Fig.28: *R. sphaeroides* mutant Q12 *Eco* RI Southern blot probed with the central 3.2 kb *Hind* III fragment of Tn5 excised from pUI800 (fig.22). The probe shows hybridization with a 16 kb *Eco* RI fragment, and not with the expected 6 kb fragment. Stringency of hybridization was high and non-specific hybridization was minimal.

The 843 cosmid dot-blots of the cosmid gene library of *Mbo* I restricted chromosomal *R. sphaeroides* DNA (Sockett and Armitage, 1991) were probed with the pEKn12-18 derived probe. Hybridization signals were produced with cosmid number 252 (fig.29) but *not* with cosmid 324, which was the cosmid that, in the previous study, had been selected from the cosmid library by hybridization with a probe derived from pEKn12-18. To check that the lack of hybridization was not caused by mis-numbering of the cosmid library, c324 was cultured from its original stock and plasmid preparations were made, dot-blotted and probed; whole pEKn12-18 was used as the positive control, which gave a strong signal. No hybridization was detected with c324.

Upon restriction of various subclones of the Q12 genome which had been used for sequencing, a number of discrepancies with the previous study were revealed including two previously unidentified *Bgl* II sites in c324. Restriction of c324 with *Bgl* II produced not the three fragments predicted (21, 9 and 11 kb) but five fragments: c.21, c.11, 6.5, 1.5 and 1.0 kb. The vector pLA2917 was purified and checked to make sure that neither of the new sites were within this component of c324; they were not. Since c324 had been the source of clones used both for complementation experiments and for sequencing in the previous study (the information from which was crucial in the present study) the insert was further investigated. The new *Bgl* II sites were found not to be in the 5.5 kb *Bgl* II - *Hin*d III fragment from which the sequencing clones were produced, but within the adjoining 14.5 kb. Another potentially serious discrepancy was the discovery of two

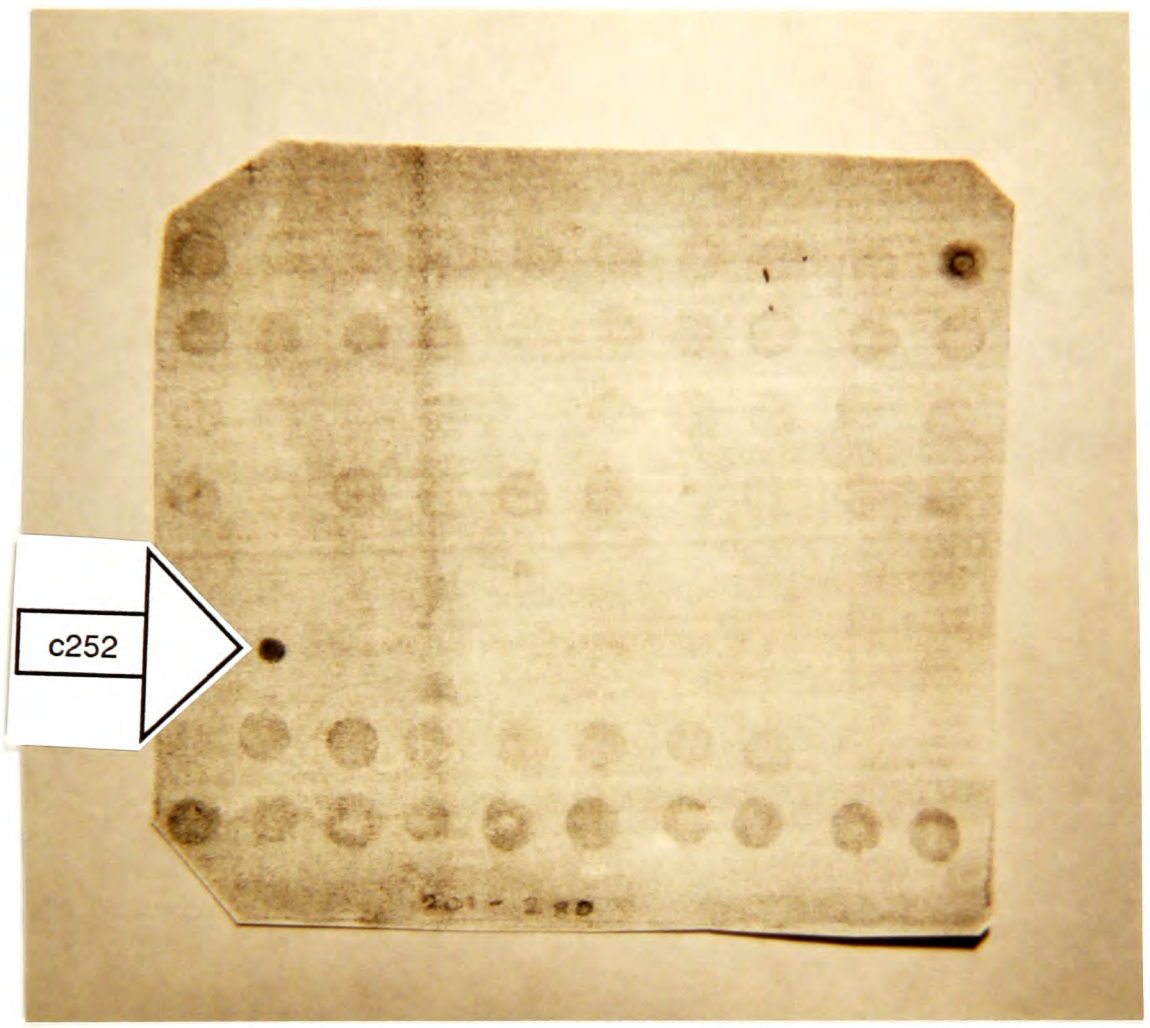


Fig.29: *Mbo* I restricted *R. sphaeroides* cosmid dot-blot probed with a DIG labeled Q12 probe derived from pEKn12-18. Hybridization is shown with cosmid 252, and not with the expected cosmid, 325. Cosmid 210 also produces a hybridization signal, but this was shown to be a non-specific reaction, possibly due to contamination. Because the results were so unexpected his experiment was repeated three times, with the same results. Hybridization conditions used were stringent.

previously overlooked *Kpn* I sites in the 1.3 kb *Sal* I - *Nru* I fragment of pWH8, which had been thought to contain most of the critical open reading frame, ORF W, and was therefore the subject of particular scrutiny including sequencing.

The above discrepancies cast serious doubt upon the subsequent complementation studies and sequencing which had been performed, and it was therefore decided to re-examine the definitive physiological and behavioural characteristics of the Q12 mutant which had initially made it worthy of investigation (see later).

3.2.2

Translational Fusions

The 1.3 kb fragment (originally a *Sal* I - *Nru* I fragment) of Q12 DNA cloned into pWH8 was thought to contain 70% of the putative chemotaxis gene ORF W, including a σ54 DNA polymerase binding site (W. Havelka thesis, Oxford, 1991). This fragment was therefore examined for the presence of a functional promoter sequence. Translational fusions of the 1.3 kb fragment of pWH8 (excised with *Eco* RI and *Hin*d III) were made in the three reading frames of the broad-host range lac Z fusion vector pUI523A (fig.30). The constructs were transformed into *E. coli* S17-1 and conjugated into *R. sphaeroides* WS8N. Transconjugants were screened on agar plates containing 30 μg/ml X-gal, 15 μg/ml tetracycline and carbon sources of varying concentrations (see Materials and Methods). No blue colonies were produced, indicating a lack of any promoter that could be recognised by *R. sphaeroides* DNA polymerases expressed under

those growth conditions.

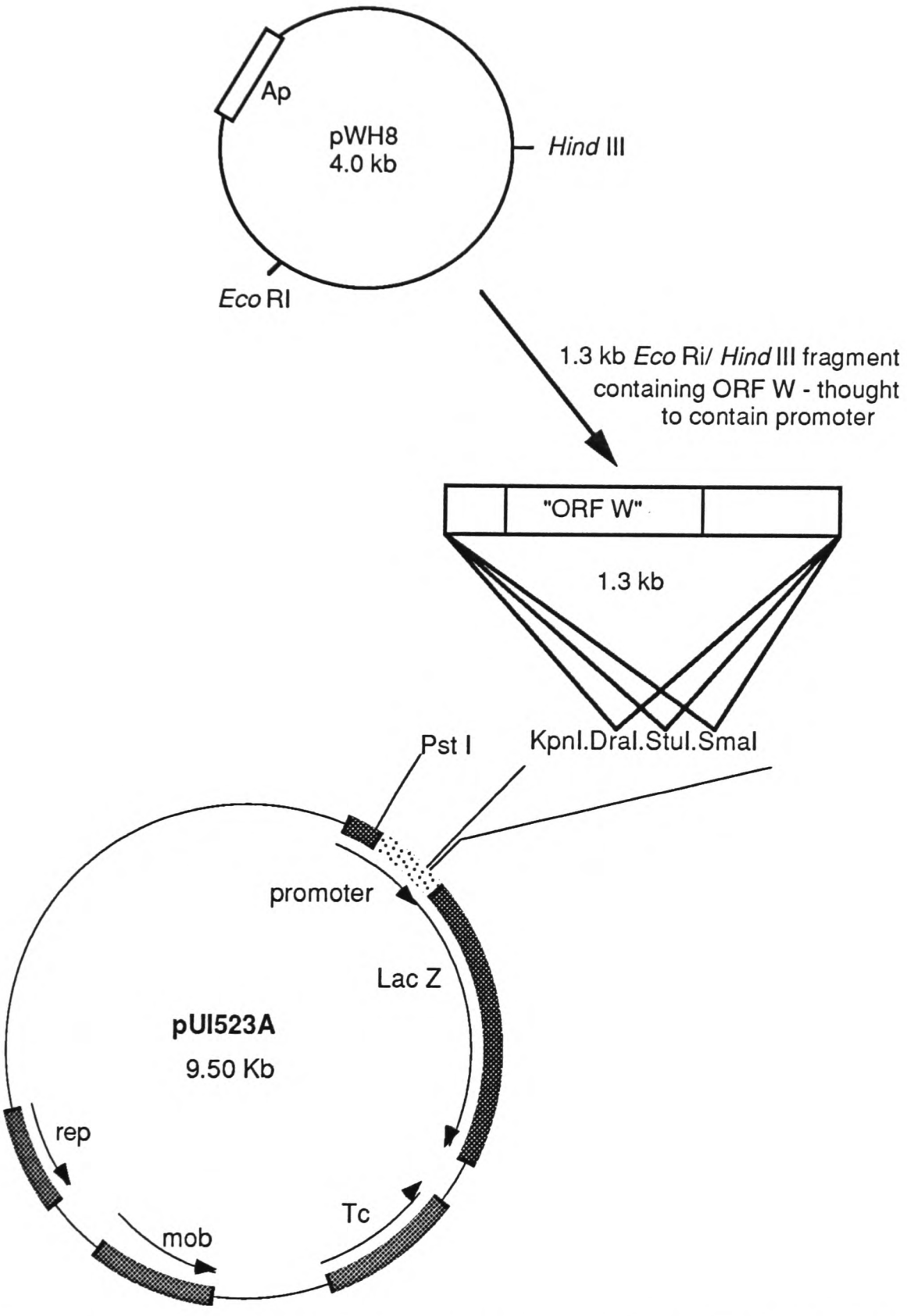


Fig.30: pUI523A, a broad host range translational (*lac*) fusion vector derived from RSF1010. (Tsu-Ning Tai *et al.*, 1988). The 1.3 kb Eco RI-*Hind* III fragment from pWH8 was cloned into the *Dra* I, *Sma* I and *Stu* I sites of the vector. These constructs were then transformed into *E. coli* S17-1 and conjugated into *R. sphaeroides* WS8N.

3.2.3

Chemotaxis and Chemokinesis of the Q12 Mutant

The most important characteristic of the putative chemotaxis mutant Q12 for the purposes of this investigation was that it should be clearly and consistently non-chemotactic under the conditions previously stated, this was therefore re-examined.

Chemotaxis assays revealed that Q12 characteristics were similar in some ways to those found previously, eg: Q12 showed no taxis to sugars or organic acids when grown on propionate (fig.31) and in no case was the accumulation response was as strong or as consistent as for wild type *R. sphaeroides*, but important discrepancies were also discovered.

Percentage motility was widely variable under plug-plate conditions and frequently fell below 10%, in these cases results were discarded. Chemotaxis by Q12 to organic acids was indisputably observed under certain conditions (fig.31). This response was affected by the choice of carbon source in the growth medium with chemotaxis being particularly inconsistent and weak when propionate was used.

The fact that Q12 is tactic to certain attractants under certain conditions and not under others suggests a mutation *not* of any common element of a sensory transduction pathway nor of the motor switching system, but towards a mutation of a component of a metabolic pathway, somehow connected with the metabolism of propionate. The loss of such a component indirectly effects the behavioural response to the substrate of

the pathway in question.

PUTATIVE CHEMOATTRACTANTS	WS8-N			Q12		
	CARBON SOURCE IN GROWTH MEDIUM					
	PROP	BUTYR	SUCC	PROP	BUTYR	SUCC
HEPES	-	-	-	-	-	-
ACETATE	+	+	+	-	+	+/-
PROPIONATE	+	+	+	-	+/-	+/-
PYRUVATE	+	+	+	-	-	-
GLUCOSE	+	-	+	-	-	-

Fig.31: Chemotactic responses of the *R. sphaeroides* Q12 mutant measured semi-quantitatively by plug-plate assay. The carbon sources used in the growth media were propionate (PROP), butyrate (BUTYR) and succinate (SUCC). The carbon source in the attractant plugs are 50 mM acetate, propionate, pyruvate and glucose. + = strong accumulation. +/- = poor accumulation. - = no accumulation.

Chemokinetic responses of Q12 were normal, as suggested previously, with the mean run speed of starved Q12 increasing more than 20% (from 16 ± 1.5 to 20 ± 2.0 μm/s) upon addition of 1 mM propionate; a similar effect was also seen upon addition of succinate, acetate, fumarate and butyrate. Computer analysis revealed that the excitation response involved not only an increase in run speed but also a decrease in

	Mean Run Length	Mean Run Speed	Mean Stop Time	Mean Stop Freq
WS8N	+53%	+24%	+29%	-20%
Q12	+5.6%	+25%	+200%	-76%

Fig. 32: Chemotactic characteristics of the Q12 mutant. Percentage change in parameters of WS8N and Q12 on addition of 1 mM sodium propionate to an equal volume of starved *R. sphaeroides* cell in 10 mM HEPES. It can be seen that Q12 exhibits chemokinesis, and that the excitation response involves both an increase in run speed and a decrease in stopping frequency.

Strain + effector	% motile	Strain + effector	% motile
WS8N + Succ:	70%	Q12 + Succ:	42%
WS8N + Prop:	67%	Q12 + Prop:	32%
WS8N + Hepes:	12%	Q12 + Hepes:	5%

Fig.33: Percentage motility. Mean Data from one day. Percentage motility measured after addition of an equal volume of 1 mM attractant (or 10 mM in the case of HEPES). Cells had been washed and resuspended in 10 mM HEPES and starved for three hours in the light at 37°C. Data shows that WS8N has a higher percentage motility under all conditions.

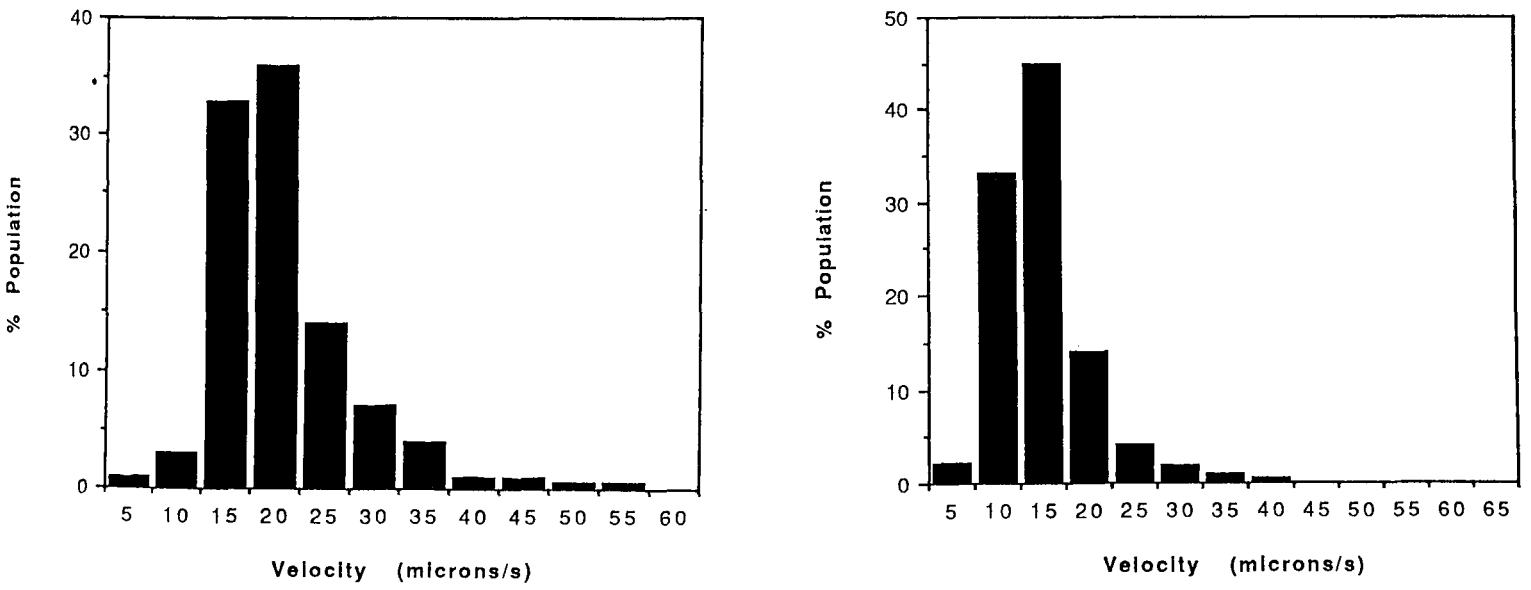


Fig.34: Graphs of mean velocity (μm/s) against percentage population for cells grown photoheterotrophically in 7 mM succinate. WS8N on the left and Q12 on the right. The Q12 mutant shows a larger proportion of slower cells.

stopping frequency (fig.32). This provided further evidence of a true chemotactic response in what had been thought to be a non-chemotactic mutant.

3.2.4

Morphology and Physiology of the Q12 Mutant

Macroscopic morphology of Q12 in succinate swarm agar was that of reduced swarming colonies, 50% to 70% the diameter of wild type *R. sphaeroides* WS8-N, this reduced size was probably, however, the result of reduced growth rate, as increased culture time resulted in larger colonies with diameters equal to wild-type. Presuming the steepness of the chemoattractant gradient formed is dependent upon the density of the growing colony, a slow growing colony will produce less of a gradient than wild-type and will therefore produce a smaller swarm over the same incubation period. As the colony size increases, the gradient, and therefore the swarm size, will increase.

Microscopically, Q12 morphology varied between the normal coccus and long (greater than eight times cell width) non-septate rods (fig.35) often displaying ballooning of cell walls and possession of inclusion granules. The inclusion granules are thought to be deposits of poly-ß-hydroxybutyrate (PHB), which is used for energy storage by *Rhodospirillaceae* (Stanier *et al*, 1959) and also may be used as a "redox dump", helping to balance the overall redox poise of the cell (Tal and Okon, 1985). The fact that these PHB granules are seen in such unusual abundance in the Q12 mutant is suggestive of a metabolic imbalance.

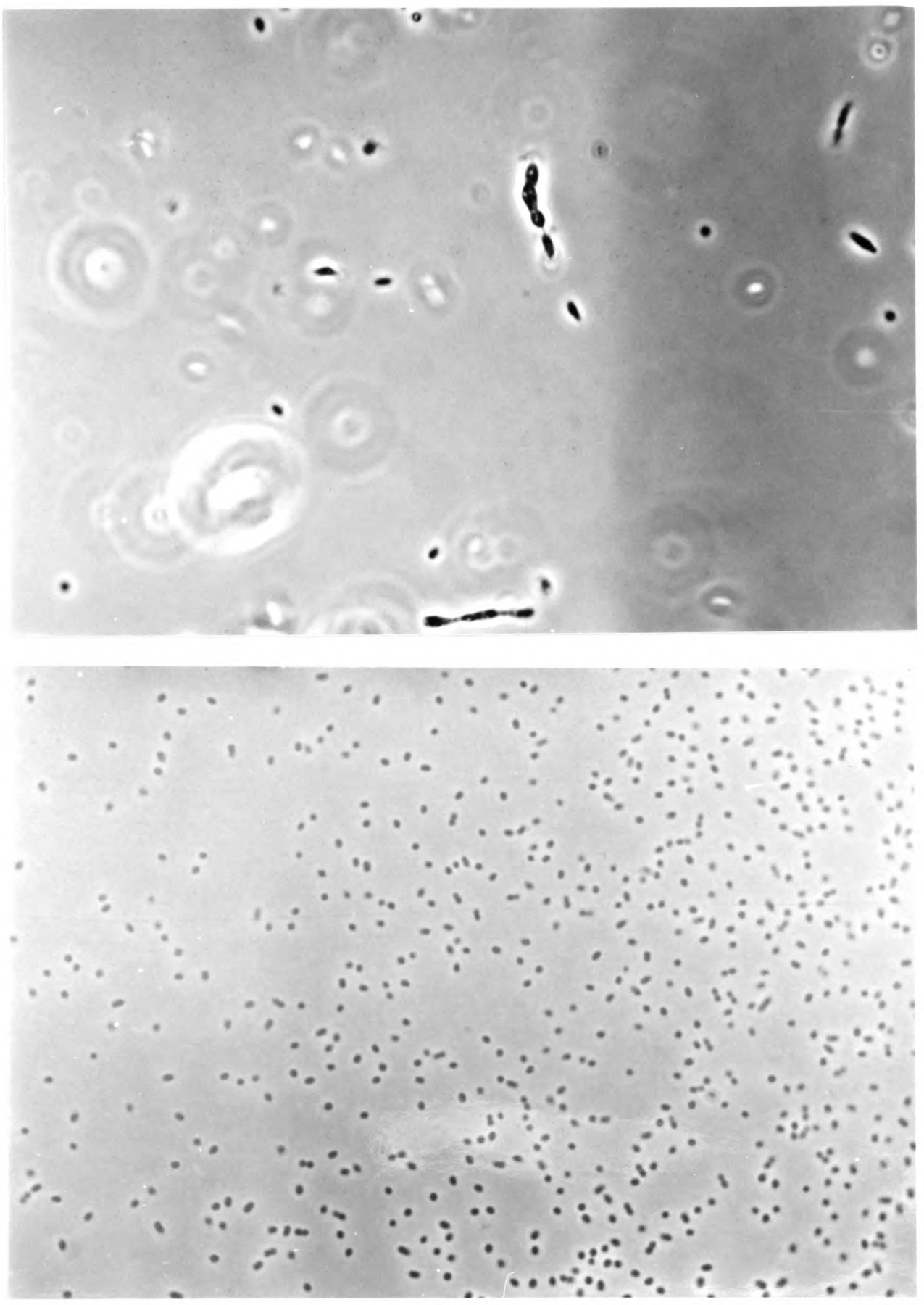


Fig.35: *R. sphaeroides* Q12 mutant microscopic morphology (x1000) exhibiting pathological, elongated, retractile cells. Wild type WS8N cells are shown below.

Wild type WS8N cells are shown below.

Physiologically, Q12 displayed marginally non-wild type pigment ratios, a lower percentage motility than WS8-N under all conditions (fig.33) and a velocity distribution with a larger proportion of slower cells (fig.34). The generation time of Q12 was about 3.5 hours (fig.36) when grown anaerobically using succinate as the sole carbon source (compared with 2.9 hours for WS8-N under identical conditions). Using propionate as the carbon source, the generation time increased to 4.5 hours. Population density at plateau phase was also less than for wild-type, particularly when grown on propionate

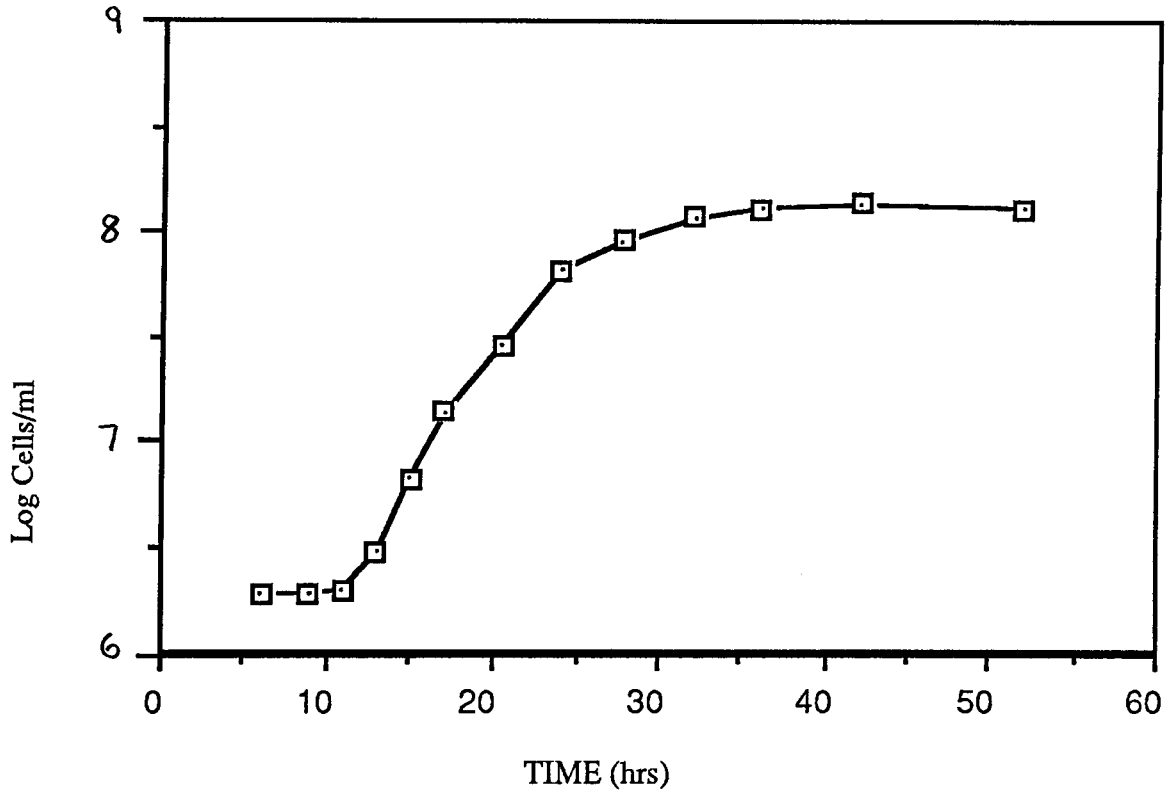


Fig.36: Cells per ml vs time for *R. sphaeroides* Q12 grown photo-heterotrophically (tungsten illumination, c.100 microeinsteins) using succinate as the carbon source (see Materials and Methods, fig. 14).

3.3 Mutagenesis of *R. sphaeroides* WS8-N

3.3.1

Transposon Mutagenesis of *R. sphaeroides* WS8-N

At this stage it was decided that although the Q12 phenotype was partially correct, it was not strictly "non-chemotactic" and it's phenotype was most probably the result of a lesion in a metabolic and not in a dedicated chemotactic pathway (see discussion). Identification of the metabolic lesion that causes the behavioural defect in Q12 was considered too long-term for a D.Phil. project and therefore no further work was performed using the Q12 mutant.

The *de novo* production of a purely non-chemotactic mutant now became the primary aim of the investigation. Transposon mutagenesis was performed using the broad host-range, mobilizable suicide vector pSUP2021 (pBR325-*Mob*::Tn5 [Hunter, 1988]) which contains Tn5. pSUP2021 was conjugated into *R. sphaeroides* WS8-N from the mobilizing strain *E. coli* S17-1 (Simon *et al*, 1983). The average mating ratio of *R. sphaeroides* to *E. coli* in these experiments was 1:20. The average ratio of reduced swarming mutants per transconjugant (first round of screening) was 1:289. The occurrence of spontaneous kanamycin resistance and of spontaneous non-swarming mutants was statistically insignificant. The number of Tn5 mutants screened was approximately 29500 from 22 separate matings. The results are summarized (fig.37) below.

Fig.37: Summary of Tn5 Mutants

c.29500 colonies were screened from 22 separate matings of pSUP2021 x WS8-N.

95 reduced swarm and very reduced swarm colonies were originally picked and colony purified three times.

51 of these reverted to wild-type swarming within three colony purifications.
Of the others:

- 32 strains remained very reduced (≤30% wt) of which:
  - 2 were motile (TAB65 and 79) and
  - 30 were non-motile; of these:
    - 29 were Fla$^-$ and
    - 1 was Fla$^+$ (TAB73)

- 6 strains remained reduced (≤60% wt) of which:
  - 4 were motile (TAB48, 49, 50 and 53)
  - 2 were non-motile; both of these non-motiles (TAB 52 and 54) were Fla$^-$.

- 5 were pigmentation mutants (TAB62, 67, 68, 70 and 96)

- 1 was a reduced swarmer *and* a pigmentation mutant (TAB51)

- 2 displayed pathological morphology (TAB51 and 53)

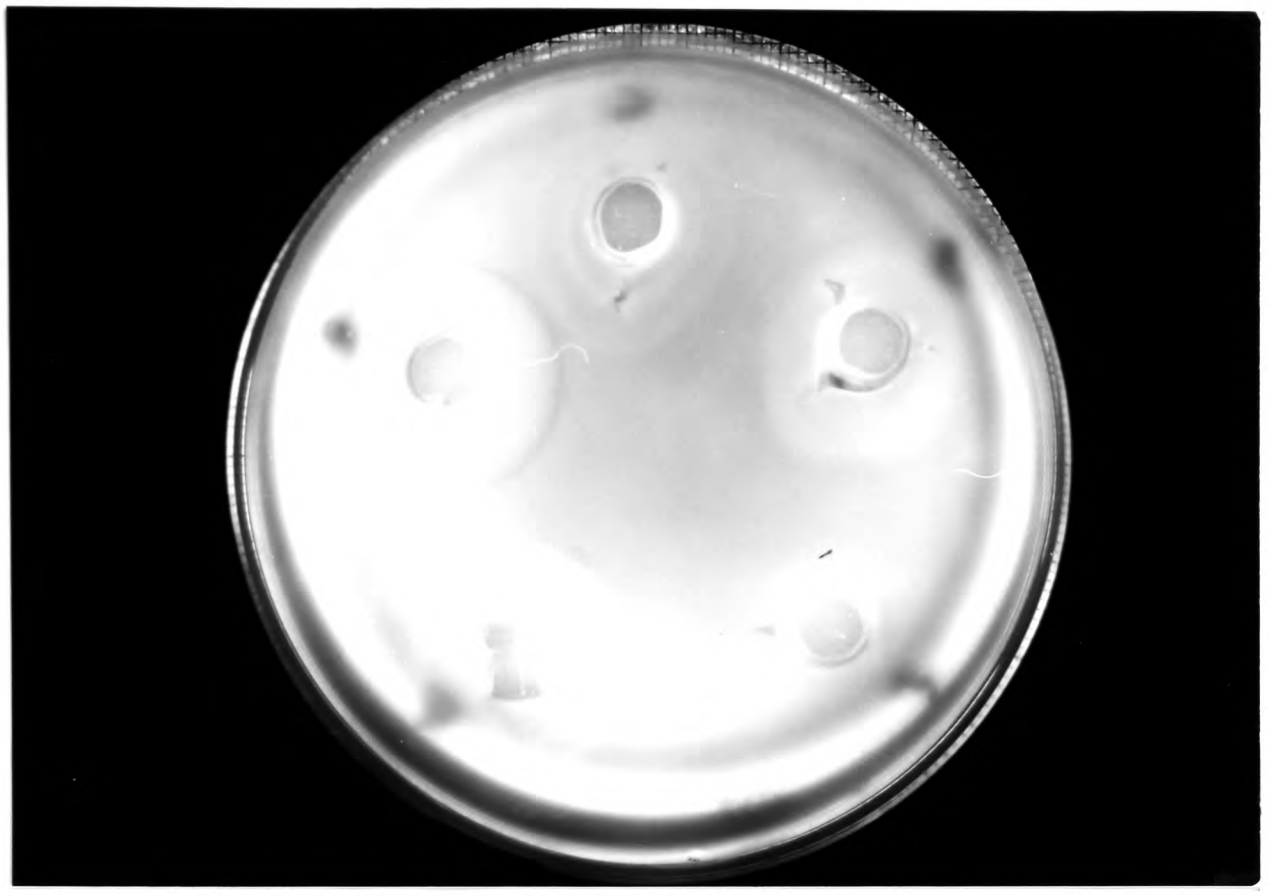
- None appeared to be motile non-chemotactic mutants

95 colonies initially demonstrated reduced swarming and were picked and colony purified three times, then grown in liquid culture (M22, succinate, naladixic acid and kanamycin) from a single colony. These were then stabbed into soft agar to check swarming morphology. 51 of those strains showed wild-type swarming at this stage and were discarded. Of the remaining strains, 32 produced very reduced swarms (≤3 mm); 6 produced reduced swarms (≤6.5 mm); 5 were pigmentation mutants which produced normal swarms (7-11 mm) and one was both a reduced swarmer and a pigmentation mutant (TAB51). All of these were checked for morphology, motility and flagellum production. Of the 38 reduced and very reduced swarmers, 32 were non-motile and only six were motile (TAB48, 49, 50, 53, 65 and 79), all of these were Fla^+. All but one of the 32 non-motiles was Fla^-, this was TAB73, which was subjected to further investigation (see later). Five of the six motile reduced and very reduced swarming mutants were examined in detail (TAB49, 50, 53, 65 and 79).

3.3.1a

Analysis of Potentially Non-Chemotactic Transposon Mutants

Computer analysis was used to investigate motility characteristics of the mutants, specifically looking for deviations from wild-type *R. sphaeroides* behaviour. Although the behaviour of mutants did differ from that of WS8-N and motility characteristics were highly variable, no consistently significant differences were found. Plug plates assays were used to determine chemotactic behaviour. All five mutants showed chemotaxis to organic acids, sugars and to amino acids (fig.38). The cause of the reduced swarming is therefore not associated with a lack of



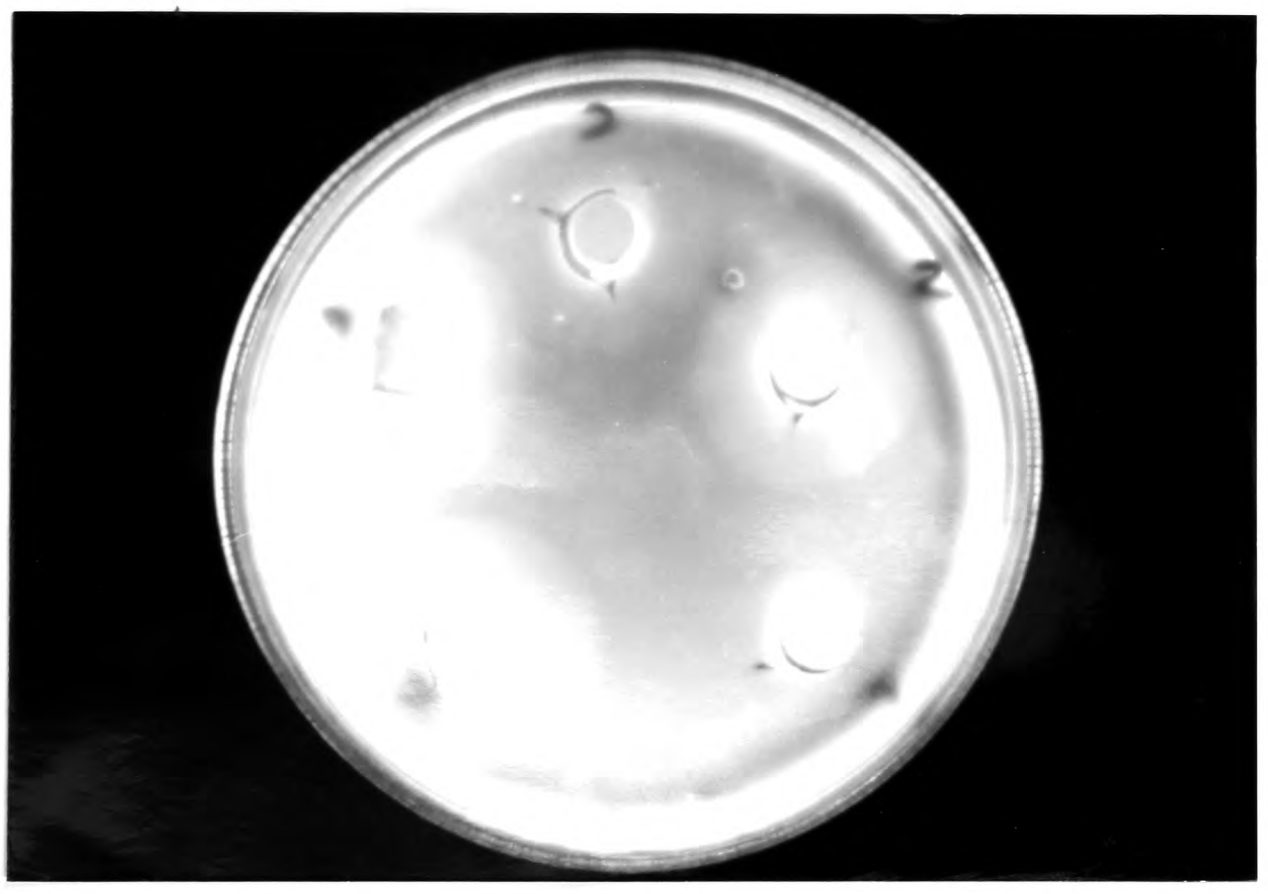


Fig.38: Plug plate assays of the reduced swarming transposon mutants TAB65 (top) and TAB79 (bottom). From the "12 O'clock" position going clockwise, the agar plugs contain: Cassamino acids, 50 mM succinate, M22, 50 mM glucose and 50 mM propionate. Accumulation is clearly visible as a disc around the plug.

chemotactic competence (as measured by any of the assays used here) and so is not a suitable subject for investigation within the scope of this study. This observation suggests that swarming behaviour may not be a suitable method for screening for chemotaxis mutants in this species.

3.3.1b

Enrichment for Chemotaxis Mutants

As, by analogy with other genera, there are probably only few dedicated chemotaxis genes in *R. sphaeroides*, and none had been identified by examining transposon mutants screened using swarm plates, it was decided to try to isolate chemotaxis mutants under conditions that should selectively enrich a population of chemotactically abnormal phenotypes.

Tube enrichment methods following mutagenesis did produce a banded pattern of cell migration (fig.39). Work with *E. coli* had suggested that the population moving up the tube (toward the chemoattractant) would be wild-type, the population remaining at the site of inoculation would be non-motile, and the population moving down the tube would be non-chemotactic. However, despite the formation of bands, sampling from those bands did not result in the isolation of a reduced swarming or of a smooth swimming sub-population. The ratio of normal to reduced swarming phenotypes was the same as that for bacteria screened on swarm plates (c. 300 : 1). Motion analysis revealed no population bias to smooth swimming and no change in stopping frequency, as would be consistent with a lack of non-chemotactic phenotypes. Assessment of

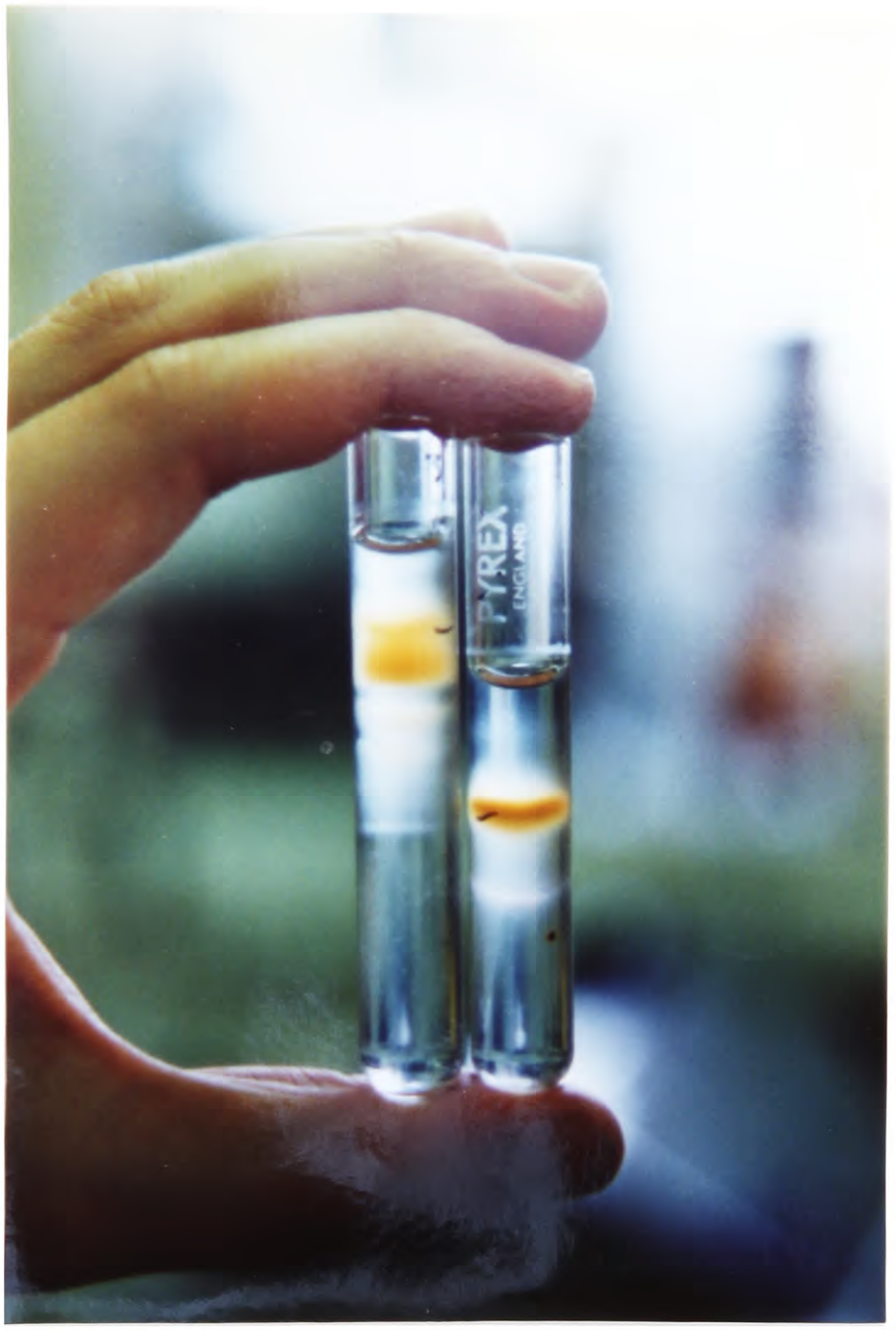


Fig.39: Tube enrichment techniques produced a banded pattern after a twelve hour incubation under photoheterotrophic conditions. [The photograph shows a variation of the technique detailed in Materials and Methods 2.4j. Here glass tubes, rather than polypropionate tubes, were used in order to clearly view the bands. The glass tubes were sealed with 2% agarose overlayed with a few drops of mineral oil; in all other respects the techniques used were identical.]

enrichment tubes was, however, obscured by the lack of a known non-chemotactic phenotype of *R. sphaeroides* with which to test the effectiveness of the enrichment procedure. Enrichment methods would have required further time consuming development and it was decided that all mutants should be diluted and screened in swarm agar. With the information available at that time, this technique, although laborious, seemed to provide a better established and ultimately more practical method of screening. With hindsight, the usefulness of the swarm-plate method of screening itself is questionable as applied to this genus.

3.3.1c

Analysis of the Non-Motile, Flagellated Mutant TAB73

The *R. sphaeroides* Tn5 mutant TAB73 was the only mutant found to possess a flagellum but to be non-motile (fig.40). Extrapolating from the equivalent situation in *E. coli*, this phenotype could have arisen either from the partial or complete deletion of motor (Mot) proteins or from the partial (but not the complete) deletion of one of the switch (Fli) proteins. The other, more intriguing possibility, was that TAB73 was the functional equivalent of an enteric "tumbly" mutant (deleted or partially deleted for either Che B or Che Z). Since, in *R. sphaeroides*, the equivalent of a tumble is a stop, the equivalent of a "tumbly" mutant would be a paralyzed strain. This line of reasoning justified the further investigation of TAB73. PARA 1, a paralysed Mot B mutant of *R. sphaeroides*, had been previously isolated (Sockett and Armitage, 1991), therefore it seemed prudent to first check that TAB73 was not

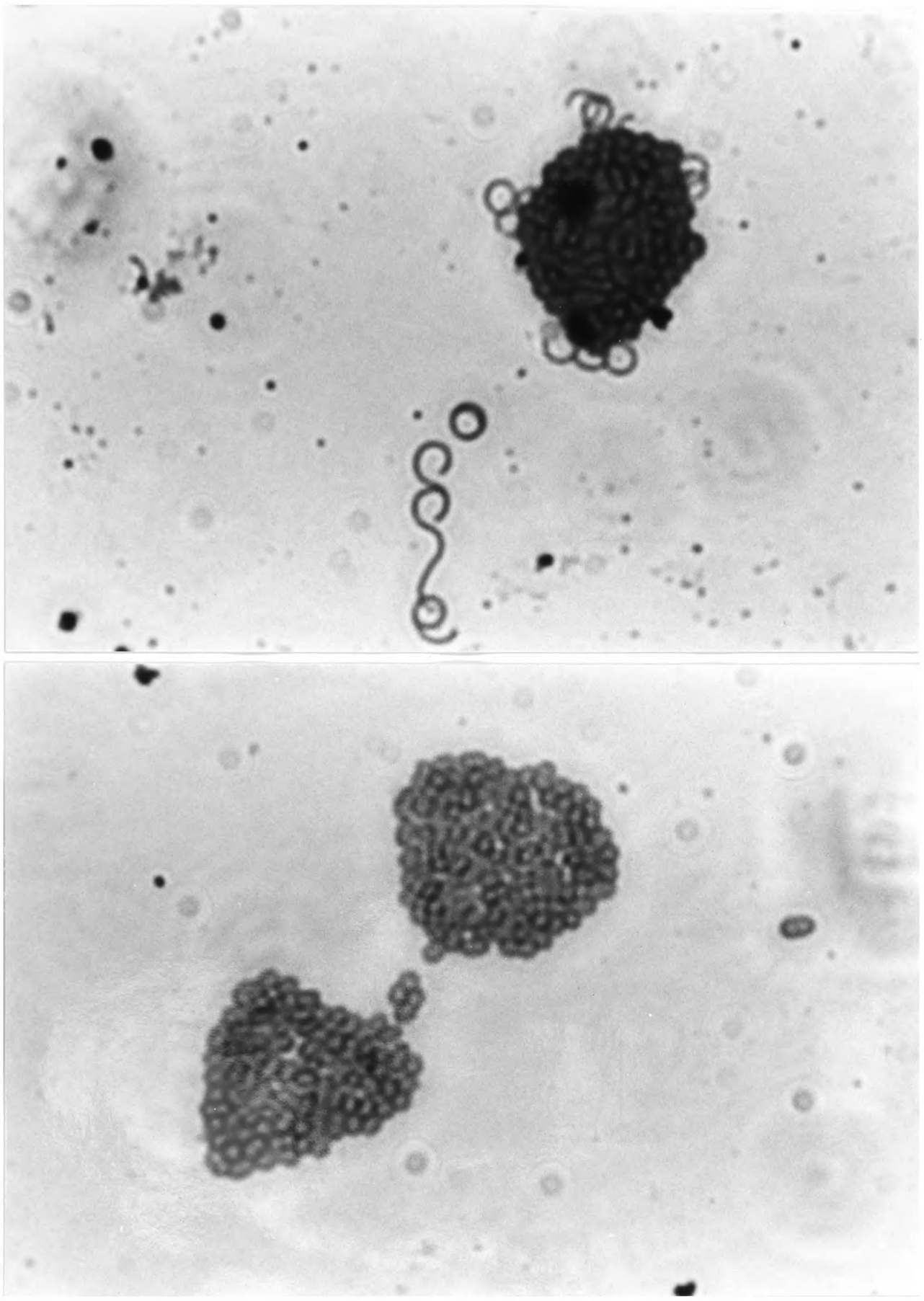


Fig.40: Flagellar stains (Materials and Methods, 2.2b) of the *R. sphaeroides* Tn5 mutant TAB73 (above) and a non-flagellated Tn5 mutant for comparison (below). TAB73 possesses a normal looking flagellum, but the flagellum is paralyzed, probably due to a Tn5 insertion in one of the motor (*mot*) genes.

genetically identical to PARA1. Microscopically, TAB73 appeared normal and did not show the characteristic oscillation exhibited by PARA1 (R. Sockett, unpublished observation). To identify the location of the transposon insertion, chromosomal DNA of TAB73 was prepared, restricted with *Bam* HI, *Eco* RI, *Sal* I and *Nar* I, run on an agarose gel and Southern blotted onto nylon. Unrestricted pUI800 (which contains Tn*phoA*) was dotted onto the filter as a positive control. Digoxygenin labelled probes (c.210 bp in size) were made by PCR using 20mer oligonucleotides derived from IS50 sequence as primers and using pMH1701 as a template (see "Materials and Methods"). Probing using an overnight hybridization and a sixteen hour colour reaction revealed hybridization signals (fig.41) as follows:

TAB73 chromosomal DNA digested with *Bam* HI	4.1 kb 3.7 kb
TAB73 chromosomal DNA digested with *Eco*RI	>12 kb
TAB73 chromosomal DNA digested with *Sal* I	6.7 kb 4.2 kb
TAB73 chromosomal DNA digested with *Nar* I	2.0 kb 1.5 kb

Restriction sites within the transposon were checked using the GCG computer programme and the EMBL database. Hybridization with two fragments of the *Bam* HI digest is expected since Tn5 possesses a centrally located *Bam* HI site. *Nar* I and *Sal* I also have a single site within Tn5. *Eco* RI does not cut within Tn5.
Disappointingly, the pattern obtained by hybridization was very similar to that obtained with PARA 1 (R. Sockett, personal communication).

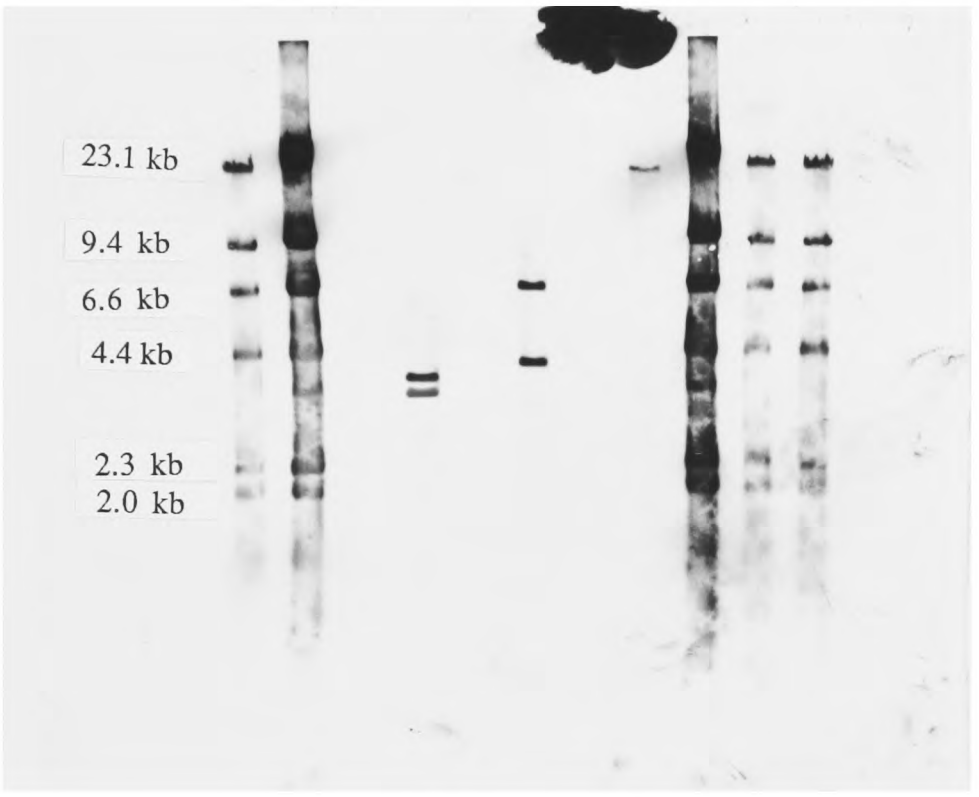


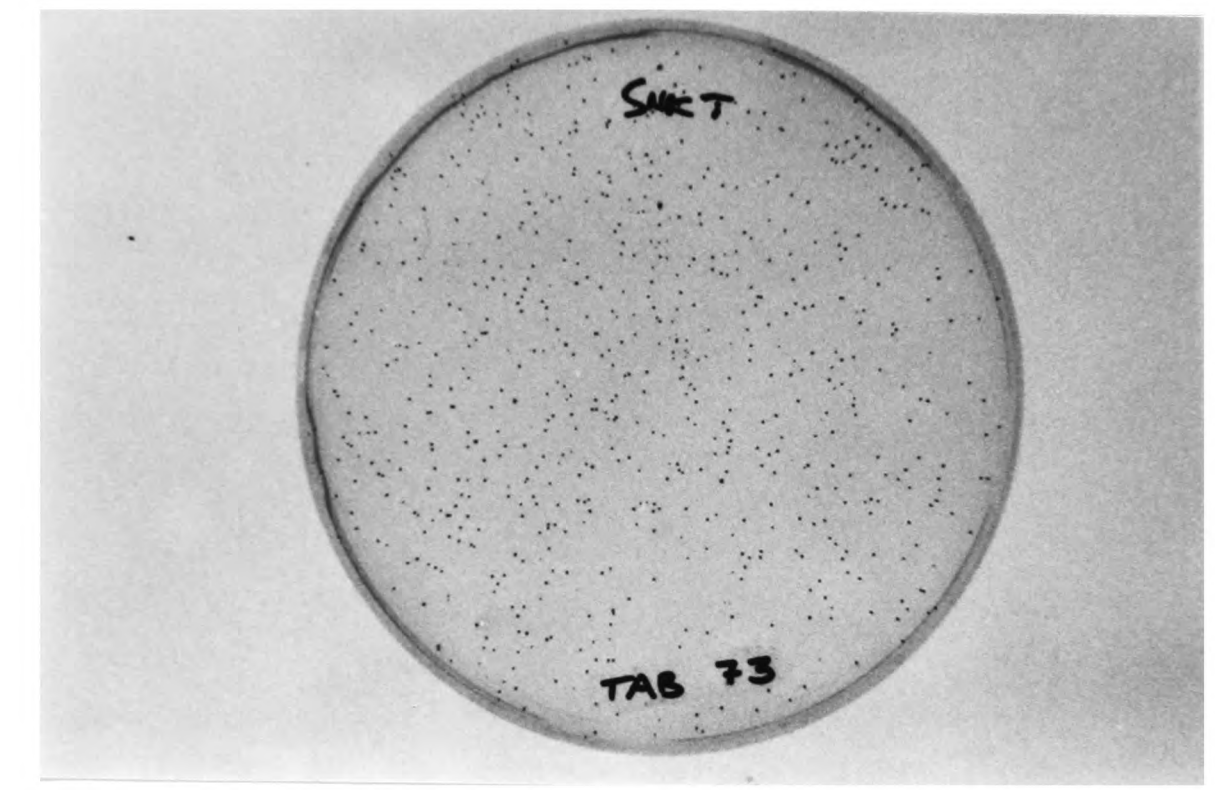
Fig.41: *R. sphaeroides* TAB73 digests *(Bam* HI, *Sal* I and *Eco* RI) Southern blotted and probed with a 210 bp DIG-labeled Tn5 probe. DIG-labeled λ *Hind* III was used as a molecular weight marker (several lanes were used because of uncertainty about concentration of λ *Hind* III). About 3 ng of unrestricted pUI800 DNA was dotted onto the membrane as a positive control, it is visible as a large black patch at the top of the gel. The hybridization conditions used were stringent, and the colour reaction was allowed to proceed for about twenty hours. The TAB73 *Nar* I digest is not shown on this gel, but all experimental conditions were identical.

The PARA 1 phenotype had been complemented by a gene on cosmid c19 and this cosmid was conjugated, via *E. coli* S17-1 into TAB73. This resulted in the restoration of motility to TAB73 (fig.42) strongly suggesting that TAB73 and PARA 1 possess lesions in the same gene (probably *mot* B). This made further investigation pointless, since PARA 1 is currently the research subject of another team.

3.3.2

Chemical Mutagenesis of *R.sphaeroides* WS8-N

No simple chemotactic mutants had been produced by transposon mutagenesis. this suggests that either the transposon was "hotspotting" or that the chemotactic signal transduction of *R. sphaeroides* was so radically different from that of enterics, that deletion of any of the chemotaxis genes of *R. sphaeroides* produced a phenotype not identifiable using techniques developed for enteric species (this is supported by the later identification of two *che* Y genes). The tendency for a transposon to become inserted non-randomly, at a particular site(s) would mean that the mutant we were seeking could never be produced by this method. It was therefore decided to use a chemical mutagenesis method, producing random point base substitutions. Base substitutions will generally cause missense mutations which will give rise to amino-acid substitutions in the resulting protein. This is a far more subtle effect than that caused by transposon mutagenesis which will usually truncate the protein leading to polar mutations in operons. Base substitutions can however also produce nonsense mutations, resulting in transcriptional termination and down-stream deletion of the protein.



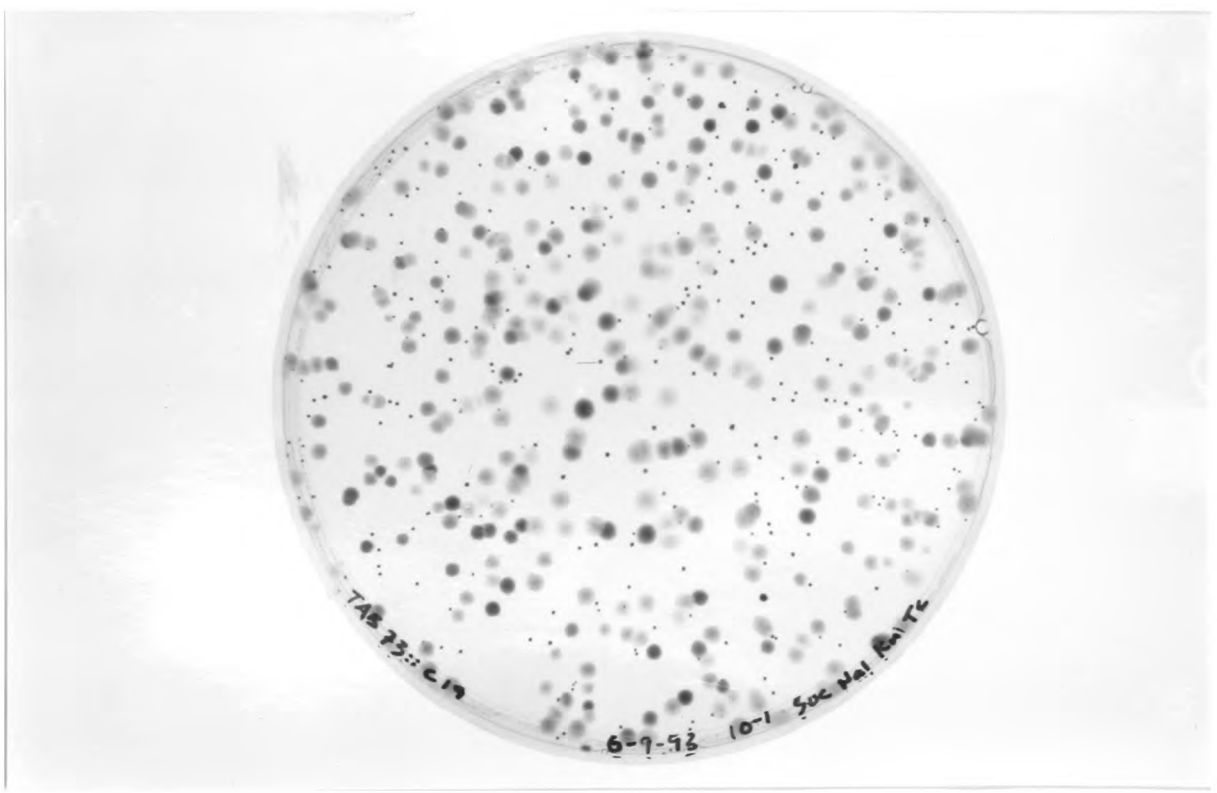


Fig.42: Complementation of the paralyzed mutant *R. sphaeroides* TAB73 with the *R. sphaeroides* cosmid c19. Non swarming, non motile TAB73 (above) regain flagellar rotation and swarming ability (below). Flagellar rotation of the complemented mutant was also observed microscopically. This complementation, together with the hybridization study, suggests that TAB73 possesses a Tn5 insertion in one of the motor genes, probably *mot* B. Swarm plates were incubated aerobically in the dark at 28°C overnight. The major carbon source in the swarm plates was 7 mM succinate.

WS8-N was subjected to EMS mutagenesis and about 4000 resultant colonies were screened. Nine (CAB1 - 9) demonstrated reduced swarming characteristics and were picked to liquid medium (M22, 5 mM succinate, 25 μg/ml naladixic acid), examined for morphology and motility, then colony purified three times and rechecked for motility and swarming morphology. Upon reisolation, only four mutants, CAB2, 6, 7 and 9 demonstrated significantly reduced swarming. All but one, CAB7, were motile. CAB2, CAB6 and CAB9 showed reduced swarming (c.15% of wild-type) which did not increase to wild-type swarming upon increased incubation. CAB2 and CAB9 populations showed a normal percentage of swimming cells, between 40% and 60%, whereas the CAB6 population varied between 1% and 60% motility.

To confirm that these reduced swarming mutants were motile in agar as well as in liquid, samples were taken directly from swarm plates. CAB2, CAB6 and CAB9 were all highly motile (40% - 60%). CAB6 and CAB9 had normal morphologies while CAB2 exhibited pathological irregular rod-shaped structures in some samples, concomitant with low percentage motility.

Chemotaxis assays using plug plates were performed on the motile, reduced swarming EMS mutants (fig.43). CAB2, CAB6 and CAB9 all showed chemotactic responses to organic acids, although CAB2 responded less strongly than the other two, this was possibly due to a lower percentage motility; the disc of accumulated cells was less dense

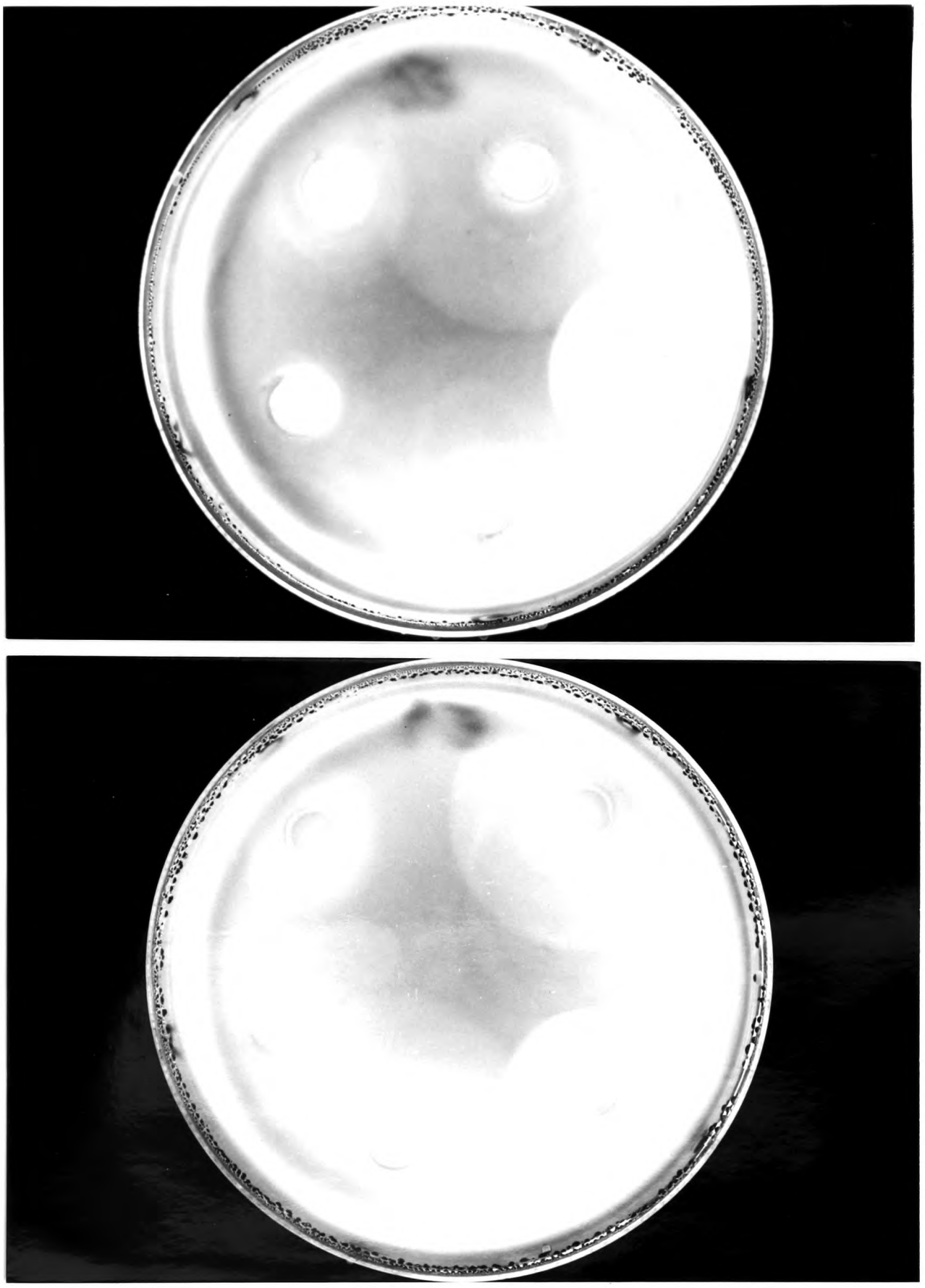


Fig.43: Plug plate assays of the *R. sphaeroides* EMS mutants CAB6 (top) and CAB9 (bottom). Both demonstrate strong accumulation towards chemoattractant plugs. From the "10 O'clock" position going clockwise, the agar plugs contain: Cassamino acids, 50 mM propionate, 50 mM succinate, 50 mM glucose and M22. Accumulation is clearly visible as a disc around the plug.

but no less broad than for CAB6 and CAB9. None responded to the M22 control. Computer analysis was performed. CAB2, CAB6 and CAB9 demonstrated no significant differences in motility characteristics when compared with wild-type *R. sphaeroides* WS8-N. None of these mutants was the functional equivalent of an enteric *che*$^-$ mutant. (The possible reasons for reduced swarming will be discussed later).

3.4 *Salmonella typhimurium fli* Gene Homologies

The flagellar motor of *R. sphaeroides* appears similar to that of enterics at the gross level ie: as seen in electron micrographs, and since clones containing the switch protein genes of *S. typhimurium* had become available to us (a gift from R. M. Macnab at Yale [See fig.26, Materials and Methods]), it was decided to look for homology to these genes in *R. sphaeroides*. The 11.3 kb clone pAMH3 contained the *S. typhimurium* genes *fli* F, *fli* G, *fli* H, *fli* I and *fli* J. The 7.5 kb clone pAMH6 contained the *S. typhimurium* genes *fli* M, *fli* N and *fli* O. The genes of greatest potential interest were *fli* G, *fli* M and *fli* N which encode proteins involved in energy transduction and switching (Kihara *et al*, 1989; Irikura *et al*, 1993) (see introduction).
Both pAMH3 and pAMH6 are based on pBR322 with the *S. typhimurium* genes cloned at the *Hind* III site (fig.26, Materials and Methods). It was decided initially to use the entire *Hind* III fragments of the the two clones as templates from which to produce DIG-labelled

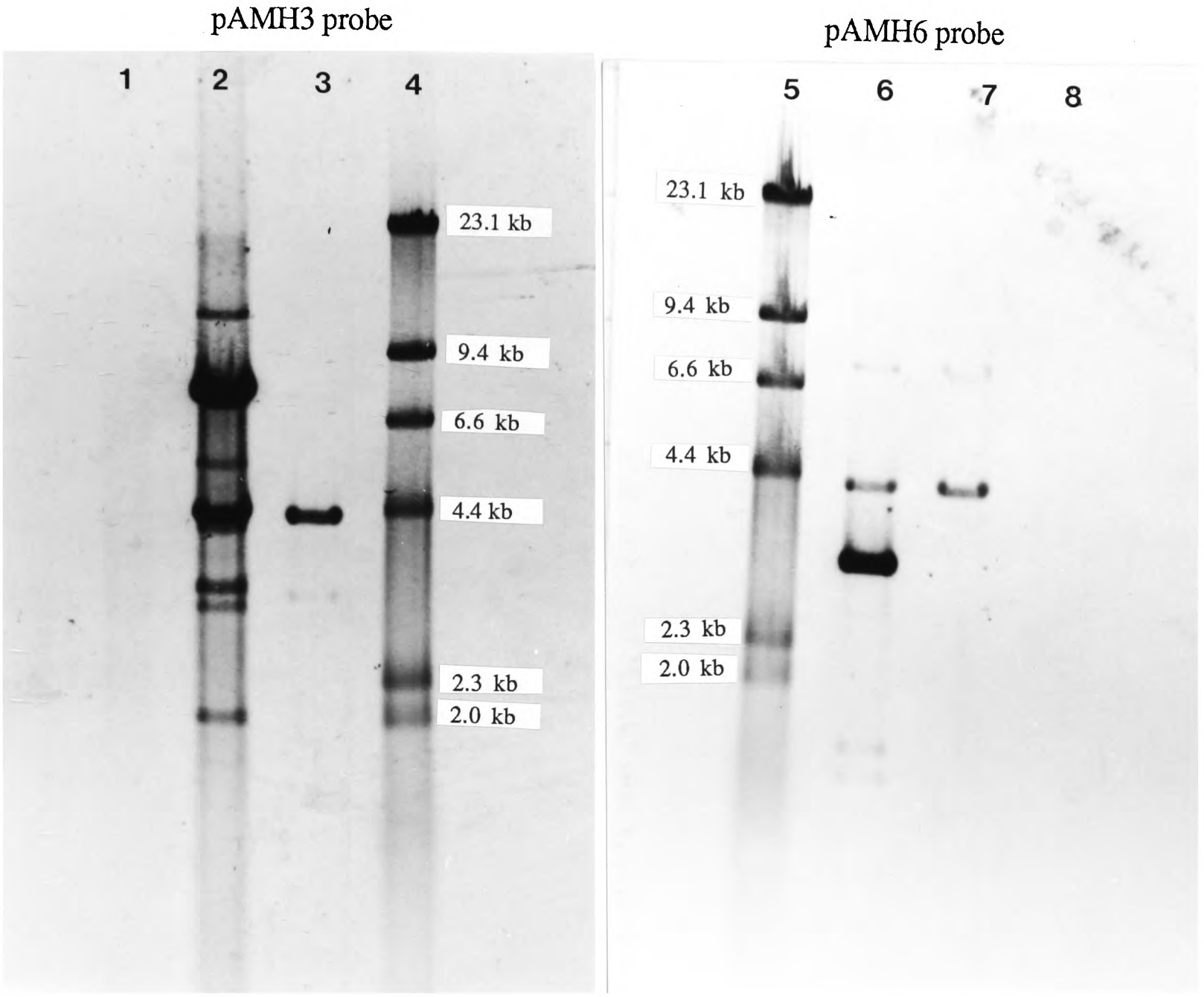


Fig.44: *R. sphaeroides* WS8N *Bam* HI chromosomal digests probed at very low stringency with *S. typhimurium fli* probes. The 7.0 kb probe derived from pAMH3 (left) produced no signal with *R. sphaeroides* DNA. Neither did the 3.2 kb probe derived from pAMH6 (right). In each case, *Hind* III digests of both pAMH3 and pAMH6 were run as controls. Because of the very low stringency conditions, a considerable amount of non-specific binding is seen, particularly with the plasmid digest from which the probe was derived (presumably due to trace contamination of the probe mixture with vector). The sister plasmid also produces a strong signal for the same reason. The lanes containing the *R. sphaeroides* WS8N *Bam* HI chromosomal digests are, however, completely clear. The colour reaction in each case proceeded for about 16 hours. Lane 1 = *R. sphaeroides* WS8N / *Bam* HI; 2 = pAMH3 / *Hind* III; 3 = pAMH6 / *Hind* III; 4 = λ *Hind* III; lane 5 = λ *Hind* III; 6 = pAMH6 / *Hind* III; 7 = pAMH3 / *Hind* III; 8 = *R. sphaeroides* WS8N / *Bam* HI.

probes. These were used to probe a Southern blotted *Bam* HI chromosomal digest of *R. sphaeroides* WS8N. *Hind* III digests of the pAMH3 and pAMH6 plasmids were run as hybridization controls.

High stringency probing (hybridization at 68°C and washing at 68°C with 0.1xSSC and 0.1% SDS) revealed no signal. The stringency was systematically reduced to a very low level (hybridization at 56°C and washing at 56°C with 0.75xSSC and 0.1% SDS). At this low stringency, non-specific binding was high, even so, there was not even the faintest hybridization signal visible in the *R. sphaeroides* lane (fig.44).

3.5 *Rhizobium meliloti mcp* and *che* Gene Homologies

While mutagenesis and *fli* gene homology experiments had been proceeding, cloned fragments of the *Rhizobium meliloti* genome (fig.25) showing some homology to enteric *che* genes, had become available from Rudiger Schmitt in Regensberg, Germany.

As the G:C ratio of *R. meliloti* is close to that of *R. sphaeroides* (c.68%), and as current evolutionary genetics puts them both in the α subgroup of proteo-bacteria (*E. coli* is in the γ subgroup), it seemed reasonable that there may be significant homology between their chemotaxis genes. One of these clones, showing homology to *che* A,

was used to probe a *R. sphaeroides* genomic digest; it hybridized with a 1.4 kb *Eco* RI fragment (M.J.Ward, this laboratory, personal communication). This fragment was cloned into pUC18 and later used to identify a cosmid, c292, in the *R. sphaeroides* genomic library.

Digoxigenin-labelled DNA probes were made, using as a template the *R. meliloti* clones pE1.5, pE2.3 and pE2.7 which carried the following *E. coli* gene homologies (see Materials and Methods and fig.25):

pE1.5 has homology to *E. coli che* W, *che* R and *che* B
pE2.3 has homology to *E. coli che* Y and to the 5' portion of *che* A
pE2.7 has homology to the portion of the *E. coli* gene that codes for the cytoplasmic portion of an MCP.

R. sphaeroides c292 DNA was restricted overnight with *Bam* HI and *Eco* RI. These c292 digests were separated by electrophoresis, Southern blotted to nylon membranes, UV fixed and hybridized (without formamide) over night with the *R. meliloti* probes. Initially, hybridization was done under stringent conditions (68°C hybridization and 68°C wash in 0.5xSSC, 0.1% SDS). No signal was obtained. Stringency was then lowered to 62°C hybridization and 62°C wash in 0.6xSSC, 0.1% SDS. Again, no signal was obtained. Stringency was further lowered to 57°C hybridization and 57°C wash in 0.75xSSC, 0.1% SDS. The results obtained are shown (fig.45a, 45b and 45c).

Under conditions of low stringency, hybridization signals were obtained

with both the pE2.3 derived probe and the pE1.5 derived probe. The degree of non-specific binding was very low producing a strong signal on a clear background. No significant hybridization was detected however, with the pE2.7 derived probe (fig.45c). The following hybridization fragment sizes were graphically calculated: pE2.3 illuminated a 1.05 kb *Bam* HI fragment and a 5.4 kb *Eco* RI fragment of c292 (fig.45b). pE1.5 illuminated a 2.7 kb *Bam* HI fragment and a 3.1 kb *Eco* RI fragment of c292 (fig.45a).

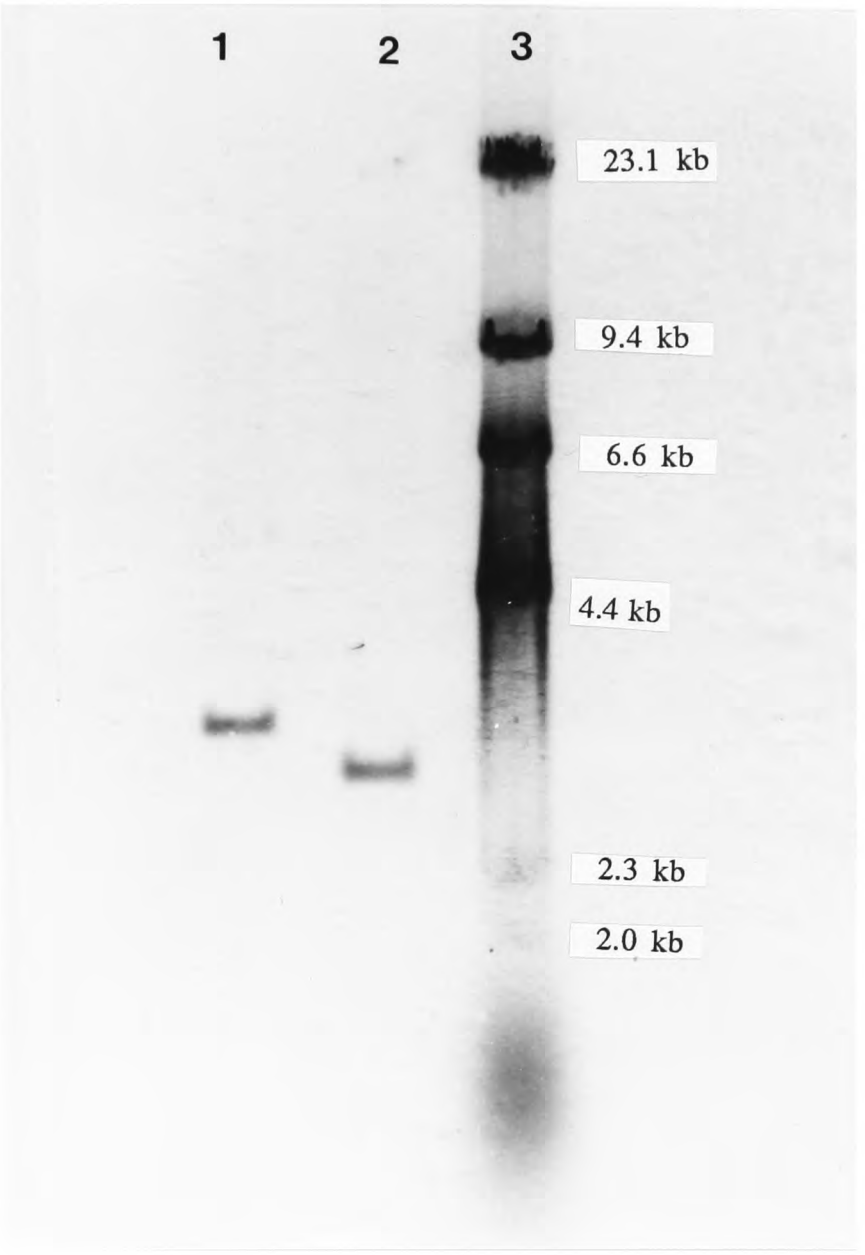


Fig.45a: Hybridization of the *R. meliloti* probe pE1.5 with a *Bam* HI digest of the *R. sphaeroides* cosmid c292 and with an *Eco* RI digest of the *R. sphaeroides* cosmid c292. Lane 1 = c292 / *Eco* RI; 2 = c292 / *Bam* HI; 3 = λ *Hind* III. pE1.5 illuminated a 2.7 kb *Bam* HI fragment and a 3.1 kb *Eco* RI fragment of c292. Stringency of hybridization was very low. The colour reaction was allowed to proceed for about 24 hours.

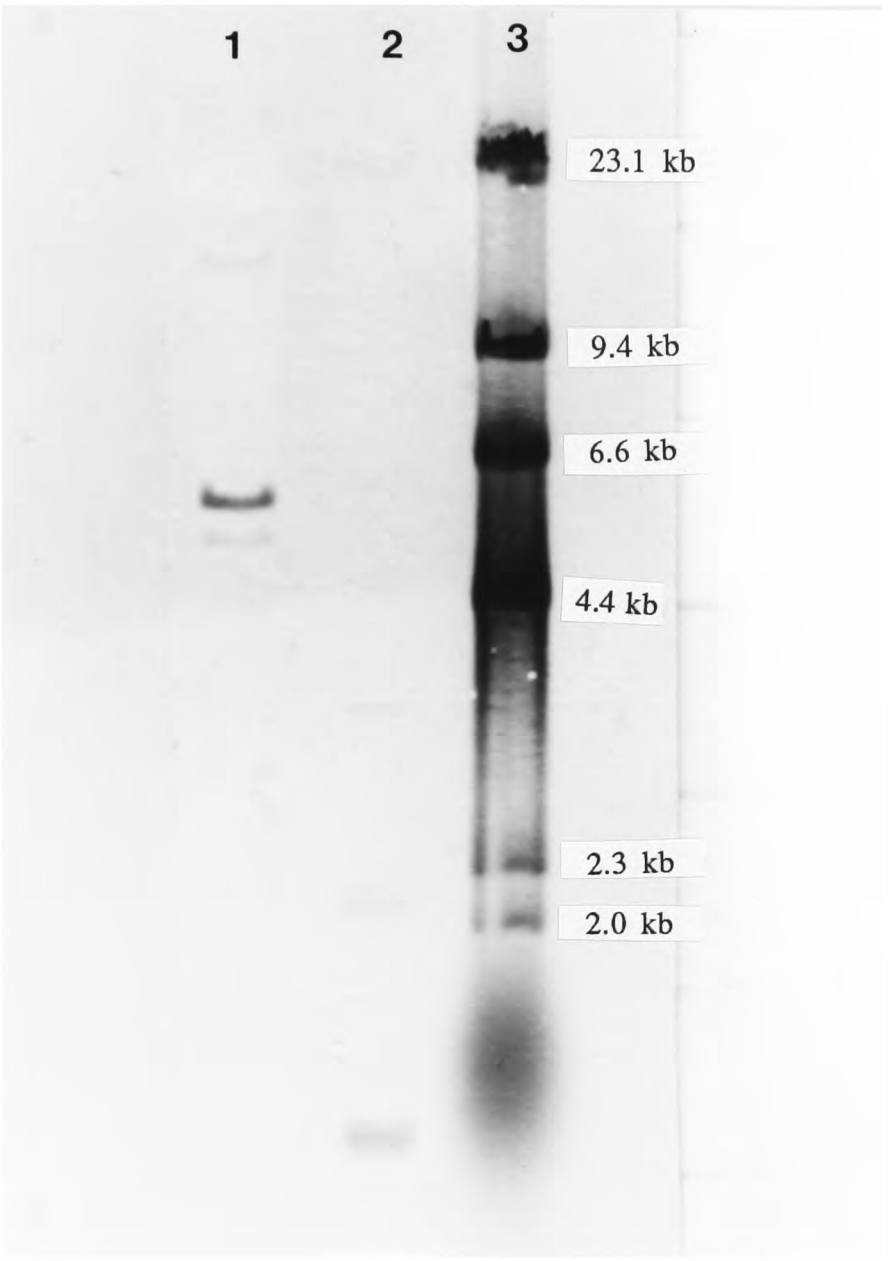


Fig.45b: Hybridization of the *R. meliloti* probe pE2.3 with a *Bam* HI digest of the *R. sphaeroides* cosmid c292 and with an *Eco* RI digest of the *R. sphaeroides* cosmid c292. Lane 1 = c292 / *Eco* RI; 2 = c292 / *Bam* HI; 3 = λ *Hind* III. pE2.3 illuminated a 1.05 kb *Bam* HI fragment and a 5.4 kb *Eco* RI fragment of c292. Stringency of hybridization was very low. The colour reaction was allowed to proceed for about 24 hours.

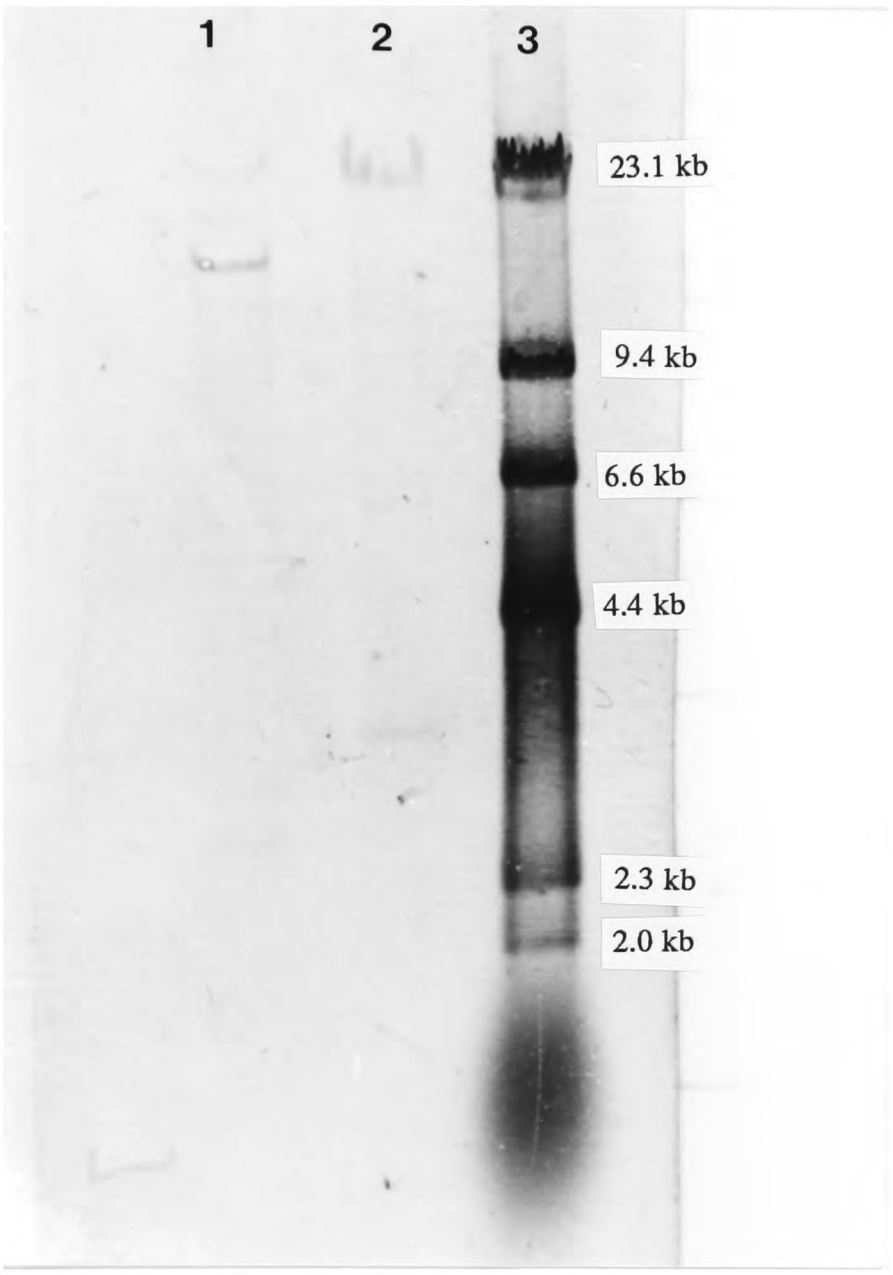


Fig.45c: Hybridization of the *R. meliloti* probe pE2.7 with a *Bam* HI digest of the *R. sphaeroides* cosmid c292 and with an *Eco* RI digest of the *R. sphaeroides* cosmid c292. Lane 1 = c292 / *Eco* RI; 2 = c292 / *Bam* HI; 3 = λ *Hind* III. pE2.7 did not produce a significant hybridization signal. Stringency of hybridization was very low. The colour reaction was allowed to proceed for about 24 hours.

3.6 Cloning and Sub-Cloning of the Chemotaxis (*che*) Gene Homologues of *R. sphaeroides*

Following the discovery of homology with *R. meliloti* chemotaxis genes, it was decided to concentrate this study upon the region of the *R. sphaeroides* genome displaying homology to the *R. meliloti* clone pE1.5, which contained the *che* W, *che* R and *che* B gene homologues (*che* A and *che* Y homologues were investigated by M.J.Ward, in this laboratory). *R. sphaeroides* c292 was partially restriction mapped (fig.46). Restriction fragments were used to produce clones and subclones containing the chemotaxis gene homologues (see below). These clones were used for sequencing.

Clones pCH1 and pCH2 were produced by cloning the 3.1 kb *Eco* RI fragment from c292 into the *Eco* RI site of pUC18. In pCH1 the insert is in the same orientation as the vector; in pCH2 it is in the opposite orientation (fig.16a, 16b). Clones pCH7 and pCH8 were constructed by cloning the 2.7 kb *Bam* HI fragment from c292 into the *Bam* HI site of pUC19. In pCH7 the insert is in the same orientation as the vector; in pCH8, it is in the opposite direction (fig.16c). This series of pUC-based clones was used not only for sequencing but also as the basis for all subsequent cloning of the chemotaxis genes of *R. sphaeroides*, including the production of suicide constructs containing transposon-interrupted genes and truncated chemotaxis gene fragments. Producing clones in both orientations facilitated restriction mapping and allowed a range of deletion sub-clones to be made.

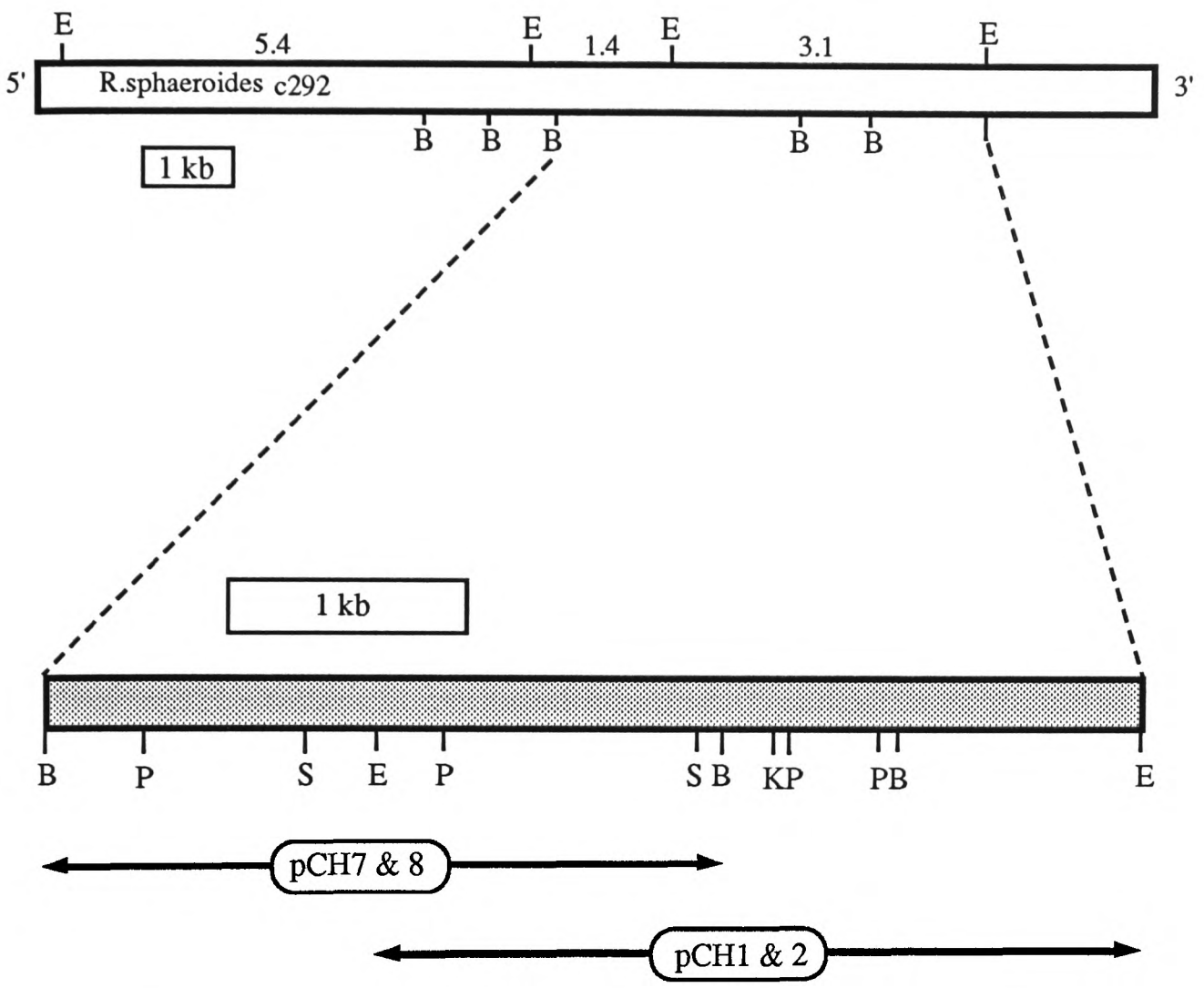


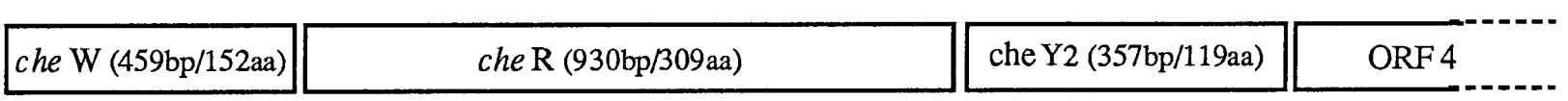
Fig.46: Partial restriction map of the *R. sphaeroides* cosmid c292 showing fragments used to construct the initial sequencing clones (pCH1,2,7 and 8) from which subsequent clones were derived. E = *Eco* RI; B = *Bam* HI; P = *Pst* I; S = *Sal* I; K = *Kpn* I.

The parent clones pCH1 and pCH2 were used to produce the sub-clones pCH3, pCH4, pCH5 and pCH6 (fig.16a and fig.16b). The construction of these subclones not only facilitated the sequencing of fragments in both forward and reverse directions, but also allowed maximum use to be made of pUC "universal" sequencing primers. pCH3 and pCH4 were produced by making *Bam* HI deletions in pCH1 and pCH2 respectively.

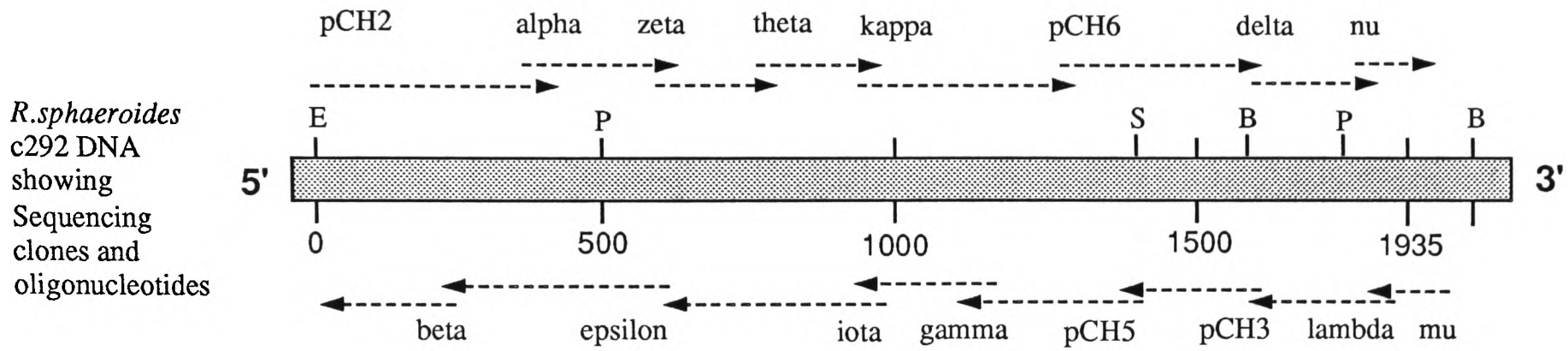
pCH5 and pCH6 were constructed by making *Sal* I deletions in pCH1 and pCH2 respectively. The overall sequencing strategy showing the role of clones, sub-clones and primers is detailed in fig.47.

3.7 DNA Sequence Determination of the Chemotaxis Gene Homologues of *R. sphaeroides*

A contiguous sequence of 1981 bp of *R. sphaeroides* DNA was revealed (fig.48) which contains four open reading frames, with a reading frame change between each.



The first open reading frame ("*R. sphaeroides* ORF W") is 459 bp long, coding for a protein of 152 amino acids. The second one ("*R. sphaeroides* ORF R") is 909 bp long, specifying a 309 amino acid protein which, unusually, starts with an N terminal valine (GTG) rather than an N terminal methionine (ATG). The valine start codon is more common in the genome of G:C rich organisms than in the genome of other organisms. The third open reading frame (*R. sphaeroides* ORF Y2) is 357 bp long, specifying a protein of just 119 amino acids. The fourth open reading frame (*R. sphaeroides* ORF 4) is the incomplete first 220 bp of a gene.



Comparitive homologies:

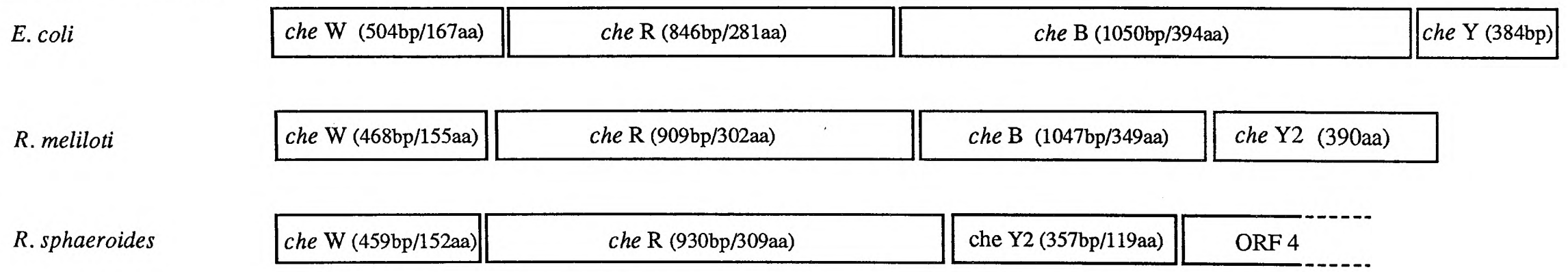


Fig.47: Partial restriction map of *R. sphaeroides* DNA on cosmid 292 showing sequencing strategy with location of oligonucleotides (alpha to nu) and original sequencing clones (pCH2 to pCH6) shown by dotted lines, orientation indicated by arrows. Comparitive homologies of open reading frames shown below in boxes with sizes of genes and of proteins given in brackets. Only a portion of the operons of *E. coli, R. meliloti and R. sphaeroides* is displayed. **E** = *Eco* RI, P = *Pst* I, S = *Sal* I, B = *Bam* HI .

```
                          SD          cheW
1   GGGGGCACGT TCTCGATGGA GTTCTGAGAT GGCGGCCGTG AGCCTGGACG
     G  G  T   F  S  M  E  F  *
                                    M  A  A  V  S  L  D
                                                  EcoRI
    AAGACCTGCG AGAAGTCGTG GCTTTCCGAA TCGGAAGTCA GGAATTCTGC
    E  D  L  R  E  V  V  A  F  R  I  G  S  Q  E  F  C

    ATTGACATCC GCGCCGTCCG CGAGATCCGC AGCTGGACGC CGACGACCAT
     I  D  I  R  A  V  R  E  I  R  S  W  T  P  T  T  I

    TCTGCCTCAC GCGCCCTCCT ATATCATCGG AGTGATCAAT CTGCGAGGTT
      L  P  H  A  P  S  Y  I  I  G  V  I  N  L  R  G

    CGGTGGTGCC GATCATCAAT CTTGCGGAAC GGCTGGGGCT CGGGTCCCTC
    S  V  V  P  I  I  N  L  A  E  R  L  G  L  G  S  L

250 GAGCCCGAGG CCCGTCACGT CATCATCATC ACCGTGGTCG AGGGTCAGGT
     E  P  E  A  R  H  V  I  I  I  T  V  V  E  G  Q  V

    CGTGGGACTG CTTGTCGATG CTGTCTCCGA CATCTTGACG CTGCCTCCCT
      V  G  L  L  V  D  A  V  S  D  I  L  T  L  P  P

    CCTCCGTGCA GCCAATGCCG AAAATTGCCT CCGAAACCAC CTTGTCCTTC
    S  S  V  Q  P  M  P  K  I  A  S  E  T  T  L  S  F

    GTGCGAGGCG TGATCACGCT GGACCAGCGG ATGCTGCGGC TGCTCGATCT
     V  R  G  V  I  T  L  D  Q  R  M  L  R  L  L  D  L

                             SD          cheR
    GATCGGAGTG CTGCCGAACG TCAGGCGGAT GATGTGAAAT ACTGGTCGCG
     I  G  V  L  P  N  V  R  R  M  M  *
                                    V  K  Y  W  S  R

500 ACAACCCCCG ACTGCAGTCC GTGACCCGCA GCTTTCTCAG GAAGACTTCC
      Q  P  P  T  A  V  R  D  P  Q  L  S  Q  E  D  F

    AGCGGATTGC GCAGCTGGCC CATGCCGAGG CGGGGCTCAC CATGCCTGAC
    Q  R  I  A  Q  L  A  H  A  E  A  G  L  T  M  P  D

    GCCAAGGAGC CGCTGGTTTA CGCCCGGCTT GCCAAGCGGC TCCGTCAACT
     A  K  E  P  L  V  Y  A  R  L  A  K  R  L  R  Q  L

    GTCCCTGACG AGCTTCACGG CCTATCTCGA CCTGATCTCG ACACCTGAAG
      S  L  T  S  F  T  A  Y  L  D  L  I  S  T  P  E

    GCCGCGATGA GCGCTCGATG TTCATATCGA GCATGACCAC CAATACGACC
    G  R  D  E  R  S  M  F  I  S  S  M  T  T  N  T  T

750 CGCTTTTTCC GCGAAGAACA TCATTTCGAA CTCCTTTCAG AACGCGTGCT
     R  F  F  R  E  E  H  H  F  E  L  L  S  E  R  V  L
```

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GCCGCCTCTG CTGGACGCGG CGCGACATGG CGCACGGGTC CGTTTCTGGT
  P  P  L   L  D  A   A  R  H  G   A  R  V   R  F  W

CCGCGGGCTG TTCGTCGGGA GAGGAGCCCT TCAGCATGGC GATCACGTTG
S  A  G  C   S  S  G   E  E  P   F  S  M  A   I  T  L

CTGGAGCTAT GCCCAGACGC CGGCCACTAT GATATCAAGA TCCTTGCCAC
 L  E  L   C  P  D  A   G  H  Y   D  I  K   I  L  A  T

CGATATAGAC CGCAAGATCC TGGGAAGGGC GCAAGCAGGC TGGTTTGCAG
  D  I  D   R  K  I   L  G  R  A   Q  A  G   W  F  A

1000 CCTCGAGCCT TGCCGCTCTG CCGGATGCGG TTCTGGCACA TCACTTCGGG
     A  S  S  L   A  A  L   P  D  A   V  L  A  H   H  F  G

CCGCCCGACC GCGACGGTCG AAGGGAGGTG TCGGCGGATC TGCGCGCCCT
 P  P  D   R  D  G  R   R  E  V   S  A  D   L  R  A  L

CGTGACCTTC AAGCCGCTGA ATCTGGTAAA GCCCTGGCCG GTGACCAGAC
  V  T  F   K  P  L   N  L  V  K   P  W  P   V  T  R

CTTTCGATGT GATATTCTGT CGGAACGTCG CGATCTACTT CGACCCCGAC
P  F  D  V   I  F  C   R  N  V   A  I  Y  F   D  P  D

ACTCAGAACC GCATCTGGCG TGGCTTCGCA AGCACGCTCG CTCCGGGAGG
 T  Q  N   R  I  W  R   G  F  A   S  T  L   A  P  G  G

1250 CCATCTCTTC ATCGGCCATT CCGAGCGGCT TTCGCACGAG GTCAGACACC
       H  L  F   I  G  H   S  E  R  L   S  H  E   V  R  H

AATTCGAAAC TGTTGGCATG ACATCATTCC GCCTGCGCGC ACCCCCGCCG
Q  F  E  T   V  G  M   T  S  F   R  L  R  A   P  P  P

                                                    SD

ACGGGCGGAC CAGGAAAGGA GATCTTTGGT GGCACTCAGG GACAGCATTA
 T  G  G   P  G  K  E   I  F  G   G  T  Q   G  Q  H  *

      cheY2
GGATCATGGT GGTCGATGAC ATGTCGACCA GCCGGGGTCT GATCATTCAG
      M  V   V  D  D   M  S  T   S  R  G  L   I  I  Q

GCATTGGAGG AATTGGGGAT CAAGAAGATC GACTTCTGCA AGGACGGCGC
 A  L  E   E  L  G  I   K  K  I   D  F  C   K  D  G  A

1500 TTCGGCCCTG CGGCAGCTGG CAGCCAATCC GGTACATCTG GTGATTTCCG
       S  A  L   R  Q  L   A  A  N  P   V  H  L   V  I  S

                                                  BamHI
ACTACAATAT GCCTGGTCTC GACGGGTTGG GCCTCCTCAA GGGGATCCGA
D  Y  N  M   P  G  L   D  G  L   G  L  L  K   G  I  R

GAGAACAAAA CCACGCAGCG GATGGGTTTC ATCCTCGTCA GCGGTACCGC
 E  N  K   T  T  Q  R   M  G  F   I  L  V   S  G  T  A

CACGCGTGAT ATCATCGAAA AGGGCCAGTC TCTCGGTATG AACAACTTCA
  T  R  D   I  I  E   K  G  Q  S   L  G  M   N  N  F
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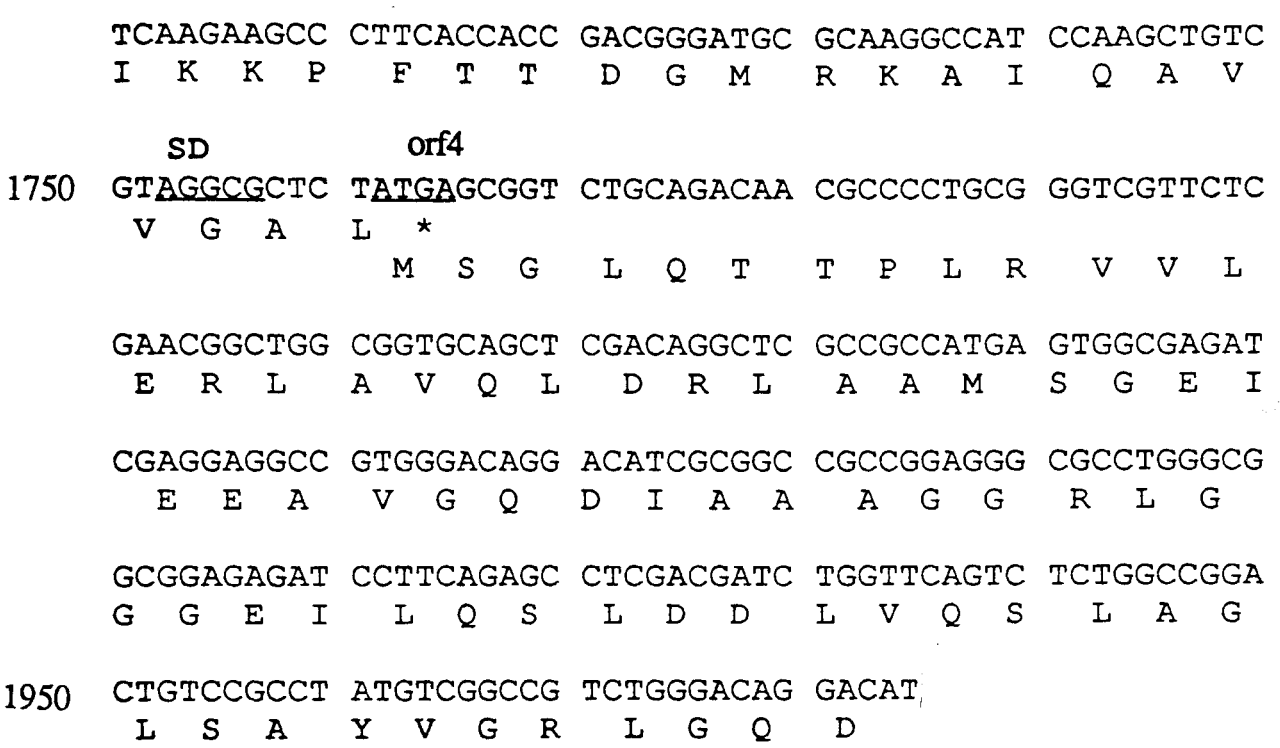


Fig.48: A 1984 bp contiguous region of the *R. sphaeroides* chemotaxis operon, sequenced from both strands, showing four open reading frames with a reading frame change between each. Translation is shown below. Putative ribosome-binding sites ("SD") are underlined. The first open reading frame, *R. sphaeroides ORF* W is 459 bp long. The second, *R. sphaeroides ORF* R, is 909 bp long. The third, *R. sphaeroides ORF* Y2, is 357 bp long. The fourth, *ORF* 4, contains the first 220 bp of a gene with no known homology.

3.8 DNA and Protein Sequence Analysis of the Chemotaxis Gene Homologues of *R. sphaeroides* and Production of Homologous Recombinants to Give Gene Disruptions

Comparative DNA and protein sequence analysis was done using a variety of software available on the 1991 version of the GCG molecular biology package (Genetics Computer Group, Wisconsin) . "Bestfit", "gap", "pileup" and "prettybox" programmes were used to compare *R. sphaeroides* DNA and protein sequences with those of the chemotaxis genes of *E. coli*, *R. meliloti* and other genera. A bank of databases was searched using the BLASTN 1.3.1MP search strategy to check for homology with other documented systems.

It was found that the *R. sphaeroides* open reading frames demonstrated very significant similarity to the enteric *che* genes *che* W, *che* R and *che* Y. (The open reading frame "*R. sphaeroides* ORF Y2", which showed similarity to enteric *che* Y also shows considerable similarity to the N terminal domain of the enteric *che* B). The open reading frames of *R. sphaeroides* showed an even higher degree of similarity with the *che* genes of *R. meliloti*, a more closely related organism (Woese, 1987). A comparison of primary protein structures revealed a higher degree of similarity (but a lower identity) between genera than did the DNA sequence comparison, indicative of conservative base substitutions. This evidence supports the theory of functional conservation between proteins. Functional sites that have been shown

to be involved in protein-protein interactions, in enzymatic activity and in the regulation of enzymic activity were searched for using the "bestfit" programme. The novel DNA sequences were also searched for the presence of putative *Rhodobacter* transcriptional promoters, for regulatory motifs and for potential Shine-Dalgarno sequences that had been identified (in most cases tentatively) by other researchers.

3.8.1

Discovery of the *R. sphaeroides che* W Gene Homologue - Analysis of this Gene and of its Projected Protein

R. sphaeroides ORF W (fig.48) showed a 46% identity to the *che* W gene of *E. coli* and a 60% identity to the *che* W gene homologue of *R. meliloti*. When translated, *R. sphaeroides* ORF W would produce a protein of 152 amino acids; this compares with 167 amino acids for the *E. coli* Che W protein and 155 amino acids for the *R. meliloti* homologue (fig.49).

The projected *R. sphaeroides* Che W protein showed a 59% similarity (and a 30% identity) to the *che* W protein of *E. coli*. When compared to the *R. meliloti* Che W homologue, it showed a 72% similarity (and a 53% identity).

The Che W protein of *S. typhimurium* (Stock *et al*, 1986) and that of *E. aerogenes* and *E. coli* (Dahl *et al*, 1989), have been shown to possess regions suggesting nucleotide (ATP/GTP) binding sites,

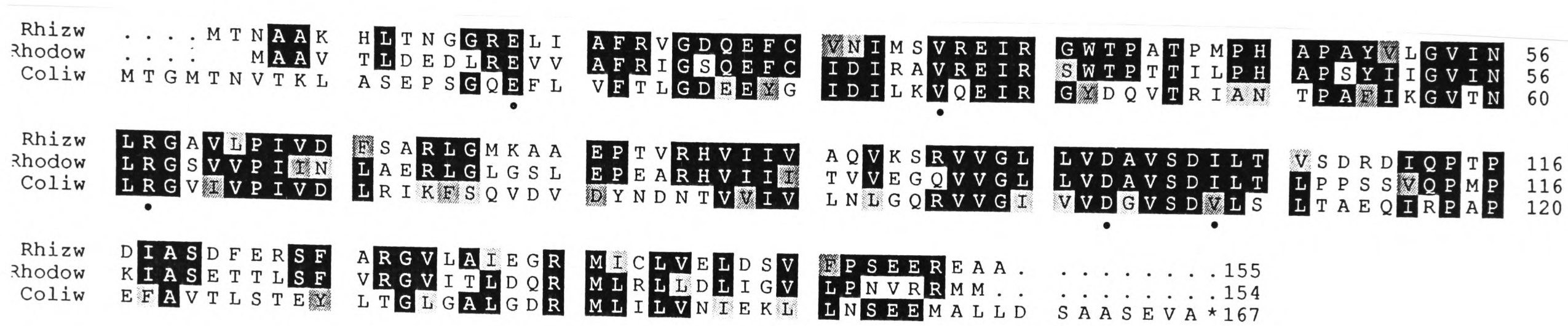


Fig.49: "Prettybox" alignment of Che W proteins from *R. sphaeroides* (Rhodow; Ward *et al*, in press), *R. meliloti* (Rhizw; Greck et al, in press) and *E. coli* (Coliw; Mutoh and Simon, 1986). Black boxes indicate identity; grey boxes indicate conservative substitution. Amino acid residues known to be essential for proper interaction between CheW and Tsr in *E. coli* (Liu and Parkinson, 1991) are indicated by a "•".

specifically the ß-α-ß motif (Wierenga *et al*, 1985). Not all known Che W proteins possess this motif however, and ones that do not include the Che W homologues of *M. xanthus* (Stock, *et al*, 1991) and of *B.subtilis* (Hanlon *et al*, 1991). This ß-α-ß motif and more extensive potential nucleotide binding sequences were searched for within the novel *R. sphaeroides* protein, but no significant similarity was revealed.

Unfortunately, the functional regions of the Che W protein are not well defined at present, but five residues have been identified as being critical to proper Che W activity. Amino acid substitutions at these sites produce an altered (smooth swimming) chemotactic phenotype in *E. coli* (Liu and Parkinson, 1991). These mutations can be suppressed by corresponding mutations in the MCP, Tsr, which suggests that the target amino acid residues are involved in an interaction between Che W and the MCP. All five of these residues were found to be conserved in the *R. sphaeroides* (and *R. meliloti*) Che W protein (fig.49). This not only strongly implies a functional role for these five amino acids residues in all three genera, but also has some very interesting implications for the mechanism of signal transduction in *R. sphaeroides*, an organism which had been thought to lack membrane-spanning MCP's.

In addition, Liu and Parkinson have used a comparison of hydro-phobicity profiles of enteric Che W (and of the transducer, Tsr) to

identify putative sites of Che W - MCP interaction. A Kyte and Doolittle hydrophobicity profile of the *R. sphaeroides* Che W was therefore produced and compared with its enteric counterpart (fig.50). The hydrophobicity profiles of the two molecules are very similar, suggesting similar folding. The five functionally important amino acid residues lie in regions of similar hydrophobicity in both the enteric molecule and its homologue in *R. sphaeroides*.

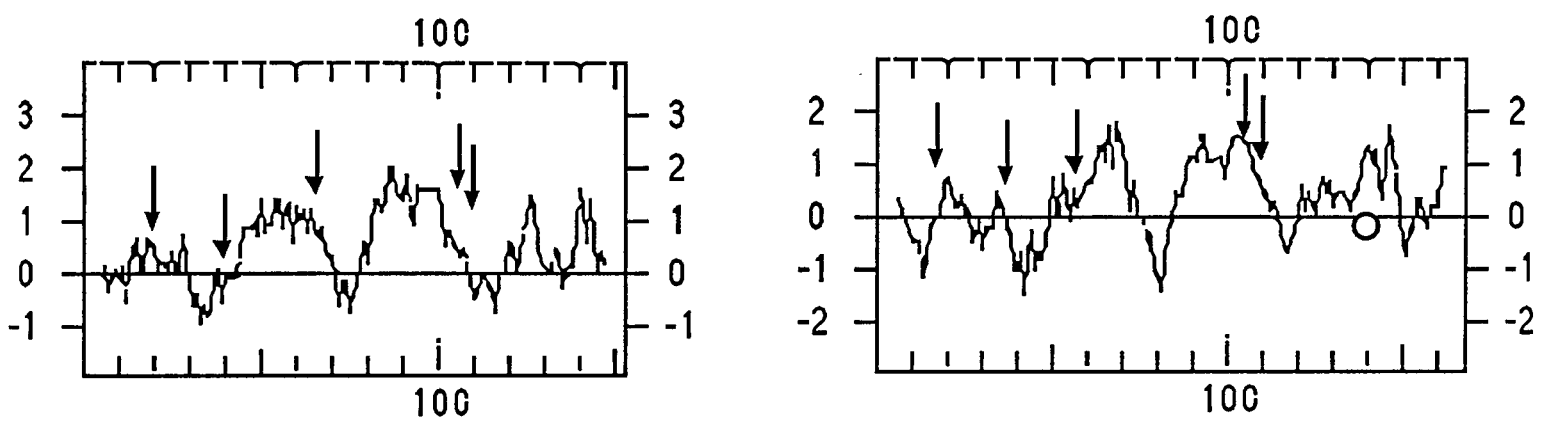


Fig.50: Kyte and Doolittle hydrophobicity plot for *R. sphaeroides* Che W (left) and for *E. coli* Che W (right). Arrows indicate the positions of amino acid residues shown to be important in the interaction between Che W and Tsr. A clear circle indicates the position of a putative (ß-α-ß) nucleotide binding site. The y-axis represents the degree of of hydrophobicity; the x-axis represents the position of the amino acid residues.

3.8.2

Overview of Production of Non-Chemotactic Phenotypes

In order to determine the function of the newly identified *che* genes of *R. sphaeroides* (discovery of *che* R and *che* Y2 is described below) it was decided to produce gene deletions which, it was hoped, would give

rise to behaviourally disturbed phenotypes resulting in a measurable loss of chemotaxis. Initially, transposon mutagenesis was used to interrupt the cloned genes which could then be homologously recombined into the genome via a suicide delivery system causing gene interruption and protein deletion. Later, internal fragments of some *R. sphaeroides* genes were cloned into a suicide vector. When homologously recombined via a single cross-over event, the construct would cause gene disruption preventing protein expression.

3.8.2a

Construction of *R. sphaeroides che* W Mutants Using the Suicide Vector pSUP202

The suicide vector pSUP202 (fig.19) is a narrow host-range plasmid which cannot be maintained in *R. sphaeroides*. Exclusion of the vector forces recombination of the homologous insert into the genomic DNA. A double cross-over event will result in only the *insert* becoming integrated into the genome. A single cross-over event will result in the integration of the entire construct (fig.51).

Prior to engineering of the pSUP202 derived suicide vectors, transposon mutagenesis was carried out on the pUC18 derived construct pCH2 which contained the *R. sphaeroides* genes *che* W, *che* R and *che* Y2. Transposon mutagenesis of pCH2 was done using mini-Tn10 (derivative 103) delivered from the bacteriophage λNK1316 (see methods, 2.4e).

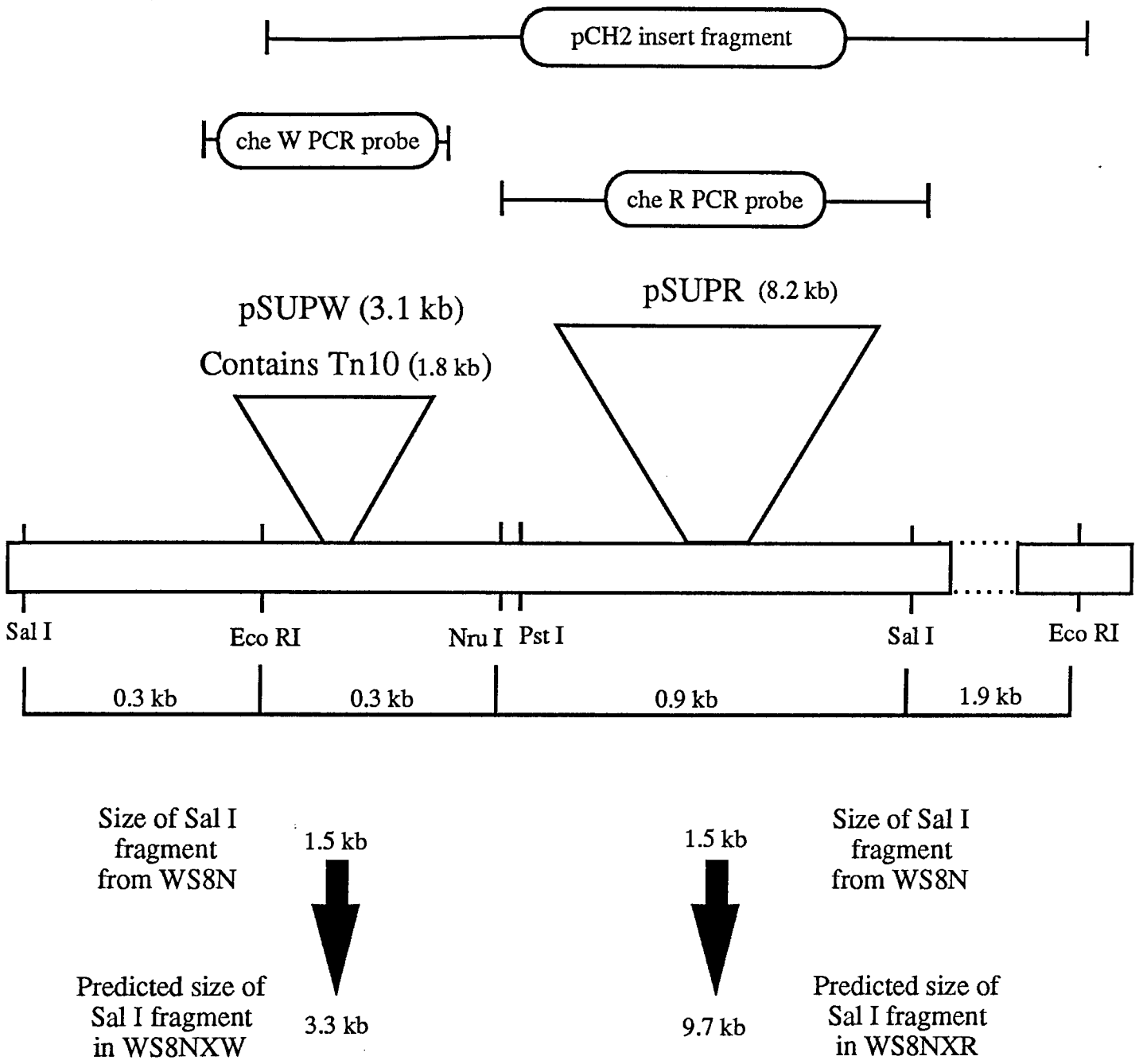


Fig.51: Schematic diagram showing plan for homologous recombination using the pSUP202-derived suicide constructs pSUPR and pSUPW. Expected size changes of the *Sal* I fragment shown. Probe fragments shown above.

About 200 mutagenised clones (showing resistance to 300 µg/ml of kanamycin) were screened by restriction. Despite the fact that the insert was larger than the vector (a 3.1 kb insert in pUC18), the transposon almost invariably integrated into the vector; ie: it was hotspotting. In

the very few cases in which Tn10 did become integrated into the insert, it was the *R. sphaeroides che* W gene that was targeted. These constructs were named the "pCH2T" series (fig.52). pCH2T21 was restricted with *Sal* I to release a 3 kb fragment containing the Tn10 interrupted *che* W gene (as well as the uninterrupted *che* R and *che* Y2 genes). This fragment was ligated into pSUP202 at the *Sal* I site, disrupting the tetracycline resistance gene. The 10.5 kb construct, called "pSUPW", (see fig.20, Materials and Methods) was kanamycin resistant but tetracycline sensitive.

pSUPW was transformed into the broad host-range mobilizing strain *E coli* S17-1 and then conjugated into *R. sphaeroides* WS8N. A double cross-over would result in the replacement of the native *che* W gene with the Tn10-interrupted *che* W gene. Such a recombinant would be kanamycin resistant (and naladixic acid resistant) but ampicillin and chloramphenicol sensitive. Single cross-over recombinants could be identified by virtue of being resistant to ampicillin and chloramphenicol as well as to kanamycin. The resultant transconjugants were called the "WS8NXW" series.

Cloning and antibiotic screening revealed pSUPW clones with inserts in both orientations, either of which was suitable for the homologous recombination step. Conjugation into *R. sphaeroides* WS8N resulted in the isolation of 54 naladixic acid resistant, kanamycin resistant transconjugants. Of these, ten were chloramphenicol sensitive

(WS8NXW 11, 26, 33, 36, 38, 41, 46, 51, 52 and 53). All the transconjugants were ampicillin sensitive, despite the fact that some of them must have been harbouring the ampicillin resistance gene. It was reasoned that the promoter of the ampicillin resistance gene of pSUP202 was not well recognised by any *R. sphaeroides* RNA polymerase and that the gene was therefore not being expressed. Ampicillin was not used as part of any further resistance screens for *R. sphaeroides*.

Chromosomal DNA preparations were made from the ten kanamycin resistant, chloramphenicol sensitive recombinants after aerobic growth with 25µg/ml kanamycin. The recombinant genome was then digested with *Sal* I, run on an electrophoresis gel overnight and Southern blotted onto a nylon membrane. This blot was hybridized using a PCR produced DIG-labelled probe consisting of the entire *che* W fragment without any flanking sequence.

It was expected that hybridization would show that the *Sal* I fragment containing the *che* W gene had increased in size from 1.5 kb to 3.3 kb due to the insertion ~~of the~~ of the 1.8 kb transposon.

The result of this hybridization can be seen in fig.54. In every case the hybridization pattern was the same. Two bands were apparent: one of 1.5 kb and one of 10.5 kb. (The WS8N / *Sal* I control produced the expected 1.5 kb band). It was clear that a simple double cross-over had not occurred. The pattern can be explained as follows. The 1.5 kb

band represents an uninterrupted native copy of the *che* W gene, while the 10.5 kb band represents the "suicide" construct, whole and maintained within *R. sphaeroides*.

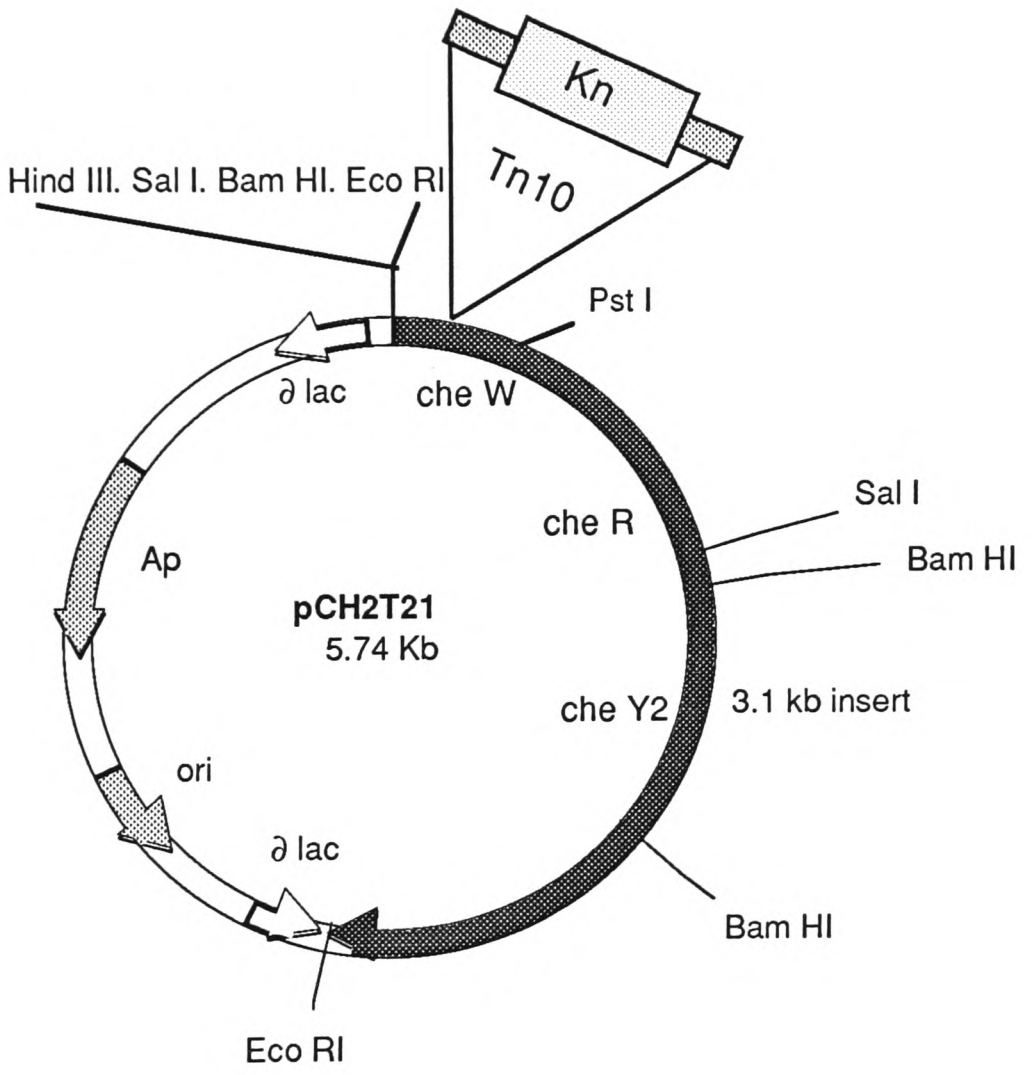


Fig.52: pCH2T21, containing the *R. sphaeroides che* W gene interrupted by mini-Tn10. The 3 kb *Sal* I fragment from this construct was cloned into pSUP202 to make the suicide construct pSUPW.

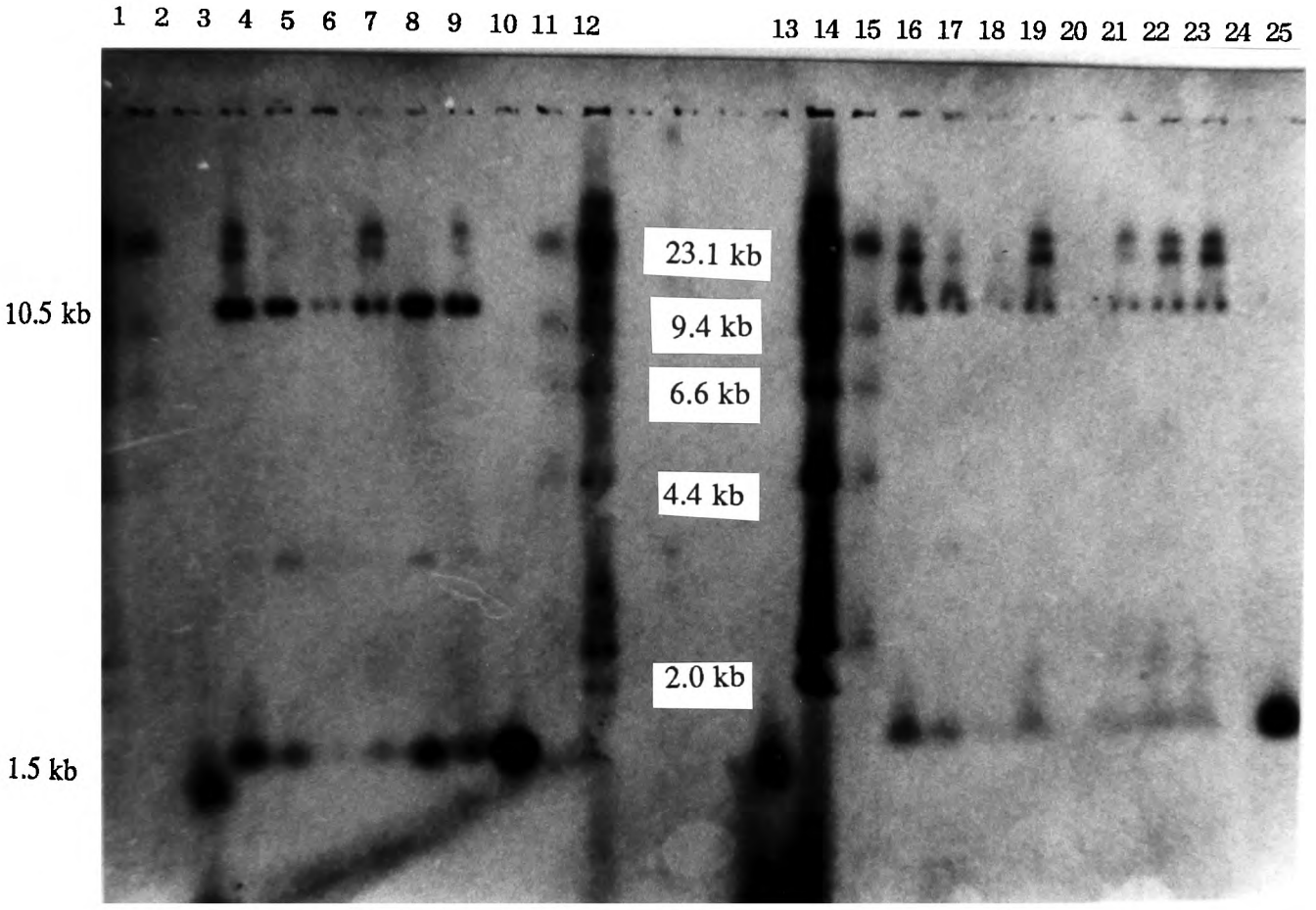


Fig.54: Hybridization pattern produced by WS8NXW *Sal* I chromosomal digests probed with a DIG-labeled *che* W probe. Each mutant digest shows two signals, one at 1.5 kb (= the *Sal* I fragment containing the native *che* W gene) and one at 10.5 kb (= the maintained suicide construct pSUPW [from which *Sal* I sites had been lost due to blunt-end cloning]). WS8N wild-type DNA and the 1.2 kb *Sal* I fragment from pCH2 (which contains the *che* W gene) were both used as positive controls. DIG-labeled λ *Hind* III was used as the molecular weight marker.

From left to right: lane 1 and 2 = λ *Hind* III (lane 1 is cut in half in the printed photograph); lane 3 = 1.2 kb *Sal* I fragment from pCH2 (positive control); lane 4 to 9 = WS8NXW/*Sal* I digests (mutant numbers 53, 52, 51, 46, 33, 11); lane 10 = WS8N/*Sal* I digest (positive control); lanes 11 and 12 = λ *Hind* III; lane 13 = 1.2 kb *Sal* I fragment from pCH2 (positive control); lanes 14 and 15 = λ *Hind* III; lanes 16 to 24 = WS8NXW/*Sal* I digests (mutant numbers 26, 36, 38, 41, 46, 51, 52, 53, 11); lane 25 = = WS8N/*Sal* I digest (positive control).

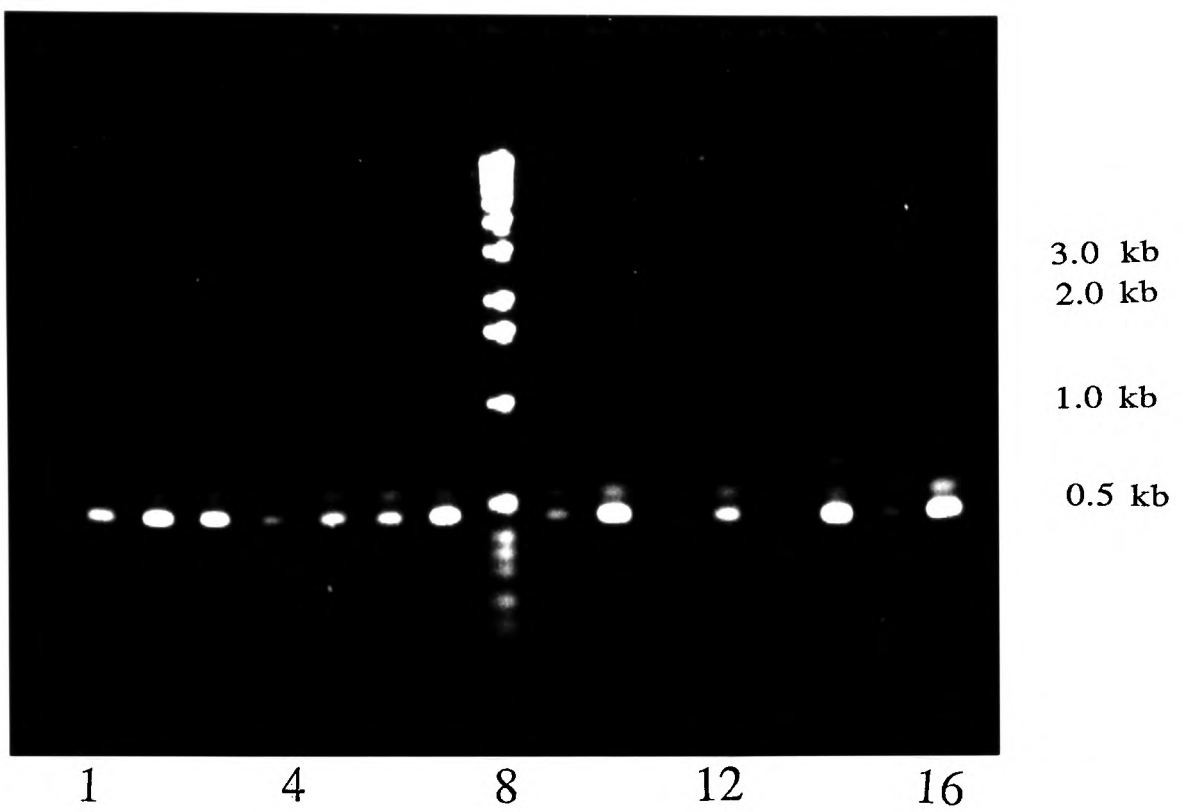


Fig.55: Verification of the presence of an intact copy of *che* W by PCR. WS8NXW mutant genomic DNA was used as a template. pCH2T21 and pSUPW plasmids (which carry the *che* W gene interrupted with a transposon) were used as negative controls. Genomic WS8N wild-type DNA was used as the positive control (two different magnesium concentrations were used in the PCR mixture, hence the different intensities of the two WS8N signals). This gel incedently also proves that the *R. sphaeroides che* W gene is interrupted in the clones pCH2T21 and pSUPW.
From left to right, the templates used in the PCR mixtures for each lane are: lanes 1 - 7 = WS8NXW (mutants numbers 1, 11, 33, 38, 41 and 46); lane 8 = 1 kb molecular weight markers; lane 9 = WS8NXW mutant number 51; lane 10 = WS8NXW mutant number 51; lane 11 = pCH212; lane 12 = WS8NXW mutant number 21; lane 13 = pSUPW; lane 14 = WS8N; lane 15 = (= 4 μg Mg^{2+}); lane 16 = WS8N (= 2 μg Mg^{2+}) For each PCR reaction mixture, 1 μl of undiluted template DNA was used. "Vent" polymerase was used in the reactions for lanes 1 to 12. "Taq" polymerase was used in the reactions for lanes 13 and 14. 2 μl or the reaction mixture was pipetted into each well of the electrophoresis gel. All nine tested mutant genomes contain a whole copy of the *R. sphaeroides che* W gene.

3.8.2b

Verification of the Presence of the Intact *che* W Gene by PCR

The presence of the intact *che* W gene was checked by PCR. Flanking oligonucleotides primers of *che* W were used and the WS8NXW mutant genomic DNA was used as a template. Genomic WS8N DNA was used as a positive control template and pCH2T and pSUPW plasmids were used as the negative control templates. If an uninterrupted copy of *che* W is present, PCR would produce a product of the appropriate size (459 bp), but if the sole copy of the gene had been interrupted by Tn10, no product would be produced. The results are displayed in fig.55. This PCR experiment confirmed that the WS8NXW mutants contained the uninterrupted *che* W gene.

3.8.2c

Phenotype of the *R. sphaeroides che* W mutant WS8NXW

Although it had been conclusively proved that the WS8NXW mutants possessed a maintained copy of the suicide vector pSUPW, plug plate assays were carried out using the mutants to definitively determine behavioural phenotype. The results (fig.56) clearly show that the WS8NXW mutants are capable of normal chemotactic accumulation in response to weak organic acids, to sugars and to amino acids.

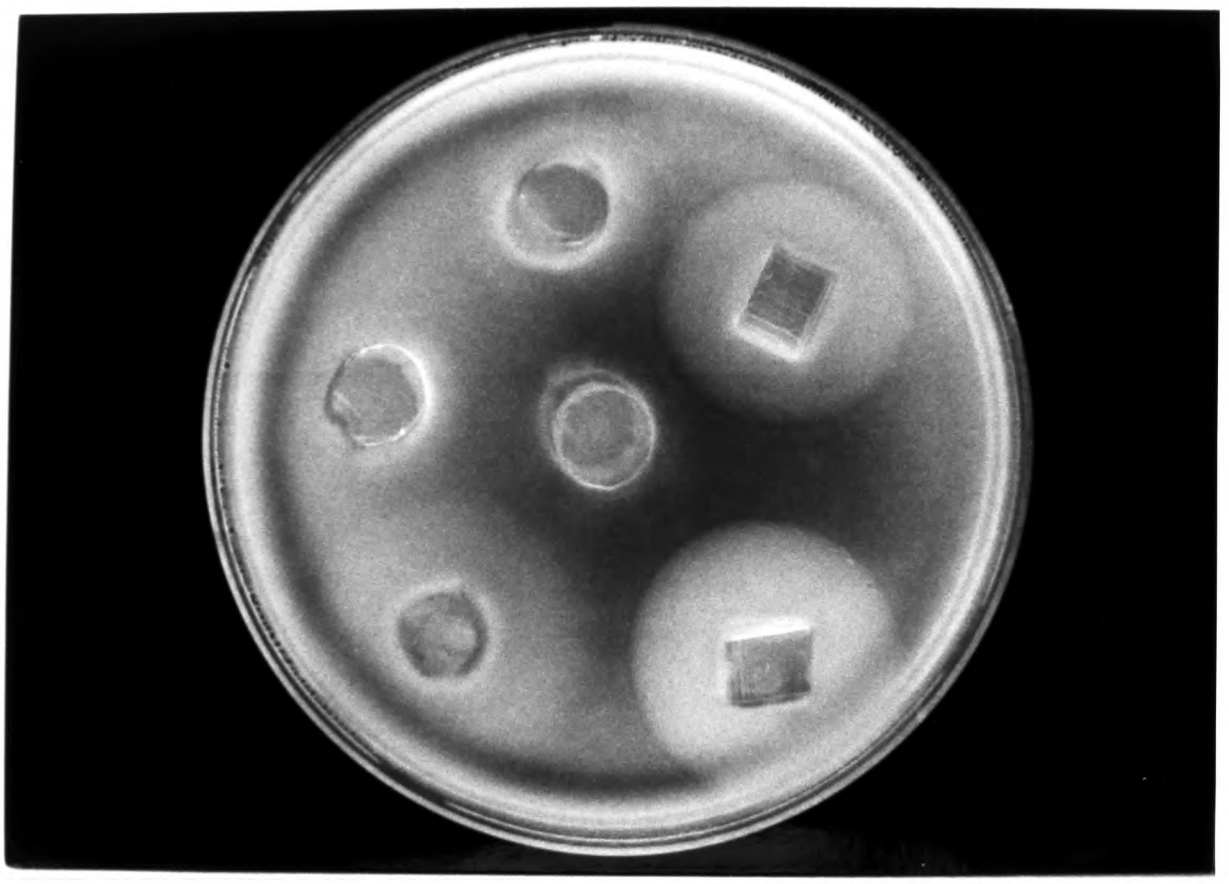


Fig.56: Plug plates of *R. sphaeroides* WS8NXW mutant showing accumulation around plugs containing 50 mM chemoattractant. From 12 O'clock going clockwise the plugs contain: succinate, acetate, fructose, glutamate, serine. The central plug containing 10 mM Na HEPES buffer.

3.8.2d

Complementation of *E.coli che* W Mutants Using the *R.sphaeroides che* W Gene

Dr. J. S. Parkinson, of the University of Utah, kindly provided us with a number of *E. coli* mutants with deleted or interrupted *che* genes. *E. coli* RP4606 contains a nonsense mutation in the *che* W gene, giving it a non-swarming phenotype. It was decided to attempt complementation of this strain with the *R. sphaeroides che* W gene.

IPTG-induced expression of *R. sphaeroides che* W would be achieved using the expression vector pTM30 (fig.22, Materials and Methods). Since no convenient restriction sties were present, and in order to avoid the inclusion of adjacent sequence, PCR cloning was employed. Forward and reverse oligonucleotide primers were synthesised for the *R. sphaeroides che* W gene and PCR was carried out, producing large amounts of the blunt *che* W gene.

The only restriction site in pTM30 that would provide the correct reading frame for the cloned gene was *Kpn* I, thus pTM30 was restricted with *Kpn* I and the resulting 5' recessed ends were blunted using the 3' exonuclease and proofreading activity of T4 polymerase. The 459 bp che W gene was then blunt-end ligated into the vector. Resultant clones were checked by restriction with *Sal* I and *Pst* I. The clone "pTMW26" (fig.57) was found to produce a 1.2 kb *Sal* I / *Pst* I fragment (containing *che* W) whereas religated vector produced only a

0.7 kb fragment. The orientation of the *che* W insert was checked by restriction with *Eco* RI (within the insert) and *Hind* III (in the vector), and was found to be the correct orientation to allow expression.

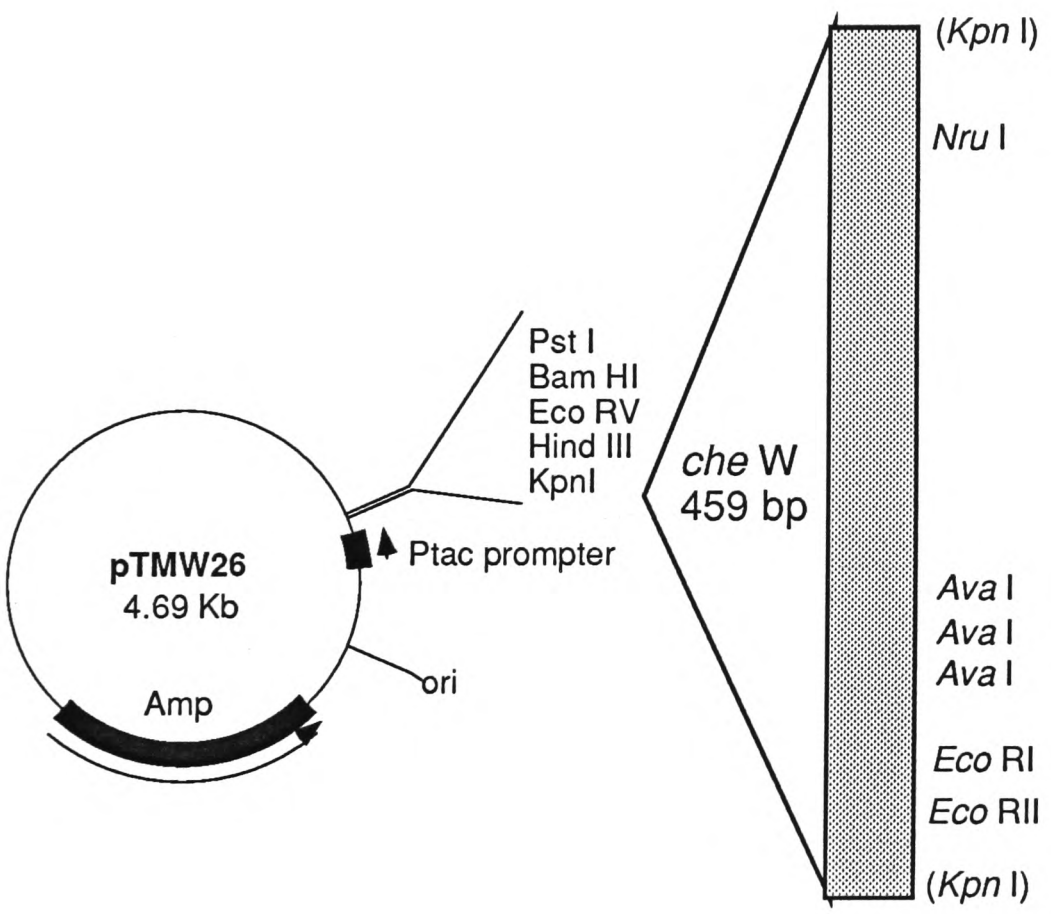


Fig.57: Protein expression construct pTMW26 was made by cloning the 459 bp PCR manufactured *R. sphaeroides che* W gene into the *Kpn* I site of pTM30. The P*tac* promoter allows controlled gene expression by induction with IPTG.

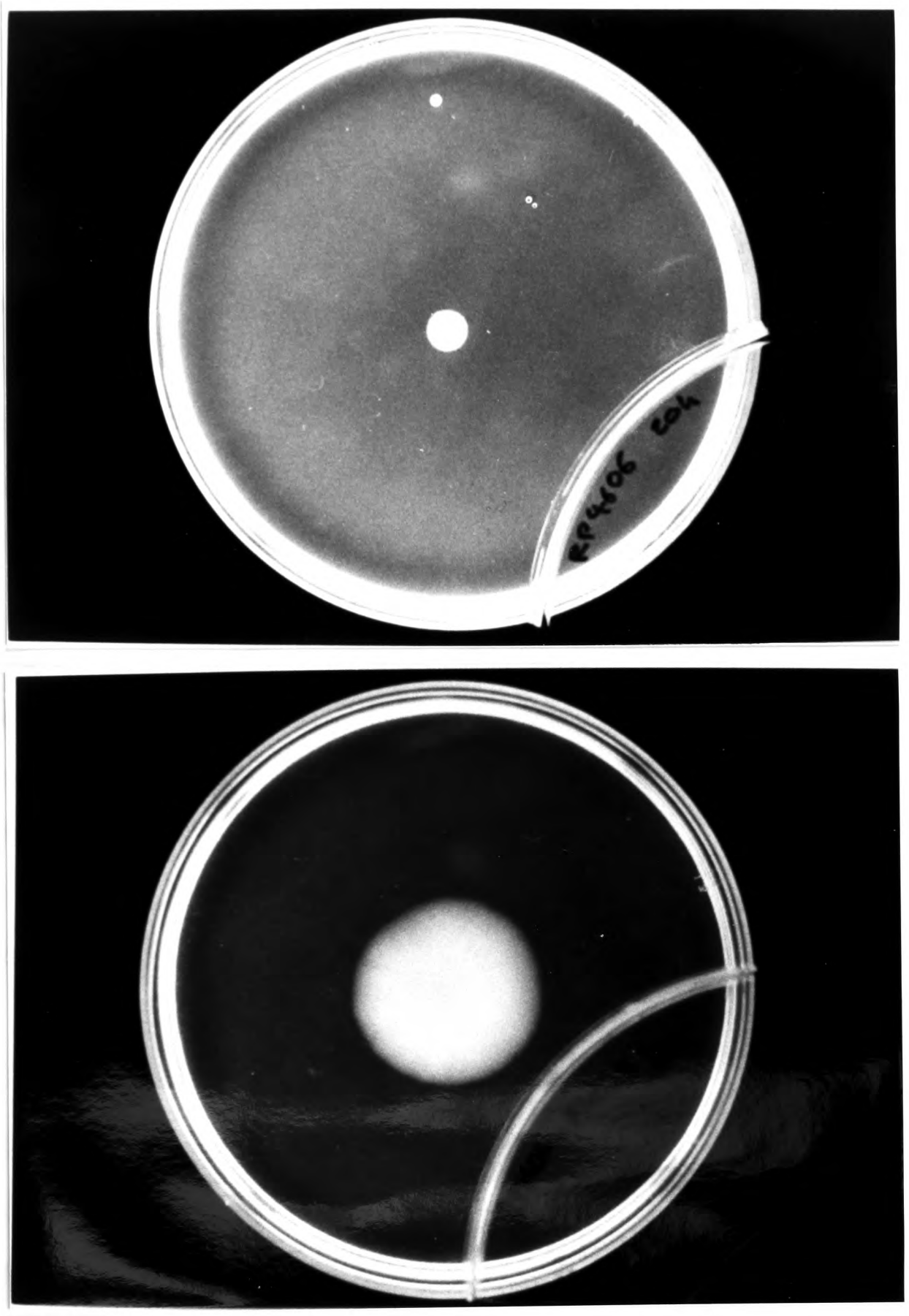


Fig.58: Complementation of the *E. coli che* W⁻ mutant RP4606 by the *R. sphaeroides che* W gene. Uncomplemented *E. coli* RP4640 (above) produced a swarm of 0.8 cm diameter after incubation for 20 hrs at 28°C. *E. coli* RP4640 that had been transformed with the protein expression construct pTMW26 (below) produced a swarm of 2.9 cm diameter after incubation for 20 hrs at 28°C. Complementation has clearly occurred. Swarm plates contained complex media semi-solidified by the addition of 0.3% agar. 40µg/ml of IPTG was present to induce expression.

pTMW26 was then transformed into *E. coli* RP4606 which had been made competent. The resultant (ampicillin resistant) transformants were assayed for swarming on 0.3% agar swarm-plates containing 40 μg/ml IPTG (Materials and Methods, 2.3e) Following overnight incubation of the inoculated swarm-plates, the untransformed mutant had produced a swarm of 0.8 cm diameter, whereas the transformed mutant had produced a swarm of radius 2.9 cm (fig.58).

Apparently, the *che* W gene of *R. sphaeroides* restores the swarming phenotype to the *E. coli che* W mutant RP4606. Such transgenic complementation strongly indicative of a similar mechanism of action for both the Che W proteins. It does not, however, necessarily imply that the Che W protein in both organisms is part of a system mediating signal transduction to the same type of stimulus.

3.8.3

Discovery of the *R. sphaeroides che* R Gene Homologue - Analysis of this Gene and of its Projected Protein

R. sphaeroides ORF R (fig.48) demonstrated a 49% identity to the *E. coli che* R gene and a 55% identity to the *R. meliloti che* R homologue. On the protein level, *R. sphaeroides* Che R showed a 58% similarity (and a 41% identity) to the Che R protein of *E. coli* and a 66% similarity (46% identity) to the Che R protein of *R. meliloti*. Upon translation, ORF R gives a protein of 309 amino acid residues; closer in length to the putative Che R protein of *R. meliloti* (302 amino

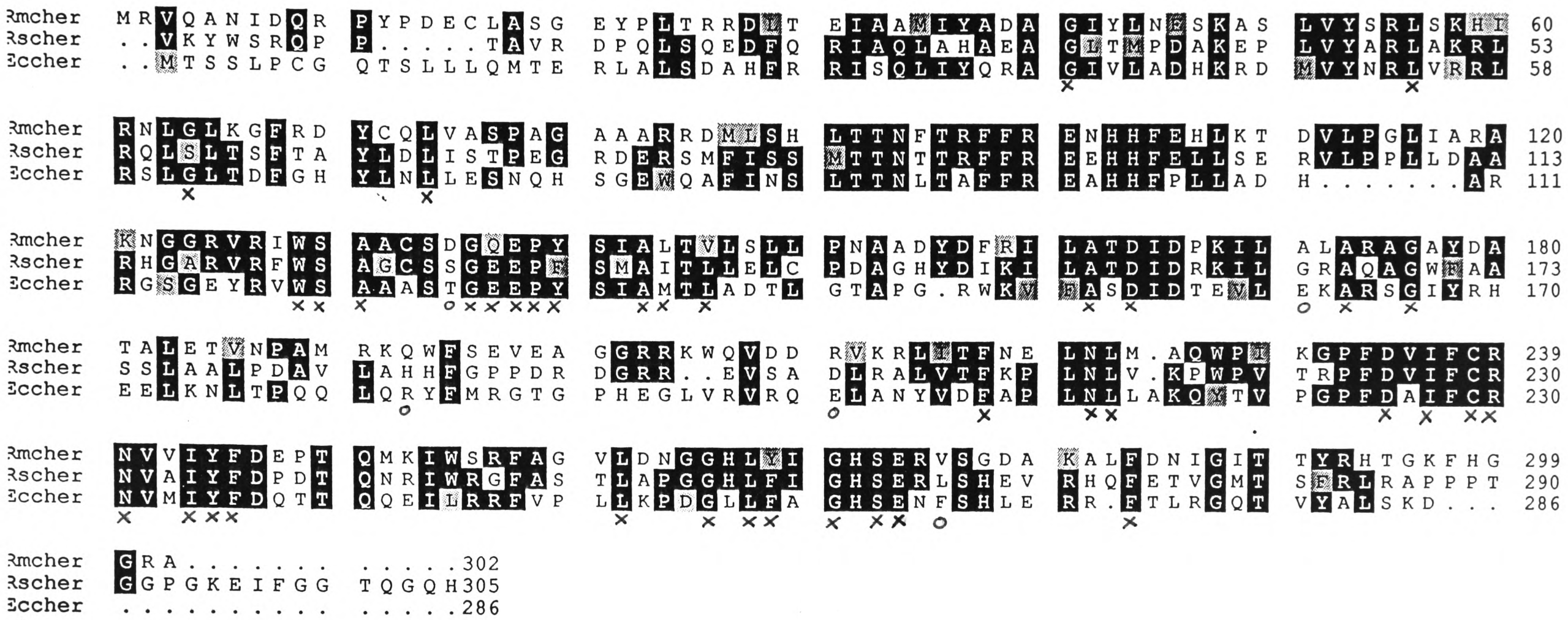


Fig.59: "Prettybox" alignment of Che R proteins from *R. sphaeroides* (Rscher; Ward *et al*, in press), *R. meliloti* (Rmcher; Greck *et al*, in press) and *E. coli* (Eccher; Mutoh and Simon, 1986). Black boxes indicate identity; grey boxes indicate conservative substitution. Amino acid residues conserved between *E. coli* Che R, B. subtilis Che R, *M. xanthus* Frz F (Kirsch *et al*, 1993) and *R. sphaeroides* Che R are indicated by a "X". Residues conserved across all named proteins with the exception of *R. sphaeroides* Che R are indicated by a"O".

acid residues) than to the Che R protein of *E. coli* (281 amino acid residues) (fig.59).

The greater degree of similarity to *Rhizobium* is probably indicative of the degree of evolutionary relatedness. This observation is in agreement with the results for the newly discovered *che* A and *che* Y genes of *R. sphaeroides* (M.J. Ward, personal communication).

As is the case for Che W, the Che R protein is not functionally well defined at present. Che R shows a large degree of primary structural variation between species and appears to be the least conserved of all the Che proteins (Simms *et al*, 1987). The residue Cys31 of the *S. typhimurium* methyltransferase enzyme encoded by *che* R is believed to be located within the active site of the enzyme and has been shown to play a central role in methyltransferase activity (Subbaramaiah *et al*, 1991). Also, cysteine residues at specific locations have been identified as being functionally important to the methyl transferase activity of Type II restriction-modification enzymes (Everett *et al*, 1990; Som and Friedman, 1991). No similarly positioned cysteine residues appear in the *R. sphaeroides* or in the *E. coli* Che R protein, neither are there any cysteine residues conserved across the *E. coli*, *R. meliloti* and *R. sphaeroides* Che R protein sequences.

A recent study (Kirsch *et al*, 1993), comparing the Che R protein of *B. subtilis* with the Che R protein of *E. coli* and the Frz F protein of *M. xanthus,* has identified 46 conserved amino acid residues, confined

mainly (72%) to four regions of high homology (fig.59). The conservation of these sites across the three genera strongly suggests a conserved functional role. The *R. sphaeroides* Che R protein displays identity at 83% of these sites with identity particularly high (88%) within the four highly conserved regions (fig.59). The significance of these regions is as yet unknown but the fact that *R. sphaeroides* Che R possesses them strongly suggests that the mechanism of action of this protein is similar to that of other Che R proteins.

3.8.3a

Construction of *R. sphaeroides che* R Mutants Using the Suicide Vector pSUP202

Construction of the *che* R mutant proceeded in parallel with *che* W mutant production (above). In the case of *che* R, Tn10 hotspotting (see above) meant that transposon mutagenesis was impracticable and it was decided to inactivate the native gene using a suicide construct containing a small internal fragment of *che* R.

The construct pCH2 was cut with *Nru* I to release a 694 bp internal fragment of *che* R. This fragment was then cloned into the blunted *Sal* I site of pSUP202, deleting the tetracycline resistance gene. The resulting 8.2 kb constructs were ampicillin resistant, chloramphenicol resistant and tetracycline sensitive and were called the "pSUPR" series (fig.21). These were transformed into the mobilizing strain *E. coli* S17-1 and then conjugated into *R. sphaeroides* WS8N. A single cross-

over was required in this case, and would result in the integration of the entire construct, conferring chloramphenicol (and ampicillin) resistance upon the recombinant strain ("WS8NXR"). The native *che* R gene would be interrupted by this recombination, preventing normal gene expression.

100 chloramphenicol resistant colonies were picked from swarm agar to succinate plates containing 3 μg/ml chloramphenicol. 98 of these isolates proved non viable and died with no sign of growth. The reason for this is unknown, although it seems reasonable to speculate that there was some problem with the activity of the antibiotic in the initial incubation step. The two transconjugants which were viable, WS8NXR1 and WS8NXR4 were cultured in liquid succinate medium and chromosomal DNA was prepared. These chromosomal preparations were restricted with *Sal* I run on an electrophoresis gel over night, and Southern blotted onto nylon. Hybridization was done using a 1332 bp PCR-derived DIG-labelled *che* R probe which had been made using the sequencing oligonucleotides λ and Z (fig.47). This probe contained homology to *che* R (as well as to a short 5' portion of *che* Y2).

It was expected that a size change would be apparent between the wild-type *Sal* I fragment (1.5 kb) and the fragment from the mutant strain containing the integrated pSUPR construct (9.7 kb) [fig.51]. This pattern was not seen. Hybridization produced bands at 1.5 kb and at 8.2 kb (fig.60).

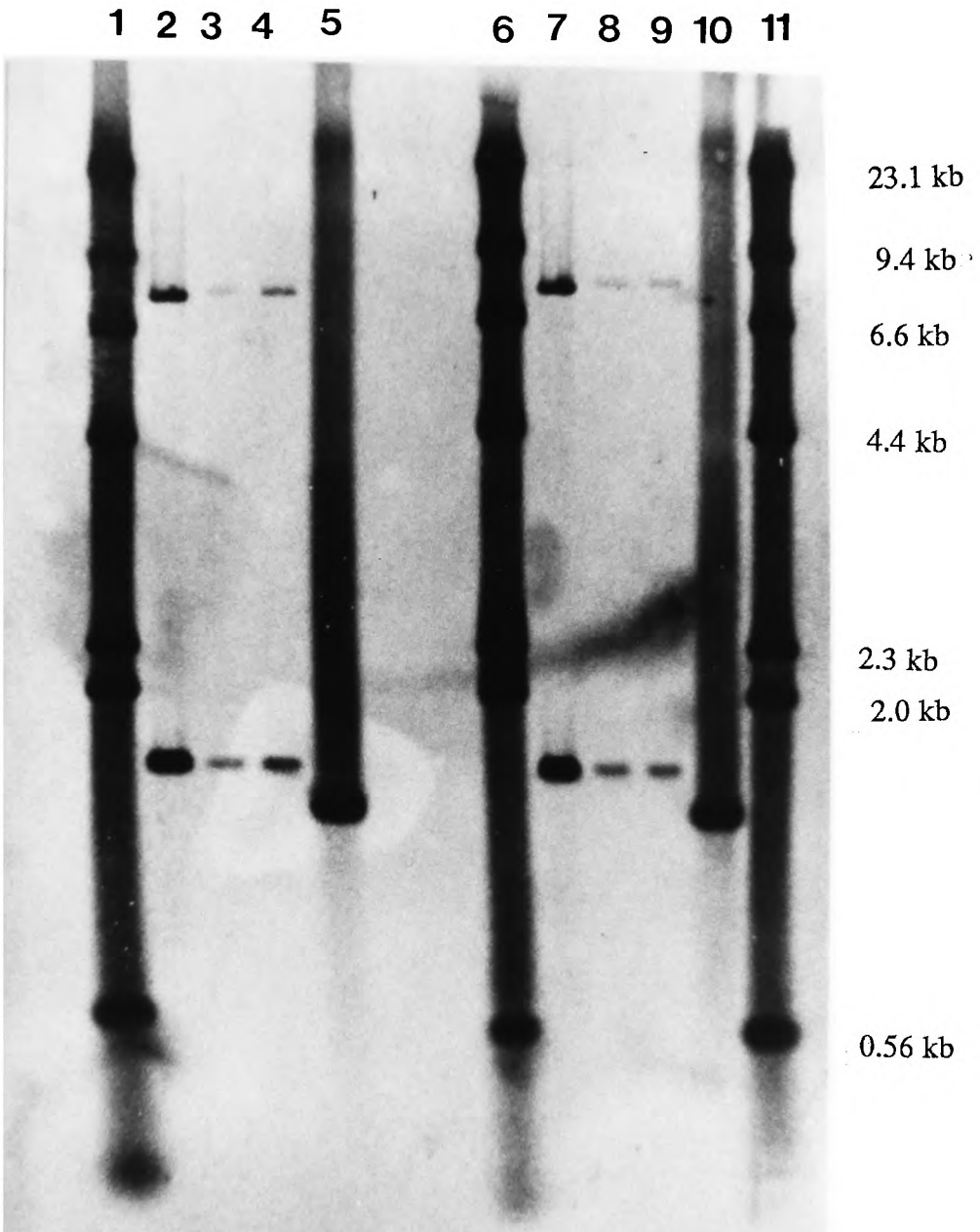


Fig.60: Hybridization pattern produced by WS8NXR *Sal* I chromosomal digests probed with a DIG-labeled *che* R probe. Each mutant digest shows two signals, one at 1.5 kb (= the *Sal* I fragment containing the native *che* R gene) and one at 8.2 kb (= the maintained suicide construct pSUPR [from which *Sal* I sites had been lost due to blunt-end cloning]). WS8N wild-type DNA and the 1.2 kb *Sal* I fragment from pCH2 (which contains the *che* W and *che* R genes) were both used as positive controls. DIG-labeled λ *Hind* III was used as the molecular weight marker.

From left to right: lane 1 = λ *Hind* III; lane 2 = wild-type WS8N/*Sal* I; lane 3 = WS8NXR1/*Sal* I; lane 4 = WS8NXR4/*Sal* I; lane 5 = 1.2 kb *Sal* I fragment from pCH2; lane 6 = λ *Hind* III; lane 7 = wild-type WS8N/*Sal* I; lane 8 = WS8NXR1 /*Sal* I; lane 9 = WS8NXR4/*Sal* I; lane 10 = 1.2 kb *Sal* I fragment from pCH2; lane 11 = λ *Hind* III.

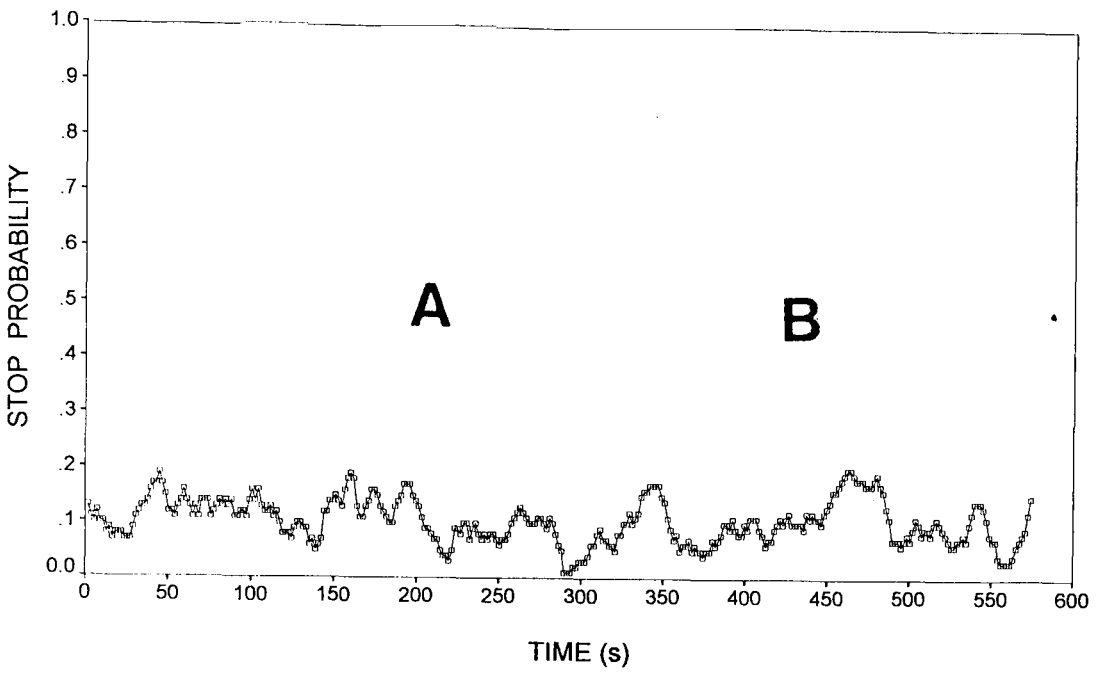
As with the WS8NXW mutants (see above), the WS8NXR mutants were shown to possess a complete and maintained suicide construct (8.2 kb), *and* an uninterrupted copy of the *che* R gene (1.5 kb), identical to that produced by the wild-type genomic digest (fig.60). .

Subsequent similar experiments using the vector pSUP202 carried out by another researcher yielded similar disappointing results, with the "suicide" construct maintained and an intact copy of the target gene being present in the transconjugant *R. sphaeroides* (M.J.Ward, personal communication). In no case in any of the experiments using pSUP202 had the target gene been interrupted. It was concluded that under the experimental conditions used, pSUP202 did not provide a convenient, high frequency delivery system for *R. sphaeroides*, and that to attempt repetition of the experiment using pSUP202 would be a waste of time. It was therefore decided to attempt homologous recombination using another, hopefully more cooperative suicide vector (see 3.8.4).

3.8.3b

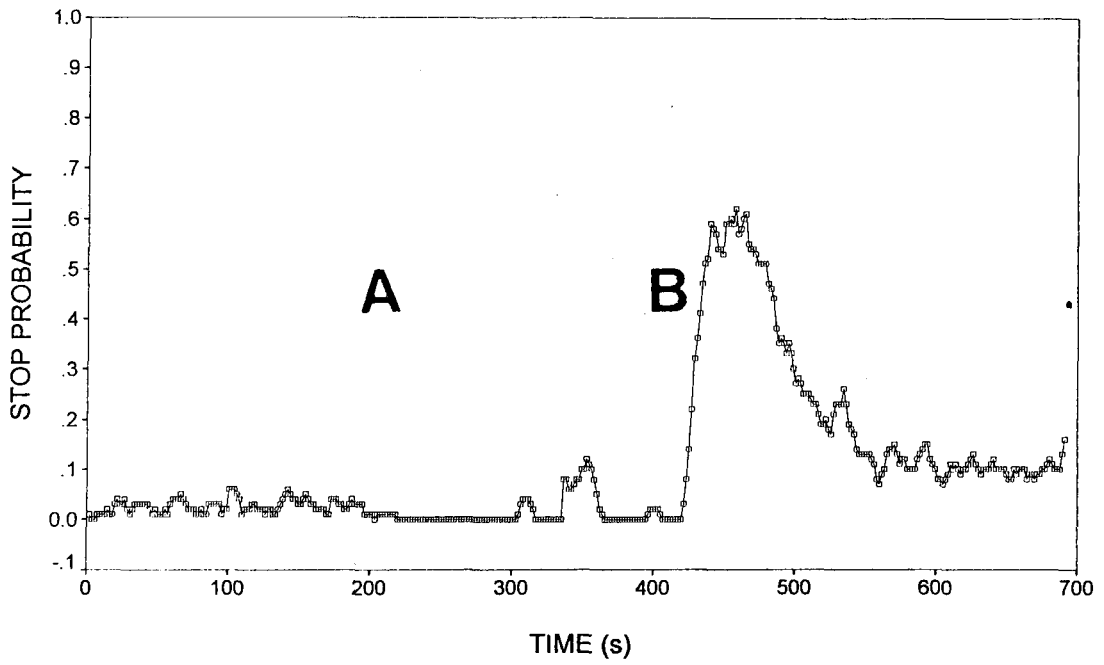
Phenotype of *che* R mutants WS8NXR1 and WS8NXR4

Prior to the hybridization experiment (above), WS8NXR1 and WS8NXR4 were analysed phenotypically using the tethered cell method (Segal *et al*, 1986). Considerable disturbance was observed in the mutants' ability to alter stopping frequency in response to addition or removal of acetate (fig.61), the mutants had a smooth-swimming bias.



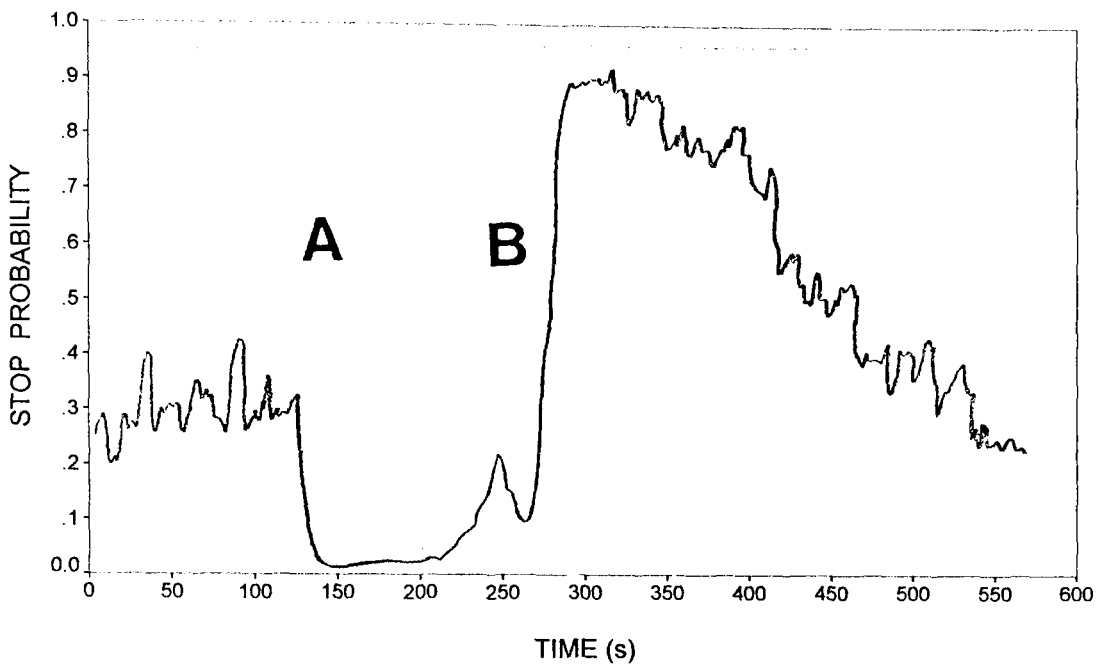
WS8NXR1

Fig.61a



WS8NXR4

Fig.61b



WS8N WILD-TYPE

Fig.61c

Fig.61: Graphs of WS8NXR1 (61a) and WS8NXR4 (61b) stopping probability per 20 second period (calculated every 2 seconds) against time (Seconds). Wild-type behaviour shown in fig.61c. The cells measured were tethered and incubated in a flow chamber which allowed precise temporal variations to be made in the chemical environment. The medium used was 1 mM Na HEPES buffer except as shown. **A**: 1 mM acetate added into the medium. **B**: Acetate removed. The mutants both show a strong smooth-swimming bias and seem to adapt very rapidly to a step down in chemoattractant concentration. A possible explanation for the behaviour of the mutant is offered in the discussion.

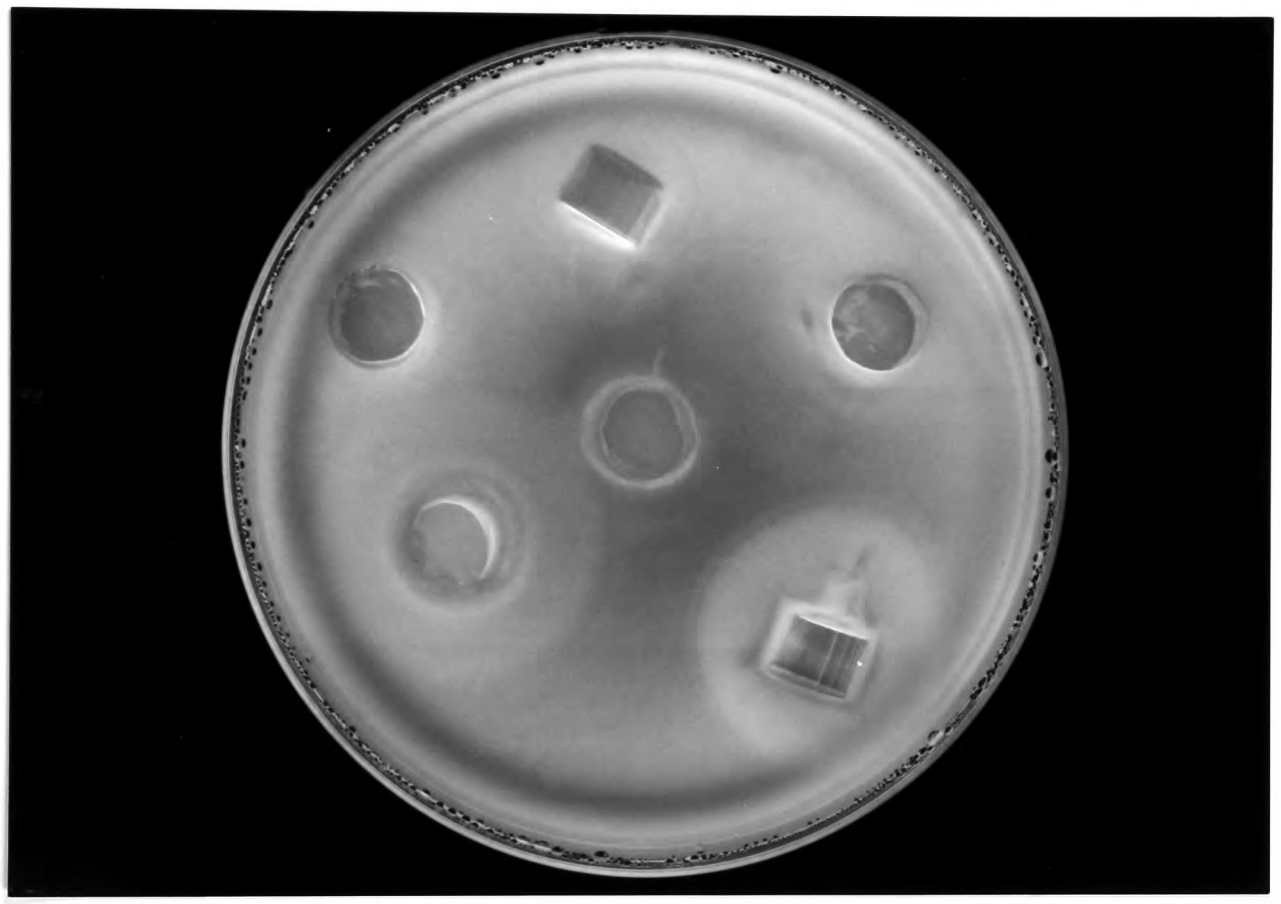


Fig.62: Plug plate assays of *R. sphaeroides* WS8NXR1 showing accumulation towards plugs containing 50 mM attractants. Form 12 O'clock going clockwise, the plugs contain: succinate, acetate, fructose, glutamate, serine. The central plug contains 10 mM Na HEPES buffer.

Such a bias should produce a non-chemotactic phenotype. Plug-plate analysis however, showed a consistent accumulation response to attractants, including sugars, amino acids and organic acids (fig.62). These results are very puzzling, not only because the clearly demonstrated alteration in stopping frequency is at odds with the results of the plug-plate assay, but also because the maintained construct, pSUPR, does not contain the complete *che* R gene. Possession of an extra entire copy of the *che* R gene could be used to explain the change in stopping frequency in terms of over expression (see discussion). On the other hand, pSUPR does contain 75% of the *R. sphaeroides che* R gene, and this may be enough to express a functional protein, at least functional enough to explain the altered adaptation response.

3.8.4

General Strategy for Construction of *che* Mutants Using the Suicide Vector pJP5603

Following the ill-fated mutagenesis experiments involving pSUP202, it was decided to produce gene disruptions in the *che* R gene, and in the *che* W gene (and in the *che* Y2 gene, see later) using the 3.1 kb, kanamycin resistant suicide vector pJP5603.

The gene disruption strategy for *che* R (and *che* Y2, see later) involved the cloning of internal fragments of the gene into pJP5603 (see below and in Materials and Methods). This construct was transformed into *E. coli* S17-1 λ*pir* and conjugated into *R. sphaeroides* WS8N. A single

crossover event would produce a kanamycin resistant mutant in which the entire construct had become integrated into the genome, interrupting the target gene and preventing expression. Since only a single crossover event was required, suitable transconjugants should be produced with a relatively high frequency (in comparison to double crossovers) and the selection procedure would be simple, requiring no sensitivity screening step (unlike for pSUP202 - derived recombinants).

The disruption strategy for *che* W involved replacing the native *che* W gene with one interrupted by Tn10 (derivative 103). The interrupted gene was delivered into WS8N using a pJP5603 - derived suicide vector transformed into *E. coli* S17-1 λ*pir* (as above).

The suicide vector pJP5603 (fig.18) is incapable of replicating in strains not carrying the π protein which is coded for by the λ *pir* gene (see methods). The choice of usable restriction sites for cloning and for the checking of constructs was limited by the presence of multiple *Pst* I, *Hind* III and *Sph* I in the vector (both within and outside the polycloning region) and of *Bam* HI and *Pst* I sites in the inserts. It was therefore decided to first clone the inserts into pUC vectors, making the pUC polycloning sites available for subsequent cloning.

3.8.4a

Construction of *R. sphaeroides che* R Mutants Using pJP5603

A 694 bp internal *Nru* I fragment of *che* R was restricted from pCH2,

purified and cloned into the *Sma* I site of pUC18 to produce "pUCδR". The insert was then cut out of pUCδR using the flanking polylinker sites *Eco* RI and *Sal* I. This fragment was cloned into the Eco RI / Sal I site of pJP5603, transformed into JM109λ*pir* and screened for by α-complementation. The resultant 3.8 kb construct "pJPδR" (fig.63) was then to be transformed into *E. coli* S17-1 λ*pir* and then conjugated into *R. sphaeroides* WS8N. Before this stage could be completed, project time expired.

3.8.4b

Construction of *R. sphaeroides che* W Mutants Using pJP5603

The mini-transposon Tn10 (derivative 103) was introduced into pCH2 (the 5.8 kb pUC18 - derived clone containing a 3.1 kb insert of *R. sphaeroides* DNA which includes the *che* W, *che* R and *che* Y2 genes) via phage λNK1316 (see Materials and Methods, 2.4e). The resultant plasmids carrying Tn10 were designated the "pCH2T" series (fig.52). The pCH2T constructs were screened by restriction, revealing a strong preference ("hotspotting") of Tn10 for the A:T rich sequence of the vector. Of about 200 clones, only two were found with the transposon inserted into the insert. These were called "pCH2T15" and "pCH2T21". The transposon-interrupted inserts from these clones were ligated into the suicide vector pJP5603 to produce the suicide clones "pJPW15" and "pJPW21". These constructs were transformed into *E. coli* S17-1 λ*pir* and thence conjugated into WS8N.

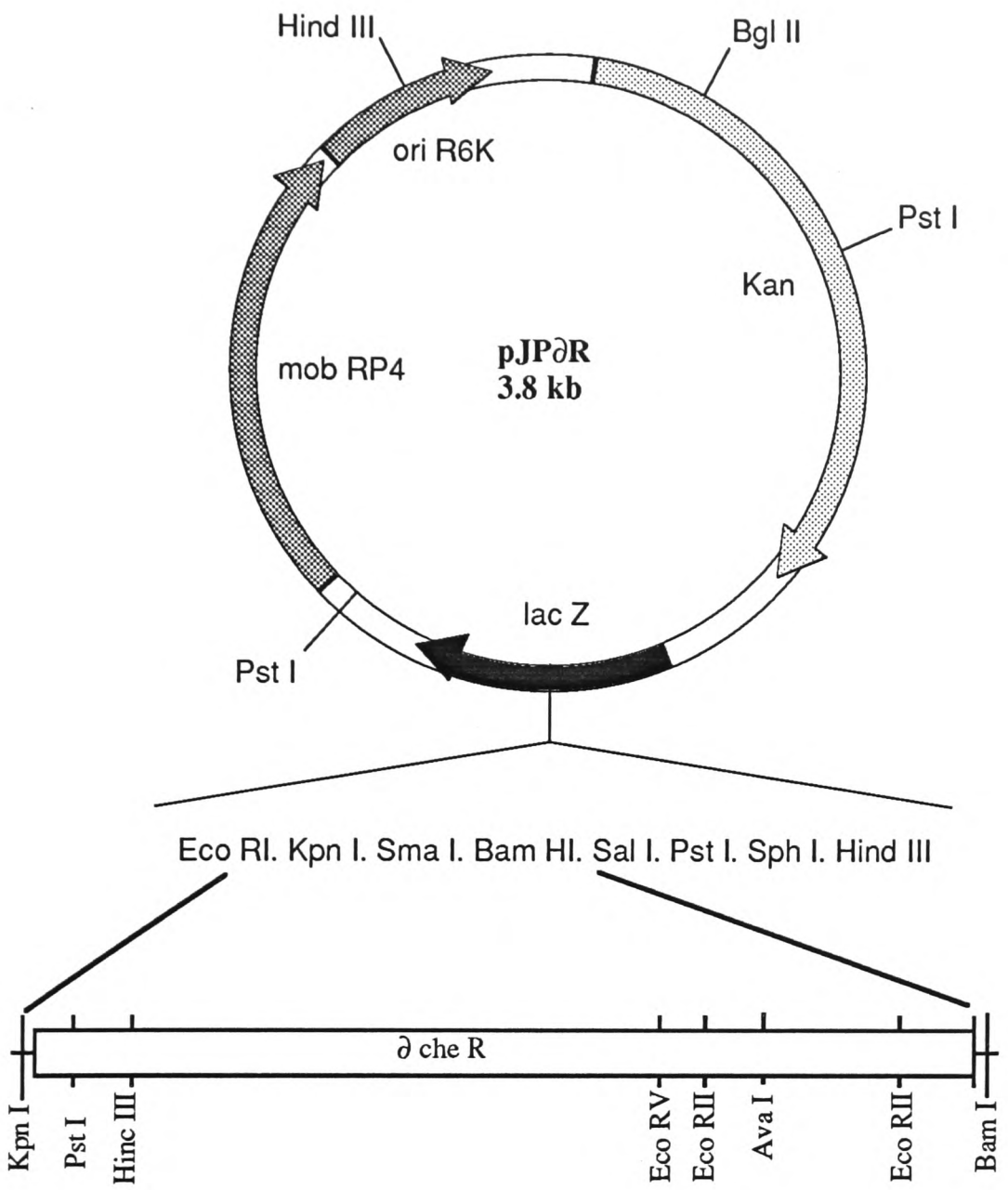


Fig.63: The 3.8 kb suicide construct pJP∂R which was constructed by ligating the *Eco* RI-*Sal* I fragment from pUC∂R (which contains a 694 bp internal fragment of *R sphaeroides che* R) into the *Eco* RI-*Sal* I site of the suicide vector pJP5603.

Resultant recombinant transconjugants (kanamycin resistant) were screened by antibiotic resistance. Two recombinants were selected for analysis: "WS8N::pJPW15" and "WS8N::pJPW21". The locus of recombination was determined by restriction of the recombinant genomes, Southern blotting and hybridization using a PCR

manufactured DIG-labeled probe homologous to the 3' end of *che* W (fig.64).

If homologous recombination had occurred as planned, the following size changes would have been seen between the wild-type and the recombinant: The illuminated *Eco* RI fragment should have increased in size from 3.1 kb to 4.9 kb; and the illuminated *Bam* HI fragment should have decreased in size from 2.7 kb to 1.0 kb (because Tn10 contains *Bam* HI sites).

Hybridization, however, resulted in the following pattern:

WS8N / *Eco* RI.....	3.1 kb
WS8N::pJPW15 / *Eco* RI	3.1 kb and 4.9 kb
WS8N::pJPW21 / *Eco* RI..	3.1 kb and 4.9 kb
WS8N / *Bam* HI.....	2.7 kb
WS8N::pJPW15 / *Bam* HI	2.7 kb and 1.0 kb
WS8N::pJPW21 / *Bam* HI...	2.7 kb and 1.0 kb

In each case, there is a whole, uninterrupted copy of the che W gene *and* a transposon interrupted copy of the gene. This means the "suicide" vector is being maintained in WS8N. WS8N must be producing a protein sufficiently analogous to the π protein to allow maintenance and replication of the pJP5603-derived plasmid. (This hybridization was done after construction of *che* R and *che* Y2 suicide vectors based on pJP5603).

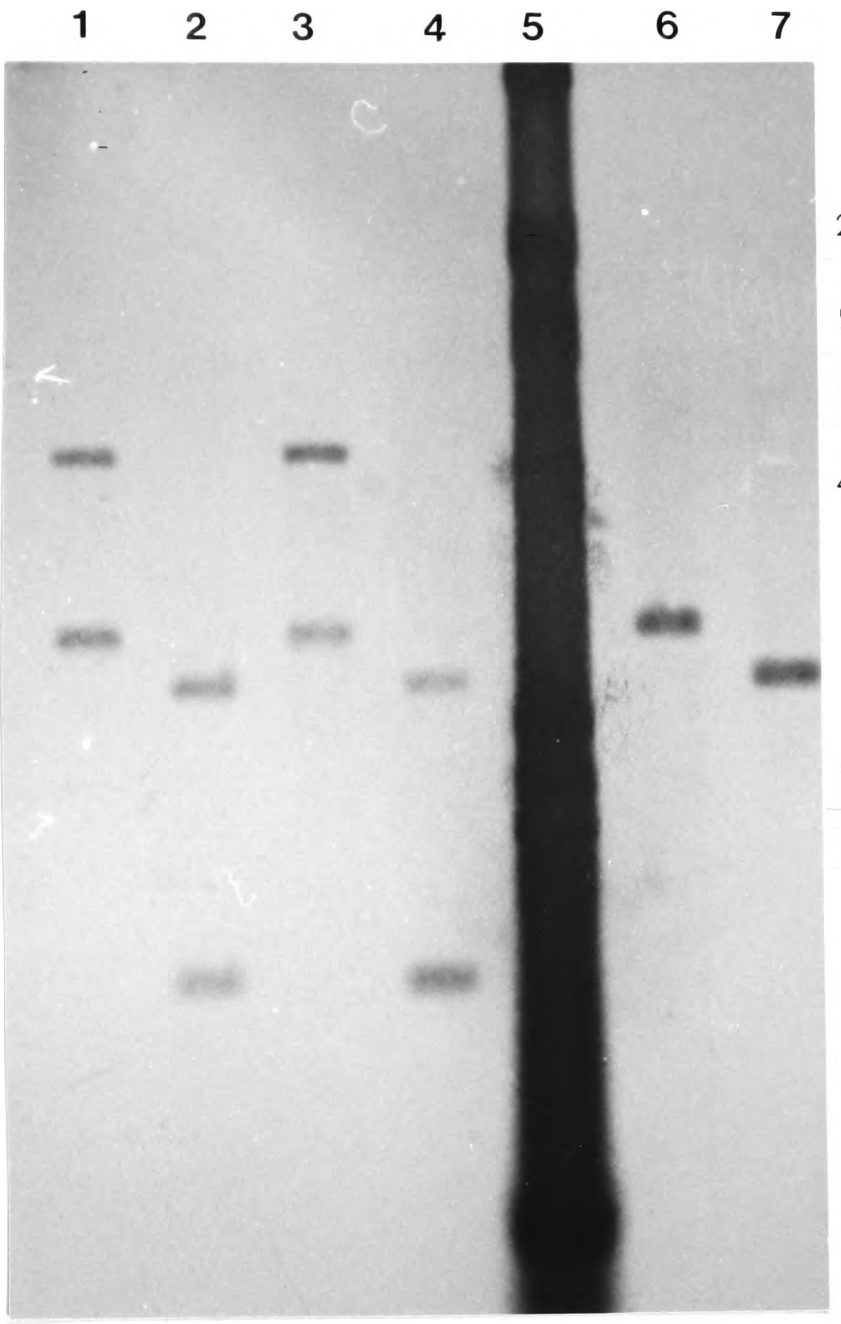


Fig.64: Hybridization pattern produced by *Eco* RI and *Bam* HI digests of the *R. sphaeroides* recombinant mutants WS8N::pJPW15 and WS8N::pJPW21. The probe was a DIG-labeled 210 bp fragment homologous to the 3' end of *R. sphaeroides che* W, made by PCR using the sequencing oligonucleotides alpha and epsilon. Conditions of hybridization were stringent, and the colour reaction was allowed to proceed for 20 hours. DIG-labeled λ *Hind* III fragments were used as molecular weight markers (although they appear over-exposed on this photograph, the bands can be seen). From left to right: lane 1 = WS8N::pJPW15/*Eco* RI; lane 2 = WS8N::pJPW15/ *Bam* HI; lane 3 = WS8N::pJPW21/*Eco* RI; lane 4 = WS8N::pJPW21/ *Bam* HI; lane 5 = DIG-labeled λ *Hind* III; lane 6 = WS8N/*Eco* RI; lane 7 = WS8N/*Bam* HI.

3.8.5

Discovery of a Second Copy of the *che* Y gene in *R.sphaeroides* - Analysis of this Gene and of its Projected Protein

By comparison with the operon structure of *E. coli*, of *S. typhimurium* and of *R. meliloti*, it had been suspected that the gene following the *che* R homologue might be the homologue of *che* B. Initial examination of the sequence data seemed to bear this out, with the projected protein of *R. sphaeroides* ORFY2 (or as it was called at this stage "ORF B") demonstrating 44% similarity (21% identity) to the *E. coli* Che B protein over the first 45 N terminal amino acid residues. This N terminal homology to Che B however, sharply deteriorated downstream.

ORFY2 (at 357 bp) was found to be considerably smaller than the *che* B gene of either *E. coli* (1050 bp) or of *R. meliloti* (the exact size of which had not been identified at that time). In fact, its size was much closer to that of a *che* Y gene, which is 384 bp in *E. coli* and 363 bp in *R. meliloti*. A *che* Y gene of *R. sphaeroides* had however, already been identified at a site paralleling the gene arrangement in *R. meliloti* and *C. cresentus* operons (M.J.Ward, this laboratory).

It thus appeared that we had either found some form of polar degeneracy of the *che* B gene or we had discovered a second copy of the *che* Y gene at a locus where *che* B was expected.

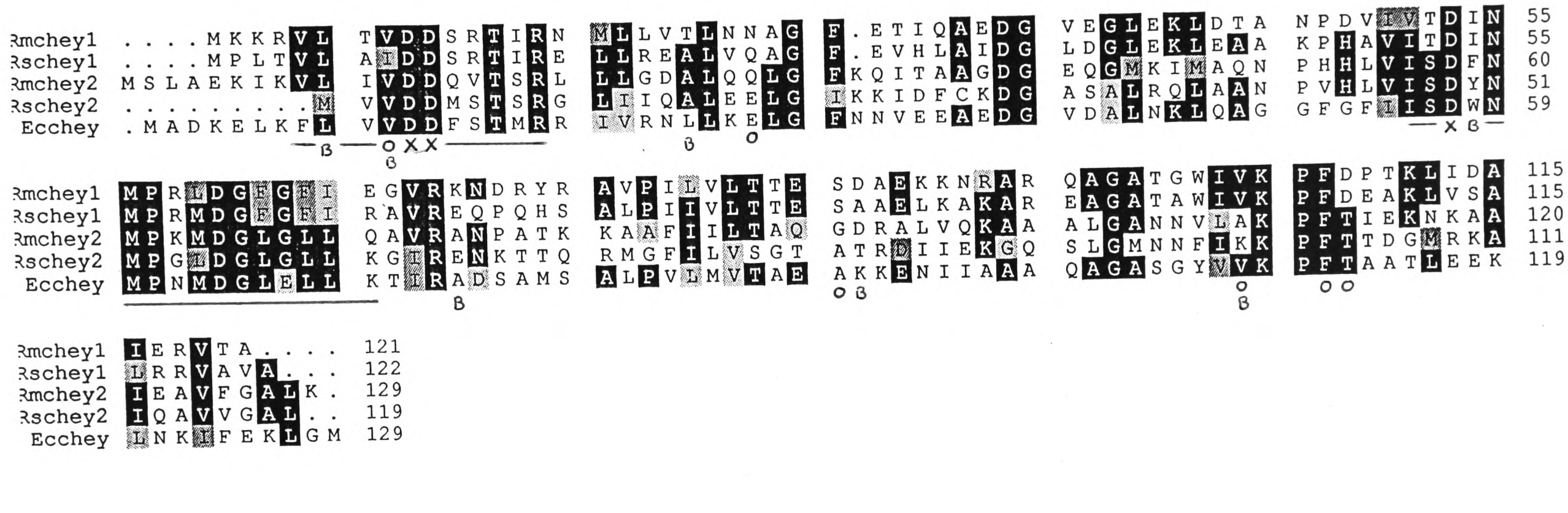


Fig.66: "Prettybox" alignment of Che Y and Che Y2 proteins from *R. sphaeroides* (Rschey1 and Rschey2; Ward *et al,* in press), *R. meliloti* (Rmchey1 and Rmchey2; Greck et al, in press) and *E. coli* (Ecchey; Matsamura *et al,* 1984). Black boxes indicate identity; grey boxes indicate conservative substitution. Regions which form the acid pocket in the mature protein are underlined (Lukat *et al,* 1990). "X" = residues known to be essential for Mg^{2+} binding (Bourret *et al,* 1990 and 1993; Lukat *et al,* 1990). "O" = residues known to be involved in communication between phospho-Che Y and the motor-switch complex (Roman *et al,* 1992). "B" = position of the seven residues that in the Che B protein would be functionally essential (only one of which, Lys^{100}, is present in the same location in *R.sphaeroides* Che Y2) [Stewart *et al,* 1993].

Detailed discussion between the group of Rudigger Schmitt in Regensburg and our group in Oxford led to the conclusion that both *R. sphaeroides* and *R. meliloti* possess a second copy of the *che* Y gene. In *R. meliloti* this gene is located after the *che* B gene (R. Schmitt, personal communication). In light of this, it appeared that in *R. sphaeroides*, the *che* B gene has been lost (at least from its "usual" locus) and that a second (but not identical) copy of *che* Y gene is present.

Upon translation, the *R. sphaeroides* Che Y2 protein displays a 55% similarity (and a 33% identity) to *E. coli* Che Y and it displays a 52% similarity (and a 27% identity) to *R. meliloti* Che Y. The greatest degree of homology is shown with *R. meliloti* Che Y2, to which *R. sphaeroides* Che Y2 shows a 70% similarity (and a 45% identity). The fact that Che Y2 is more similar to the *E. coli* Che Y protein than to the *R. meliloti* Che Y protein is surprising, but perhaps most surprising, is that the Che Y2 protein shows only a 53% similarity (and a 30% identity) to the *che* Y protein from *its own* genome, less than the homology shown for the *E. coli* Che Y protein.

A dendrogram, which compares homology and shows the degree of evolutionary relatedness between proteins was prepared for the Che B, Che Y and Che Y2 proteins sequences of *E. coli*, *R. meliloti* and *R. sphaeroides*. From this, it is apparent that *R. sphaeroides* Che Y2 is most closely related to the *R. meliloti* Che Y2 protein, showing

considerable evolutionary divergence from the progenitor it shares with the Che Y proteins of *R. sphaeroides* and of *R. meliloti* (see below).

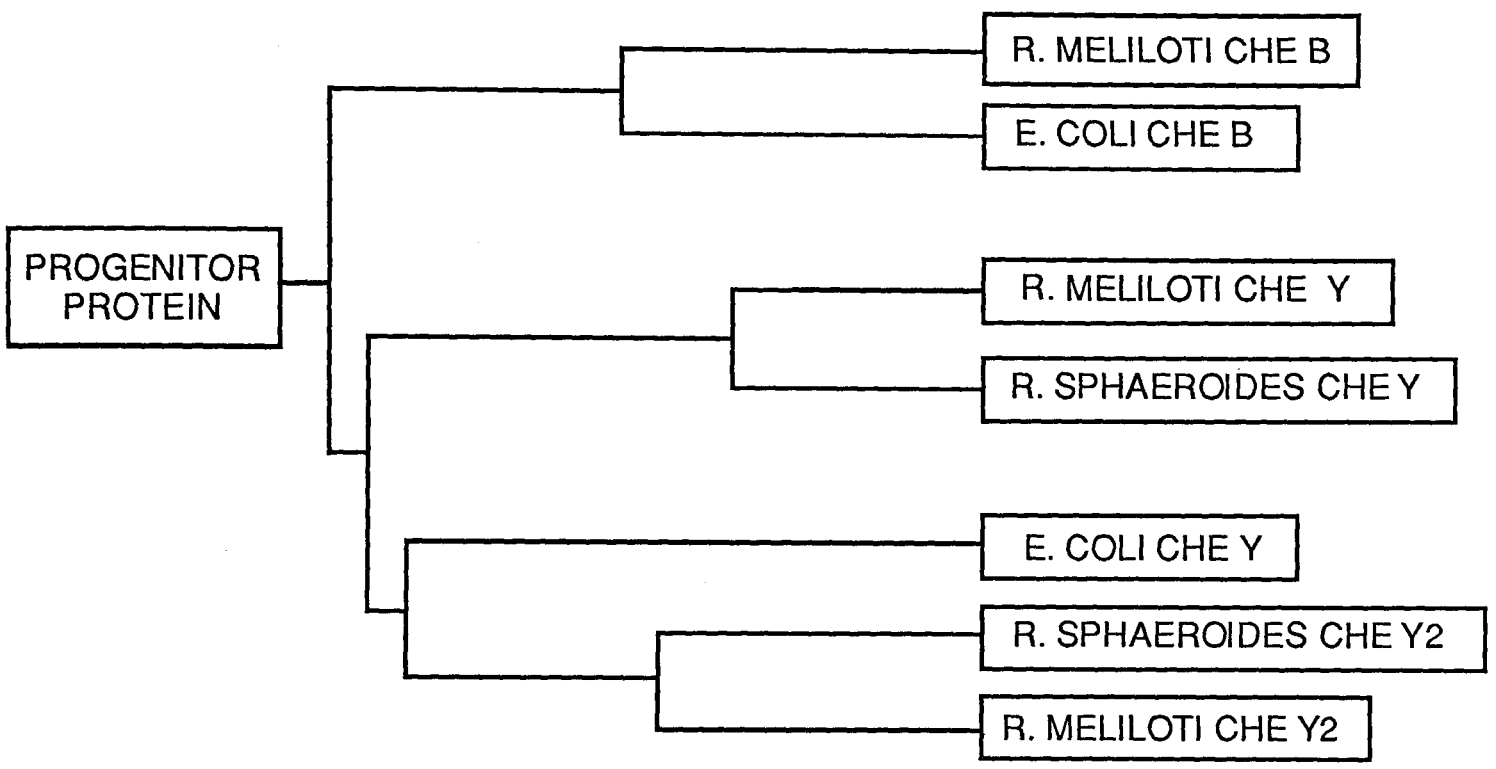


fig.65: Dendrogram comparing homology and schematically representing evolutionary relatedness of the Che B, Che Y and Che Y2 proteins of *E. coli*, *R. meliloti* and *R. sphaeroides*. The *R. meliloti* Che Y2 protein shows a closer relationship to the *R. sphaeroides* Che Y2 protein than to either the *R. sphaeroides* of *R. meliloti* Che Y proteins.

In order to further resolve the functional identity of the Che Y2 protein, it was investigated for the possession of functionally important sites previously identified in the enteric Che B and Che Y proteins. Stewart had recently identified seven ~~of~~ amino acid residues (Asp^{11}, Arg^{42}, Glu^{58}, Arg^{73}, Glu^{91} and Lys^{107}) in the Che B protein of *E. coli* which

are involved in methylesterase action (Stewart, 1993). Of these, Asp^{11}, Arg^{42}, Glu^{58}, and Lys^{107} are also involved in phospho-Che A dependent phosphorylation of Che B. The *R. sphaeroides* Che Y2 protein was found to lack all but one (Lys^{107}) of these residues (fig.66).

The Che Y protein of enterics has been the subject of considerable scrutiny recently, and eleven amino acid residues have been identified as being functionally important in *E. coli* and in *S. typhimurium* (Blat and Eisenbach, 1994; Bourret *et al*, 1993; Lukat *et al*, 1990; Bourret *et al*, 1990; Lukat *et al*, 1991; Roman *et al*, 1992). The *R. sphaeroides* Che Y2 protein possesses nine of these eleven residues (fig.66). These residues are involved in Che Y interaction with phospho-Che A, in Che Y phosphorylation (Asp^{57}), in acid pocket formation, Mg^{2+} binding and in signal transduction to the switch complex (fig.66). The two regions of residues which are believed to come together to form the acid pocket structure in the mature protein (Lukat *et al*, 1990) are highly conserved across genera and are also found in the *R. sphaeroides* Che Y2 protein. (fig.66).

3.8.5a

Construction of *R. sphaeroides che* Y2 Mutants Using pJP5603

Having deduced that "ORF B" was in fact the *che* Y2 gene of *R. sphaeroides* (analogous to the newly discovered *che* Y2 gene of *R. meliloti*), it was decided to produce disruptions in the gene in order to investigate its function. For this mutagenesis, the suicide vector pJP5603 was used. The general strategy was the same as that used for

the pJP5603 mutagenesis of *R. sphaeroides che* R (see above) and the experiments proceeded contemporaneously.

A 221 bp internal *Kpn* I / *Sal* I fragment of *che* Y2 was restricted from pCH2, purified and cloned into the *Kpn* I / *Sal* I site of pUC19 to produce "pUCδY2". The insert from pUCδY2 was then removed using *Eco* RI and *Sal* I polylinker sites, cloned into the *Eco* RI / *Sal* I site of pJP5603 and transformed into JM109λ*pir*. This 3.3 kb construct, "pJPδY2" was then to be transformed into S17-1 λ*pir* and then conjugated into *R. sphaeroides* WS8N. Before this experiment could be completed, project time expired.

3.9 DNA Motifs and Homology with Other Systems

3.9.1

Promoter Motifs

Putative *Rhodobacter* promoter consensus motifs and regulatory motifs derived from other studies were searched for throughout the entire novel *Rhodobacter* sequence. Target motifs included the enteric σ70- and σ32- and σ54-dependent promoter sequences which have been shown to be recognised by the *R. sphaeroides* RNA polymerase holoenzymes αββ'σ 93 and αββ'σ 32 respectively (Karls *et al*, 1993). No meaningful similarities were found with most of the putative promoter or regulator motifs, and what homology there was, was usually present at inappropriate locations and was therefore considered to be

coincidental, especially in light of the length of the consensus sequences in question. One potentially significant homology was found between a putative regulatory region of the *R.sphaeroides* cytochrome c_2 gene and a region located at the 3' end of *R. sphaeroides che* Y2. The sequence on the cytochrome gene is located 54 bases upstream of a start site and is thought to contain regions important in transcriptional regulation, specifically, sites for trans-acting regulatory elements and a -35 like motif (MacGregor *et al*, 1991). The region of homology extends over 31 bases (CATCCA..[19 bp]..GAGCGG), lying at the 3' end of *che* Y2 and slightly overlapping *Rhodobacter* ORF 3.

3.9.2

Similarity with Other Systems

Although in this study the newly discovered genes of *R. sphaeroides* have only been compared with their counterparts in *E. coli*, *S. typhimurium* and *R. meliloti*, they do show homology to a lesser extent with genes from other (recorded) chemotaxis systems and in the case of ORF Y2, with other members of the "Che Y superfamily" of response regulator proteins (for a review of this superfamily see Voltz, 1993). The *R. sphaeroides* proteins show considerable similarity to the chemotaxis system proteins of the swarming bacterium *M. xanthus* (Blackheart and Zusman, 1985; Blackheart and Zusman, 1986; McBride *et al*, 1989; McCleary and Zusman, 1990). The *R. sphaeroides* Che W protein shows a 53% similarity (and a 27% identity) to the *M. xanthus* Frz A protein. The *R. sphaeroides* Che Y2 protein shows a 48%

similarity (and a 23% identity) to the C terminal of the *M. xanthus* Frz E protein, which is known to possesses homology to the Che Y proteins of enterics (the N terminal being homologous with the Che A proteins of enterics). *R. sphaeroides* Che R also shows some homology to Frz F, which codes for a methyltransferase. The similarity between these proteins is not as great as for Che W and Che Y2 and occurs in two fairly small patches, one of 23 and one of 43 amino acid residues. Although these regions contain residues which are conserved between genera, the functional significance of these residues is as yet unknown.

4. DISCUSSION

The *R. sphaeroides* Q12 Mutant

Although potentially very interesting as a metabolic mutant, Q12 was found not to be a suitable mutant for the current investigation of chemotaxis for the following reasons:

- i) Q12 was not a "true" non-chemotactic mutant, because taxis was observed towards acetate and butyrate.[1]
- ii) Q12 showed a highly pleiotrophic phenotype, suggestive of a metabolic lesion.
- iii) Q12 displayed highly variable behaviour, reduced growth rate and pathological cell morphology, all of which make results hard to interpret.
- iv) Errors in the previous work, (including the misidentification of the chromosomal *Eco* RI restriction fragment in which Tn*pho*A had been inserted) had lead to the production of erroneous clones and misleading, unreliable results.

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[1] It should be noted, however, that the discovery of a second copy of *che* Y could change this interpretation. The existence of a dual chemotaxis signal pathway could explain taxis to one substrate but not to another. Model 2 (see later) would be consistent with this interpretation of the Q12 data. The intermediary metabolism of *Rhodobacter* species, however, is complex and not very well understood, and investigation of the effects of such a metabolic lesion on the tactic behaviour of the organism would be a major, long-term project and would best be done when the Che Y1 donor is identified.

The *che* Genes of *R. sphaeroides*

Three chemotaxis genes, *che* W, *che* R and *che* Y2, previously unknown in *R. sphaeroides*, were located and sequenced (Ward *et al*, in press). *Che* W and *che* R are homologues of the classic "*che*" system genes of *E. coli* and of other enterics. *R. sphaeroides che* Y2 is also fairly closely related (55% similarity) to the *E. coli che* Y gene, but shows far greater relatedness (70% similarity) to the recently discovered, second *che* Y gene (che Y2) of *R. meliloti* (Greck *et al*, in press).

The function of the second copy of the Che Y protein in either organism is unknown, but here we hypothesize that it is the response regulator of second, parallel chemotaxis signal transduction pathway. The presence of a dual chemotactic sensory transduction pathway explains the behaviour of wild type *R. sphaeroides* and the problems encountered in the production of mutants using methods developed for enteric genera which have a single sensory transduction pathway.

R. sphaeroides in now known to possess *che* A, *che* W, *che* R, *che* Y and *che* Y2 genes. *Che* B, which in enterics and in *R. meliloti*, is located downstream of and adjacent to *che* R, appears to be missing from the *R.sphaeroides* chemotaxis operon. Also, a *che* Z homologue has not, as yet, been found (this is similarly the case for *R. meliloti*).

MCP's have not been conclusively identified in *R. sphaeroides*, but

ORF 2, an open reading frame downstream of and adjacent to *che* R, has been sequenced and expressed. The ORF 2 protein shows partial similarity to the enteric MCP Tsr. This homology, however, is confined to the signalling domain of the MCP, and there is no homology with receptor or transmembrane domains (Ward *et al*, in press). Despite the tentative nature of this evidence, the structure of the *R. sphaeroides* Che W protein, as well as the presence of Che A, supports the possibility of *ORF* 2 coding for a functional Tsr-like protein (discussed below). The negative results of transposon mutagenesis also can be interpreted to support the presence of MCP-like proteins in *R. sphaeroides* (discussed below).

Recent protein work shows that antibodies raised against the 60 kDal protein of *ORF* 2 hybridize with a cytoplasmic protein of *Caulobacter crescentus*, a closely related α-group bacterium (P. Hamblin, this group, personal communication). It may be that all α-group bacteria possess MCP-like sensors which are cytoplasmic, unlike enteric bacteria in which the MCP's are membrane spanning. The operon order in *C. crescentus* also appears to be the same as that in *R. sphaeroides* and *R. meliloti* (both α-group bacteria); this similarity of organization suggests that the chemotactic signal transduction system of α-group bacteria may be fundamentally and importantly different from that of the enterics (γ-group bacteria).

The Che W Protein of *R. sphaeroides*.

The projected *R. sphaeroides* Che W protein shows strong structural conservation across genera, displaying a 59% similarity to *E. coli* Che W and 72% similarity to *R. meliloti* Che W (fig.49). In size, *R. sphaeroides che* W is also more similar to the *R. meliloti* protein than to the *E. coli* protein, being only 1.9% smaller than *R. meliloti che* W and 9.0% smaller than *E. coli che* W.

The Kyte and Doolittle hydrophobicity profile of *R. sphaeroides che* W is substantially similar to that of *E. coli che* W (see results, 3.8.1). Local hydrophobicity strongly influences folding and therefore mature protein structure. Assuming structure and function are related, then similar hydrophobicity profiles would lend support to the hypothesis that the two proteins have similar functions. Also, key functional amino acid residues appear at locations of similar hydrophobicity, making it probable that they are located similarly with respect to their environment in both *E. coli* and *R. sphaeroides*. [This evidence is only supportive, but dissimilar profiles would have provided considerable evidence against similarity of function].

The *che* W protein of *R. sphaeroides* does possess all five of the amino acid residues known to be essential for *che* W activity in *E. coli* (Liu *et al*, 1991). These residues are thought to facilitate interaction between *che* W and the MCP, Tsr, as point mutations at these sites are complemented by corresponding mutations in the MCP.

The conservation of these residues suggests that *R. sphaeroides che* W is functionally the same as *E. coli che* W and that *R. sphaeroides che* W interacts with an MCP-like protein possessing a very similar architecture to that of Tsr, at least on the cytoplasmic (signalling domain) side. This evidence is therefore consistent with ORF2 coding for a functional MCP-like, cytoplasmic protein with a signalling domain similar in structure and function to that of Tsr. Also, the presence of Che A, Che Y and Che Y2 supports the possibility of *R. sphaeroides* Che W functioning in a similar way to the protein in *E. coli*, to produce an "MCP"/Che W/Che A/Che Y(2) complex (Hazelbauer *et al*, 1993, Schuster *et al*, 1993, Gegner *et al*, 1992).

The most persuasive evidence for the conserved mechanism of action and conserved functional role of *R. sphaeroides* Che W, is its ability to complement the non-swarming *E. coli* Che W$^-$ mutant, RP4606 (Results, 3.8.2c), producing a swarming phenotype. The ability of *R. sphaeroides* Che W to substitute for *E. coli* Che W proves that it can act to facilitate complex formation between the enteric MCP and the histidine protein kinase, Che A, allowing signal propagation via Che Y to the motor/switch complex. [2]

The *che* R Protein of *R. sphaeroides*

The *che* R protein of *R. sphaeroides* displays a 58% similarity with *E. coli che* R and a 66% similarity with *R. meliloti che* R. Again, the

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[2] Since this work was done, experiments involving the overexpression of *R sphaeroides* Che W in *E. coli* have strengthened the case for complementation and therefore for similarity of function of the Che W protein between the two genera.

R. sphaeroides protein, at 309 amino acid residues, is more similar in size to the *R. meliloti* homologue (303 residues) than to the *E. coli* protein (281 residues).

The very strong homology (88% identity) shown within the four highly conserved regions of the *che* R protein (fig.59) (Kirsch *et al*, 1993a) argues convincingly that the mechanism of action of *R. sphaeroides che* R is identical to that of *che* R in other genera ie: it is a methyl-transferase.

In *E. coli, che* R mediates adaptation to an increase in chemoattractant concentration. If, as seems quite possible, *R. sphaeroides* possesses *che* R but lacks *che* B, then by analogy with enterics, one would expect it to show adaptation only to an increase in attractant and not to a decrease. *R. sphaeroides*, however, shows a chemotactic response and subsequent adaptation only to a *decrease* in chemoattractant concentration and does not adapt to an increase in chemoattractant concentration (Poole and Armitage, 1988; Packer *et al*, in press).

The fact that *R. sphaeroides che* R acts as a methyltransferase does not necessarily mean that it serves exactly the same *function* as the *che* R of *E. coli*, especially in view of the fact that behavioural and genetic evidence presented here (and in other studies) suggests that the chemosensory pathway of *R. sphaeroides* is different in many respects to that identified in *E. coli*.

In *B. subtilis*, an organism that possesses both *che* R and *che* B proteins, it seems that the function of the two proteins is reversed from that in enteric bacteria. *B. subtilis* Che R (a proven methyltransferase) seems to promote adaptation to *repellent* stimuli while Che B promotes adaptation to increases in chemoattractant concentration (Kirsch *et al* 1993a; Kirsch *et al* 1993b). The reversed role of Che R and Che B in *B. subtilis* suggests an altered interaction between the MCP and Che A or between phospho-CheY and the motor.

The lack of adaptation in *R. sphaeroides* to an increase in attractant, and its demonstrated adaptation to a decrease in attractant is, therefore, not theoretically inconsistent with the presence of Che R and the absence of Che B in this organism. It is, however, possible that a *che* B gene will be found somewhere on the genome, and this possibility is currently being investigated.

The tethered cell phenotype of the *R. sphaeroides* Che R mutants WS8NXR1 and WS8NXR4 (Results, 3.8.3b) is interesting, and can be argued to support the theory that Che R in *R. sphaeroides* acts to mediate adaptation to a step down in chemoattractant concentration. In this experiment it was shown that the suicide vector (pSUPR), which contained the Che R fragment, was stably maintained in *R. sphaeroides*, and that there was a whole, native copy of the *che* R gene present. Despite this, the "mutants" still showed a radically

disturbed phenotype when tethered. WS8NXR1 showed a strong smooth swimming bias (fig.61a), with an average stopping probability of about 0.1 stops per 20 second period (this compares with a probability of about 0.3 stops per 20 second period for wild type cells). WS8NXR4 demonstrated a very strong smooth swimming bias (fig.61b), with a stopping probability hovering around zero. Addition of 1 mM acetate to either mutant produced no detectable response. Removal of chemoattractant did not produce a response from WS8NXR1, but did produce a sudden increase in stopping probability in WS8NXR4, similar to the response of a wild type cell. Adaptation, however, seemed to occur very rapidly, with the stopping probability returning to background levels within about 150 seconds, which compares with a wild-type adaptation time of about 650 seconds.

This behaviour could be explained if the pSUPR were expressing the cloned fragment of *che* R, and if that fragment was sufficient to code for a functional or semi-functional Che R protein (the clone contains 75% of the *che* R gene). If this protein mediates adaptation to a step down in chemoattractant concentration, and is being overexpressed in these mutants, then the expected phenotype would be smooth swimming. After removal of an attractant, one would expect such a mutant to adapt much more rapidly than the wild-type. WS8NXR4 behaves in precisely this manner. WS8NXR1 may be adapting so rapidly that the response to chemoattractant removal (the increase in stopping probability) is attenuated before it becomes apparent to the

computer, which measures stopping probability over a twenty second period. The inferences drawn from the results of this experiment are obviously highly speculative, considering that only 75% of the gene is present, and there is no biochemical evidence to support the belief that the fragment of the che R gene is being expressed. Nonetheless, such an explanation does present itself as a possibility, which if correct, would be strongly supportive of the theory that *R. sphaeroides* Che R acts to mediate adaptation to a step down in chemoattractant concentration and not to a step up in chemoattractant concentration.

The Che Y2 Protein of *R. sphaeroides*

Perhaps the most important finding in this project was the identification of a second copy of the che Y gene in *R. sphaeroides*, subsequently also identified in *R. meliloti*.

The Che Y2 protein of *R. sphaeroides* displays a 70% similarity to *R. meliloti* Che Y2 (and a 55% similarity to *E. coli* Che Y). The degree of evolutionary relatedness between the Che Y, Che Y2 and Che B proteins of *E. coli, R. meliloti* and *R. sphaeroides* is shown in the dendrogram (fig.65). Che Y2 shows conservation of important functional residues (fig.66) including those involved in phosphorylation (Asp^{57}), interaction with Che A, acid pocket formation and Mg^{2+} binding (Asp^{12}, Asp^{13}). This structural homology implies functional conservation between genera. The fact that the sites of interaction with Che A have been conserved also has implications for *R. sphaeroides* Che A, suggesting it has a similar structure and function to its enteric counterpart.

Two Hypothetical Models Of The Signal Transduction Pathway In *R. Sphaeroides*

Using the elements identified two models of a chemotaxis signal transduction pathway can be reasonably hypothesised that explain the behaviour of *R. sphaeroides* and the results of this study (fig.67 and fig.68).

It is proposed (in both models) that a dual chemotaxis pathway exists with both phospho-Che Y and phospho-Che Y2 acting independently at the switch/motor complex to increase rotational stopping probability.

In both models the phospho-Che Y2 pathway (the "Y2 branch") is analogous to the classical signal transduction pathway of enterics, except that the sensor molecule is not a trans-membrane MCP. The sensor may still be an MCP in as much as it is methylated by Che R to promote adaptation to a signal, also, function is proposed to be very similar to that of an enteric MCP, transducing the primary (cytoplasmic) signal to Che A, via direct interaction with Che A and Che W.

The phospho-Che Y pathway (the "Y1 branch") is less well defined than the Y2 branch, and so less amenable to simple analogy with other systems. How Che Y functions is not known. It may lie at the end of a corresponding MCP-Che pathway, or interact with the known Che A, or

it may be phosphorylated by a protein unrelated to Che A. Since Che Y *is* a response regulator, and since almost all response regulators are associated with an identified, corresponding histidine protein kinase, it seems reasonable to hypothesize the existence of a corresponding histidine protein kinase, "Che A*".(Che A* is essential for the functioning of Model No.2 and convenient in Model No.1).

Model No.1

In Model No.1 (fig.67), the Y2 branch acts just like the classic "che" system of enterics. An increase in the concentration of specific products of organic acid catabolism is sensed at the distal end of the Y2 branch by a Tsr-like cytoplasmic sensor. The Tsr-like protein undergoes a conformational change, promoting the formation of a complex between the sensor, Che W, Che A and Che Y2. The kinetics of Che A autophosphorylation is increased, Che A becomes phosphorylated, and a signal is transmitted to Che Y2 by phosphorylation at Asp^{57}. Phospho-Che Y2 then acts at the motor/switch complex to interrupt rotation.[3]

The dominant stimulus for the Y1 input branch in Model No.1 could be any class of attractant to which *R. sphaeroides* is attracted other than

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[3] Recently, the *che* A and *ORF* 2 genes of *R. sphaeroides* have been deleted, producing mutants which seem to respond normally to organic acids, but which have an altered tactic response towards sugars. This suggests that the Y2 branch is involved in sensing sugars and not organic acid metabolites. Such evidence supports Model No.2 in favour of Model No.1, at least in respect to the class of attractant sensed by the different branches.

organic acids, eg: sugars, amino acids, ammonia or even metals such as rubidium. Certain elements of *R. sphaeroides'* behaviour suggest that it can respond to general metabolic signals as well as to specific attractants. Unlike *E. coli, R. sphaeroides* responds only to substances that are currently growth-limiting (Poole and Armitage, 1989). Also, *R. sphaeroides* shows aerotaxis and phototaxis, both of which are thought to be electron-transport dependent and MCP-independent (Armitage, 1992). The Y1 branch may respond to a general metabolic signal, related to the overall energy state of the cell (eg: pmf or [ATP]) or the signal may be related to the rate of electron transport by the monitoring the redox poise of a central component of the electron transport. Ubiquinone is a particularly attractive candidate for this sort of indicator molecule, as it is the one component common to all branches of the electron transport chain of *R. sphaeroides* (fig.12).

Whatever the stimulus, it will be sensed by Che A*, possibly via the interaction of a specific cytoplasmic sensor protein, "Sensor*", and will be transmitted to Che Y by phosphorylation at Asp^{57}.
"Sensor*" is not strictly necessary for the functioning of Model No.1, but it would allow reciprocal crosstalk between different branches of the model and is included as a default element of this hypothesis in deference to the classical "che" system model.

In a variation of Model No.1, Che A* and Sensor* are done away with

altogether, and Che Y becomes autophosphorylated directly by a low molecular weight phosphate-donor such as acetyl phosphate. *In vivo* acetyl phosphate concentration varies greatly depending on growth medium and stage of growth cycle (McCleary and Stock, 1993) and it has been shown that acetyl phosphate will rapidly donate a phosphate group to *E. coli* Che Y in the presence of Mg^{2+} *in vitro* (Lukat *et al*, 1992). *In vivo*, it has been demonstrated that in the absence of Che A, *E. coli* can be made to tumble rapidly by exposure of the cells to acetate (Wolfe *et al*, 1988). This "acetate effect" was previously thought to be due to acetylation, but is now believed to be due to an increase in intracellular acetyl phosphate concentration (McCleary *et al*, 1993). The kinase activity of Che A is not required for Che Y phosphorylation, since Che Y possesses autophosphorylation activity (Lukat and Stock, 1993). Acetyl phosphate links acetate metabolism with the Krebs cycle and is a key metabolic intermediate in a range of metabolic pathways; it has been invoked as a possible global regulator in enterics (McCleary *et al*, 1993; McCleary and Stock, 1993). [4]

In Model No.1, the presence of dual Che A proteins can explain the normal swarming on weak organic acid plates of the Che A^- mutant (Ward *et al*, in press). Crosstalk between the Tsr-like sensor and the Che A* protein allows the chemotaxis signal to bypass the deleted Che A protein. The Tsr-like protein would form a complex with Che W,

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[4] Fumarate has also been shown to influence motor switching (Barak and Eisenbach, 1992), but in these experiments cell envelopes were used, and Che Y was not present, this suggests that fumarate somehow works directly at the motor/switch complex. The mechanism of this interaction is unknown.

Che A* and Che Y (or Che Y2), increasing Che A* auto-phosphorylation, and the signal would be passed from phospho-Che A*, via Che Y (or Che Y2) to the motor to produce stopping.

The phenotype of the Che A^- mutant could also be explained in Model No.1 without invoking crosstalk, if the Y1 branch is used to sense general metabolic signals. In this case, the fact that organic acids or the products of their metabolism could not be sensed directly by the Y2 branch because of the deletion of Che A, would not cause a loss of taxis to these chemoattractants. Metabolism of the substrate would cause increased concentrations of high energy intermediates and increased electron transport which would be sensed by Che A*, initiating a tactic response via phospho-Che Y.

Model No.2

In Model No.2 (fig.70), the products of catabolism of organic acids, are sensed at the distal end of the Y1 branch by Che A*. Sensing may require the presence of a separate, MCP-like sensor molecule, or Che A* may be the primary sensor in this pathway. In most two component regulatory systems, the histidine protein kinase molecule is a membrane-spanning sensor which samples the external environment, transmitting information directly to the response regulator by aspartate phosphorylation. Che A* may likewise be the primary sensor for this branch of the sensory transduction pathway, although it would be located in the cytoplasm, and not in the membrane. Che A* will

autophosphorylate, promoting Che Y autophosphorylation, and phospho-Che Y will then act at the motor to cause a pause in rotation.

In Model No.2, the Y2 branch senses any attractant other than organic acid catabolites, via the primary sensor of this branch, the Tsr-like protein. The signal is transmitted in the classical way from Che A to Che Y2 to the motor/switch complex.

In Model No.2, the fact that a Che A^- mutant of *R. sphaeroides* responds normally to weak organic acids (Ward *et al*, in press) is explained by the presence of two completely separate chemotaxis signalling pathways. An MCP-like sensor at the distal end of the Y1 branch is not required for this model. Crosstalk is not involved. Interruption of the Y2 pathway by the deletion of Che A simply does not interfere with the flow of information through the Y1 branch.

If Model No.2 is correct, then finding the class of substance that stimulates the Y2 pathway is simply a matter of screening the Che A^- mutant against different known attractants in mini-swarm agar. If, however, Model No.1 is correct, a non-chemotactic mutant could only be created: i) by producing double mutants in the dual elements of the separate pathways (the best target for this would be the *che* Y and *che* Y2 genes, since we have both genes currently cloned); or ii) by targeting the primary receptor (the Tsr-like protein of "sensor*") of either branch of the signal transduction pathway. This approach would

require the assumption that the primary receptors are functionally unique, and do not sense the same ligands.

The Role of Che W in Both Model No.1 and Model No.2

In both models, Che W fulfills it's classical role in facilitating complex formation between the sensor, Che A (or Che A*), and Che Y (or Che Y2). This interaction increases the autophosphorylation kinetics of Che A (or Che A*), leading to an increase in the concentration of phospho-Che A (or phospho-Che A*).

The Role of Che R in Both Model No.1 and Model No.2

Both of the above models would allow adaptation to a step down in the concentration of whatever attractant is sensed by the Tsr-like sensor. Adaptation to a step up in concentration will not occur. Computer modelling has shown that such an an adaptive response will cause accumulation, though at a slightly slower rate than for an organism which adapts to both increases and decreases in attractant concentration (Packer *et al*, in press). The fact that *R. sphaeroides* adapts only to a step down in attractant concentration, and may also lack Che Z, may help explain this organism's behavioural heterogeneity and lack of an "all-or-none" response. The lack of Che Z and Che B would result in slower adaptation and less frequent adaptation for *R. sphaeroides* than for *E. coli*. Because of relatively slow adaptation, and given that the microenvironment is heterogeneous, there will be a lag in adaptation of members of the population to changes in the environment. The population would therefore be behaviourally diverse and would require

a relatively long time in a homogeneous environment to reach behavioural heterogeneity.

The Absence of Che Z in *R. sphaeroides*

The absence of Che Z in both of the above models necessitates an explanation of how *R. sphaeroides* is able to rotate it's flagellum at all, without getting "trapped" in a permanent rotational pause state, which would be the equivalent of the tumbly bias seen in the Che Z^- mutant of *E. coli* (Stewart *et al*, 1988). It does not seem likely that competition between the two phospho-Che Y proteins is responsible for resumption of rotation, but a difference in binding kinetics between components could explain it.

Reduced affinity between Che Y (or Che Y2) and the phosphate group would cause increased dissociation of phospho-Che Y(or phospho-Che Y2) which would therefore become dissociated from the switch (or not have a chance to bind at the switch), allowing continued flagellar rotation. Phospho-Che Y in *E. coli* has been shown to spontaneously dephosphorylate with first order kinetics giving a rate constant of about 2 min^{-1} (Stock, 1991), and *E. coli* $CheZ^-$ mutants are not 100% tumbly, so a relatively minor change in structure (and therefore binding kinetics) would probably be enough to allow the desired balance between rotation and stops. The fact that *R. sphaeroides* takes a relatively long time to adapt to a step-down in attractant concentration, four to five minutes (fig.61b), may be related to the lack of Che Z,

adaptation being dependent on spontaneous phospho-CheY(or phospho-Che Y2) dephosphorylation. *R. meliloti*, like *R. sphaeroides*, is a member of the alpha subgroup of bacteria, and also possesses a unidirectional motor. As with *R. sphaeroides*, no *che* Z gene has been found in *R. meliloti*. It may be that Che Z is not required for unidirectional motor rotation.

Why Did Transposon Mutagenesis Fail to Produce Mutants Non-Chemotactic to Organic Acids ?

The lack of non-chemotactic mutants as a result of transposon mutagenesis can be explained by four possible phenomena: i) The transposon Tn5 was hotspotting. ii) Functional redundancy between sensor molecules made production of the desired phenotype highly improbable. iii) The fact that one pathway responds to organic acid catabolites and that the other responds to a general metabolic signal meant that mutants non-chemotactic to organic acids could not be made by the interruption of a single gene. This would have made production of the desired phenotype highly improbable. iv) Elements essential to producing a chemotactic response to succinate were also essential for flagellum assembly or for the viability of the cell.

• i) The simplest and most likely scenario is that Tn5 is prone to hotspotting. Although Tn5 generally has a relatively low insertional specificity, it has been shown to integrate with high frequency into naturally occurring sequences showing homology to the Tn5 termini

(Bruijn and Lupski, 1984: six out of six investigated insertions were found to be at the same locus). Mini-Tn10 (derivative 103), a transposon specifically designed to maximize randomness of transposition (Materials and Methods, 2.4e) was clearly shown to hotspot, becoming preferentially inserted into the vector DNA, which had a relatively low G:C content in comparison to the *R. sphaeroides* insert (Results, 3.8.2a). Tn5 may be inserting at specific sites displaying similarity to the Tn5 termini, or may simply have a preference for A:T rich regions, infrequent in the *R. sphaeroides* genome (68% G:C). If this were the case, the target gene may never have been interrupted, explaining the lack of non-chemotactic phenotypes.

• ii) If hotspotting is *not* occurring, then considering the number of transposon mutants screened (about 30,000), the probability of hitting any one gene would have been very high; in fact, assuming only one transposition per kanamycin resistant cell, the probability of *not* targetting the desired gene would be ten-to-one against. [Including the results of an identical experiment done by another researcher, the improbability of such a result rises to twenty-to-one against (M.J. Ward, this laboratory, personal communication)]. Despite this, all of the mutants screened (that had functional flagella) were able to accumulate in response to chemoattractants when assayed on plug plates (Results, 3.3.1a). The flow of chemotactic information would therefore have had to bypass the deleted protein to act at the flagellar switch.

Crosstalk between the sensor and Che components of the dual chemotaxis pathway cannot explain the lack of non-chemotactic mutants produced by transposon mutagenesis. If the primary sensors (in either model) are *functionally unique* components, then once they are deleted, the cell will not be able to respond to the corresponding effector. The lack of non-chemotactic mutants could be explained if there was functional redundancy between the primary sensors. If both primary sensors (Tsr-like protein and "sensor*") were sensitive to organic acid metabolites, then knocking out one or the other of them would not produce a non-chemotactic phenotype. In this case, to produce a non-chemotactic mutant would require simultaneous interruption of two specific genes, which would be highly unlikely. Although the probability of interrupting one gene is only about 3.3 x 10^{-4}, the probability of interrupting two specific genes simultaneously would be about 1.1 x 10^{-7} (assuming two transposition events per cell), making it highly improbable that such a mutant would have be produced during this study. The fact that there are two branches of the sensory pathway in *R. sphaeroides* does not mean that each branch is activated by a single, dedicated sensor. It is quite possible that both branches may be activated by either sensor. In enterics, different MCP's activate the same components of a common sensory transduction system. The same could be occurring in *R. sphaeroides*, but with the added complication of a dual Che protein pathway.

• iii) As proposed for Model No.1, the Y1 branch could be used to

sense general metabolic signals. In this scenario, even if the Y2 branch, which specifically senses the products of organic acid catabolism, is interrupted by transposon mutagenesis, the organism would still respond to the presence of the attractant via a metabolically derived signal sensed via the Y1 branch. Metabolism of the substrate would cause increased concentrations of high energy intermediates and increased electron transport. This metabolic signal would act as a stimulus, detected by "sensor *" (or directly by Che A*), and Che Y would become phosphorylated acting at the motor/switch complex to produce a rotational stop. Thus directional changing would *not* be prevented, despite the lesion in the Y2 branch, and such mutants would *not* display an altered (reduced swarming) phenotype on mini-swarm agar plates and would go unnoticed in screening procedures.

• iv) Lastly, it is theoretically possible, though unlikely, that the machinery facilitating chemotaxis to organic acids (and therefore swarming on mini-swarm agar plates) is an integral part of the motor and cannot be deleted without producing a non-flagellate or paralyzed mutant. This would be the case if Che Y were a structurally essential part of the motor/switch complex, which directly sensed the products of succinate catabolism or the metabolic effects of such catabolism. In the same vein, if the machinery involved in chemotaxis to organic acids were an integral part of the metabolic machinery, again, detecting catabolites or the metabolic effects of catabolism, then such sensory components could not be deleted without producing a non-viable mutant.

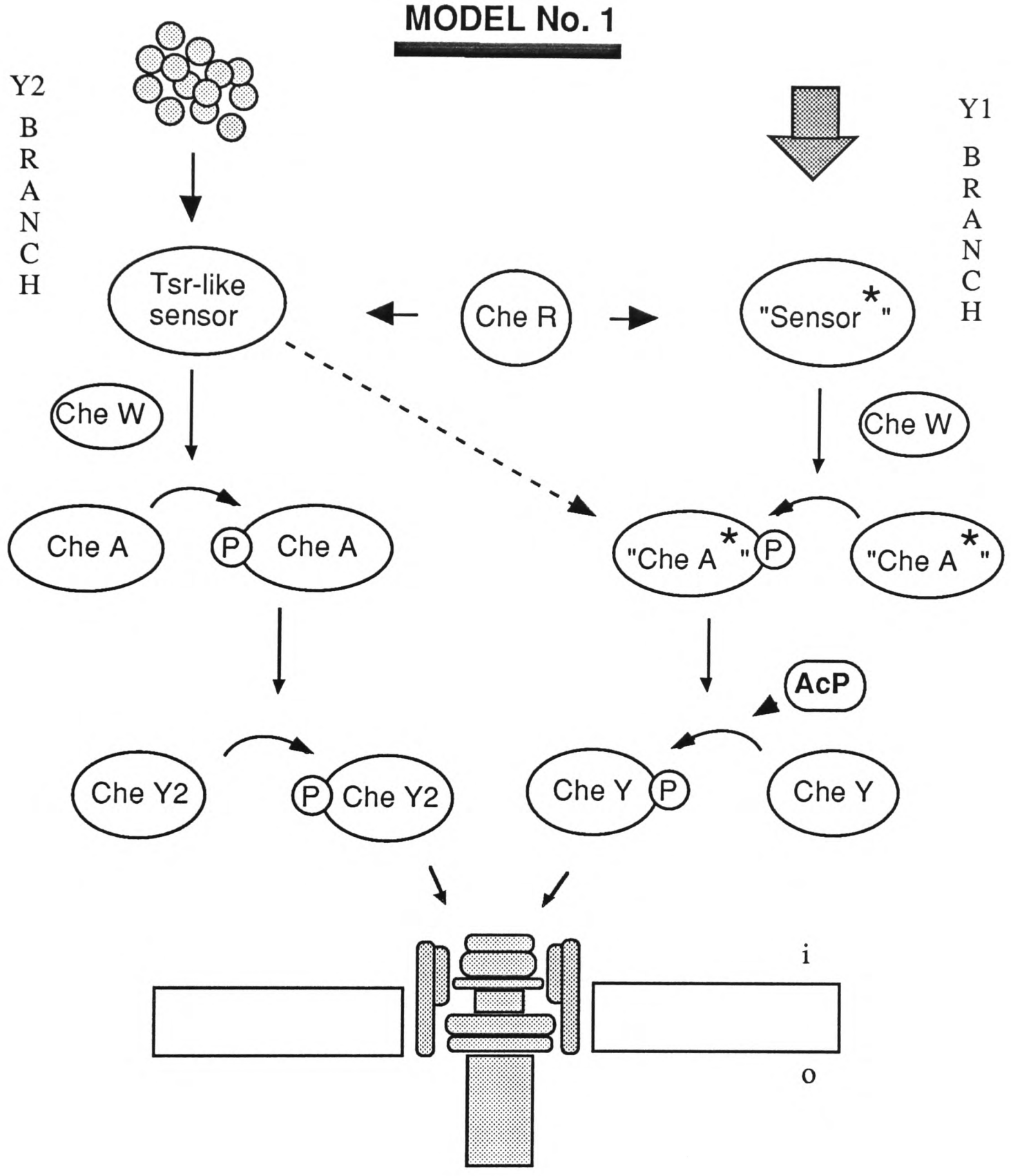

Fig.67: Model No.1 of chemotactic sensory transduction in *R. sphaeroides*. ⊛ = catabolic products of organic acids. ⛛ = tactic signal other than the catabolic products of organic acids. May be a "metabolic" signal related directly to rate of electron transport or to the general energy state of the cell (pmf or [ATP]). **AcP** = acetyl phosphate, acting directly to phosphorylate Che Y. Elements in " " are hypothetical molecules not yet shown to exist in *R. sphaeroides*. Dashed line symbolizes crosstalk (although only shown between the Tsr-like sensor and Che A*, crosstalk could occur between any sensor and histidine protein kinase or between any histidine protein kinase and response regulator molecule in this model.

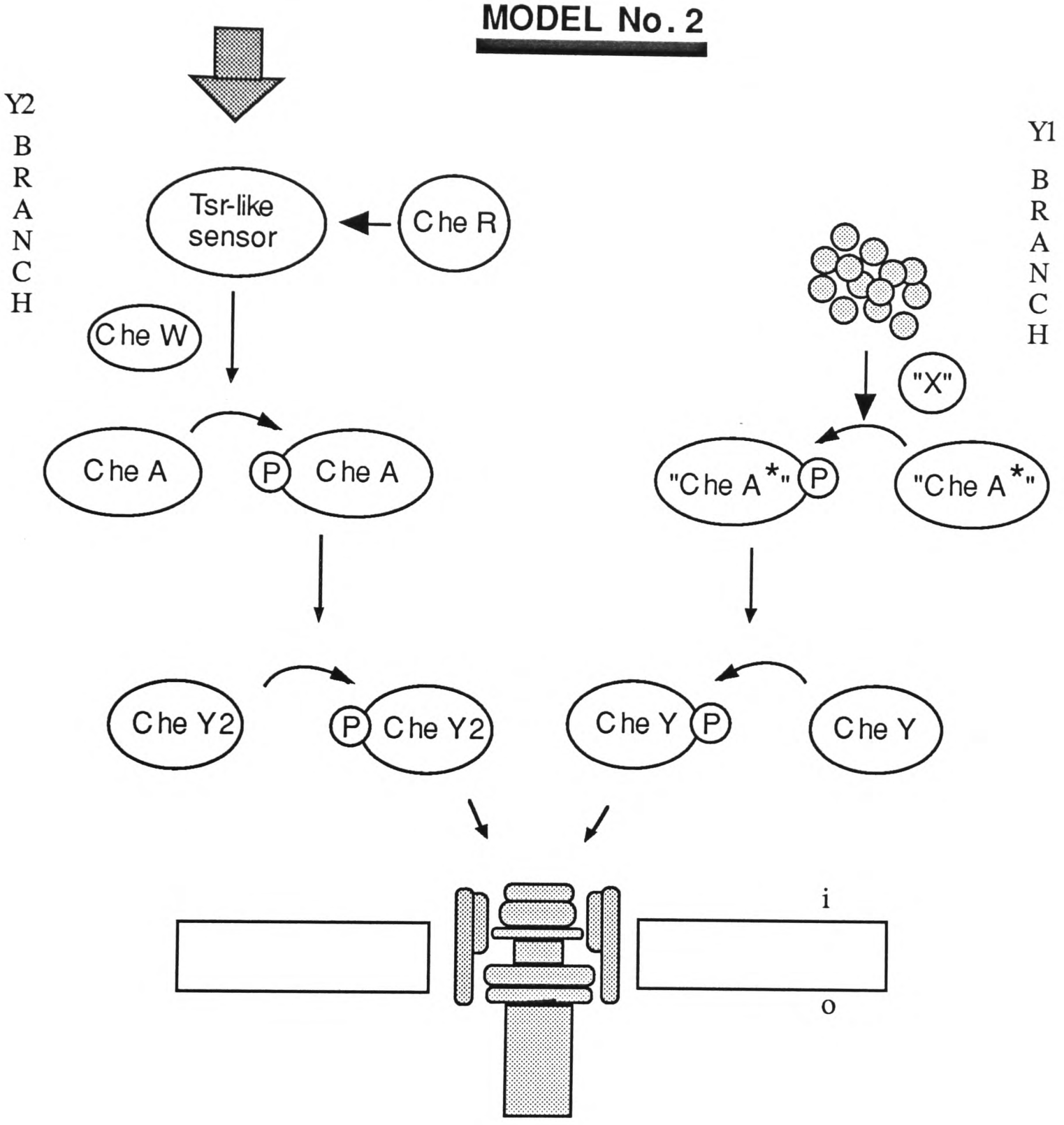

Fig.68: Model No.2 of chemotactic sensory transduction in *R. sphaeroides*. ⊛ = catabolic products of organic acids. ⛛ = tactic signal other than the catabolic products of organic acids. May be a "metabolic" signal related directly to rate of electron transport or to the general energy state of the cell (pmf or [ATP]). X = hypothetical sensor molecule. Crosstalk is not necessary for this model to explain the results of *R. sphaeroides* mutagenesis, but the model does not require the absence of crosstalk, and it may occur as in Model No.1.

Significance of This Study

The evidence from this study shows that the chemotactic signal transduction system of *R. sphaeroides* is importantly different from that of enteric bacteria. This difference accounts for the wild-type behaviour of *R. sphaeroides*. It also explains both the difficulty in obtaining behavioural mutants and the inadequacy of traditional screening techniques (mini-swarm agar plates) developed for the behavioural characterization of enteric mutants.

Rhodobacter, like *Rhizobium*, *Caulobacter*, *Azospirillum* and *Agrobacterium* species, are all members of the α-group of bacteria, whereas *E. coli* and *S. typhimurium* are members of the γ-group of bacteria. *R. sphaeroides*, *R. meliloti* and *C. cresentus* all share similar behavioural characteristics and all appear to have the same operon structure. The chemotactic operons of *Azospirillum* and *Agrobacterium* species have not yet been thoroughly characterised, but both show behaviour patterns that are similar to *R. sphaeroides* and to *R. meliloti* but that are different from enterics: populations are behaviourally heterogeneous and do not demonstrate the all-or-none response characteristic of enterics, and these organisms also demonstrate chemokinesis.

It may be that what has been discovered is a chemosensory system, different from that found in enterics, but common to a wide range of environmentally important organisms (the α-group of bacteria). This

signal transduction system possesses two branches which feed signals to the motor/switch complex. The branching of the pathway, as well as the possible lack of Che B and Che Z (as explained in the hypotheses above) explains the wild-type heterogeneity of *R. sphaeroides* behaviour, as well as the apparent lack of behavioural mutants found using mini-swarm screening techniques developed for studies focusing on enteric organisms.

Future Work

The most productive experiments that suggest themselves for future work are:

1. Finish sequencing the *R. sphaeroides* chemotaxis operon and determine whether MCP's are present, and if so, how many are present. If MCP's are found, it will be important to determine whether they become methylated and demethylated in the same fashion as their enteric counterparts.
2. Delete both Che Y and Che Y2 in the same *R. sphaeroides* mutant and assay for chemotaxis.
3. Delete the MCP-like protein in *R. sphaeroides* and screen the resultant mutant to determine whether this molecule is a unique sensor for some attractant.
4. Overexpress each of the cloned Che proteins in *R. sphaeroides*; examination of resultant phenotype and comparison with the overexpression mutants in other genera may produce evidence of *in vivo* of function.

5. Express and purify the cloned Che proteins of *R. sphaeroides;* examine their relative phosphorylation kinetics *in vitro* and relate that to *in vivo* behaviour.

6. In the light of this investigation, it is possible that the Q12 mutant possesses an important lesion in the metabolic and/or sensory pathway that integrates at the motor via Che Y. Once the Che proteins have been purified to allow *in vitro* biochemical studies, it would be interesting to identify the protein deleted in Q12, as it certainly has a function in chemotaxis and may be closely associated with Che Y.

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