An evaluation of the Agrobacterium tumefaciens Virulence gene system as a potential diagnostic test for Neuroblastoma.

Palmer, Adrian Charles Vernon

Adrian Charles Vernon Palmer.

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Abstract.

Neuroblastoma is a common pediatric cancer, the prognosis for which is markedly dependent upon the progression of the disease at the time of diagnosis. It has been argued that a mass screening programme for all infants would aid early detection of neuroblastoma and reduce mortality. Neuroblastoma is unusual amongst childhood cancers since the basis for such a test exsists - otherwise asymptomatic patients excrete abnormal amounts of specific phenolic compounds in their urine. The presence of these metabolites at elevated levels is taken as diagnostic of the disease. A number of pilot screening programmes in different parts of the world have shown that a quick, inexpensive and reliable method of screening is needed. One candidate for this is a test based upon the responses of *Agrobacterium tumefaciens* to compounds with structures similar to those produced as a result of tumour metabolism.

This bacterium responds to such phenolic ligands chemotactically and by induction of virulence gene expression. Data presented in this work shows that phenolics secreted by neuroblastoma tumours are incapable of inducing virulence gene expression but are capable of acting as chemoattractants. The role of phosphorylation in VirA/G mediated phenolic chemotaxis is investigated. Evidence is presented that phosphorylation of Vir and G is required for chemotaxis. A novel, highly reproducible and comparible measure of bacterial chemotaxis, the chemotactic index is derived and applied.

Sp.

Declaration.

No part of this work has been previously submitted for a degree at this or any other university. All the material is my own original work except where otherwise stated.

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This work is dedicated to my parents and to Julie. I should like to thank them for their support and understanding - it is with regret that one realises how much has been taken for granted when it is too late to say "thank you".

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Abbreviations.

4-MUG	4-methylumbelliferyl-ß-D-glucuronide
a.k.a	Also known as
A.t.	*Agrobacterium tumefaciens*
AS	3'-5' Dimethoxy-4'-hydroxyacetophenone (Acetosyringone)
AS-Br	Bromo-acetosyringone
ASOH	Hydroxy-acetosyrinogone
bp	Base pairs
CAT	Computer aided tomography
CAT	Chloramphenicol acetyl-transferase
CI	Chemotactic Index
CT	Computerised tomography
ddH_2O	Double distilled water
dNTP	deoxynucleoside triphosphate
ddNTP	dideoxynucleoside triphosphate
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
E.c.	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethylmethane sulphonate
EtOH	Ethanol
GCFID	Gas chromatography flame ionisation detection
GCMS	Gas chromatography mass spectroscopy
HA	Hydroxylamine
HPLC	High performance liquid chromatography

HVA	3'-methoxy-4'-hydroxyphenylacetic acid, (Homovanillic acid)
Kb	Kilobase pairs
MIBG	Metaiodobenzylguanidine
Mes	2-(N-morpholino)-ethanesulphonic acid
MOPS	(3-[N-morpholino] propanesulphonic acid)
MU	4-methylumbelliferone
NG	N-methyl-N'-nitro-N-nitrosoguanidine
NMR	Nuclear magnetic resonance spectroscopy
NSE	Neuron specific enolase
NTA	Nitroacetic acid
OH-AS	Hydroxy acetosyringone
ONPG	*o*-nitrophenyl-ß-D-galactoside
p.d.	Potential difference
PKU	Phenylketonuria
RNase	Ribonuclease
RT	Room temperature
RTP	Room temperature and pressure
SDS	Sodium dodecyl sulphate
SSC	Saline sodium citrate
TLC	Thin layer chromatography.
Tris	[2-amino-2-(hydroxymethyl) propane-1-3-diol, (tris)]
T DNA	Transfer DNA
Ti plasmid	Tumour inducing plasmid
UV	Ultra violet
VMA	3'-methoxy-4'-hydroxyphenylacetic acid (Vanillylmandelic acid)

w.t.	Wild type
w/v	Weight for volume
X-GAL	5-bromo-4-chloro-3-indolyl-ß-D-galacto pryanoside

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1. Introduction.

1.1 Neuroblastoma.

Neuroblastoma was probably first described by Virchow in 1864, the present name being ascribed in 1910 by Wright. It is one of the most interesting paediatric malignant neoplasms, with a wide spectrum of possible outcomes, including complete spontaneous remission, maturation to benign ganglioneuroma and death of the patient (Smith and Castleberry, 1990). The disease is usually congenital (Carlsen, 1988), arising *in utero* as a result of loss of regulation of cell division (Sutow, 1958, Rubin, 1968, Wilson & Draper, 1974, Birch *et al*, 1980, Carlsen, 1988). Neuroblastoma is the most common malignant neoplasm of infancy and the most frequent extracranial solid tumour of childhood (Garvin *et al*, 1990). Prognosis is markedly dependent on a variety of factors (Evans *et al*, 1987), becoming poorer with increasing tumour burden and dissemination (Carlsen, 1992).

1.1.1 Origin Of Neuroblastomas.

Primary neuroblastomas appear at many sites in the body. The presence of histopathologic features such as rosettes and fibrils with similarity to developing adrenal tissue led Wright to propose in 1910 that the tumour arose from the primitive neural crest (Wright, 1910). There is a wide spectrum of cell types in tumours described as neuroblastoma, some tumours being composed of small round cells almost indistinguishable from other so called small, round cell tumours cells such as Ewing's sarcoma and rhabdomysarcoma, whilst other tumours are so well differentiated as to be called ganglioneuroma, ganglion cells comprising the majority of the tumour (Evans *et al*, 1980). The disease is classified as a subgroup of peripheral primitive neuroectodermal tumours (PNET) (Denher, 1986, Shimada, 1992).

The anatomic sites of neuroblastoma origin and biologic growth are well explained by the embryology of neuroblast development (Smith and Castleberry, 1990). The Neurocristopathy concept (Schummacher, 1992, Schmike, 1980) asserts that certain diseases originate from an aberration of the development, maturation and migration of neural crest cells.

The nervous system develops from pluripotent stem cells (Smith and Castleberry, 1990) in the embryo and cell differentiation continues into puberty (Carlsen, 1992) . The embyonal ectoderm thickens to form the neural plate. This invaginates to establish the neural groove, which closes along the midline, forming a tube and eventually giving rise to the central nervous system. The neural crest separates from the neural tube, giving a plate from which sensory ganglia, autonomic ganglia, melanocytes, Schwann cells, the chromaffin system and the leptomeninges arise. Neuroblasts migrate from the neural crest to the central ganglia flanking the spinal column, the thoracic and abdominal prevertebral ganglia and the postganglionic cells of the adrenal medulla. Here they synapse with sympathetic preganglion fibres. In the 8mm embryo, primitive sympathetic cells can be found in the aortic regions. The differentiation of neuroblastoma cells mirrors that of neuroblasts. The undifferentiated embryonal cells with a large nucleus and almost no cytoplasm have a dispersed chromatin pattern and no nucleolus. Cells are tightly packed and arranged diffusely. As neuroblasts develop to form processes, rosettes and pseudorosettes arise. Finally as the cells become recognisable as mature ganglia dendrites appear, the cytoplasm coalesces, Nissl substance becomes visible and a nucleolus becomes visible in the now vesicular nucleus (Smith and Castleberry, 1990). Embryonic neuroblasts, some with mitotic capability, and cells at all stages of differentiation may be found in the maturing nervous system (Carlsen, 1992). The distribution of the sympathetic nervous system mirrors the sites of primary tumours, most commonly in the adrenal glands (over 50% of cases) and less frequently in other endocrine glands, the peritoneum, retroperitoneum, thymus, heart,

mediastinium, abdominal connective and soft tissue, and other sites in the brain, head, neck, thorax, abdomen and pelvis (Smith and Castleberry, 1990, Hanawa *et al*, 1990). On rare occasions a case is encountered with more than one primary tumour (Felici *et al*, 1990). Tumours can appear concurrently or in sequence. When tumours arise in relatively rapid succession metastasis is expected. True multiple primary tumours more frequently arise together or at intervals of several years (Evans, 1980).

Neuroblastoma is thought to arise according to the Knudson 2 stage mutational model (Knudson and Strong, 1972, Moolgavkar & Knudson, 1981, Knudson, 1985, Knudson, 1989) due to its age distribution (Carlsen, 1992). This model proposes emancipation from regulation of cell division due to single mutations in several groups of genes, division promoting oncogenes and tumour suppressing (anti-oncogenes) genes. *N-myc* (Schwab *et al*, 1983) has been proposed as the neuroblastoma oncogene, whilst the as yet unidentified tumour suppressor is thought to map to 1p36.1-2 (Weith *et al*, 1989). The *ret* proto-oncogene is also specifically expressed in neuroblastoma cell lines and thought to play a role in tumour biology (Ikeda *et al*, 1990). For carcinogenesis to occur, mutation is required in both genetic groups in the same cell. Mutations need not arise at the same time, and if such a mutation is received by a cell early in development and passed on to many daughters, the change of a cell receiving a second "hit" greatly increases (Marshall, 1991). Non malignant and regressing cancers are explained as arising due to one "hit" in this model (Knudson and Meadows, 1980, Carlsen, 1992). In hereditary cancers, the first mutation is always germinal (Knudson and Meadows, 1980). Knudson (1980) estimates that 20% of neuroblastomas arise from an inherited predisposition. Gilbert and Balaban-Malenbaum (1980) note that such inherited mutations are dominant but Brodeur (1990) estimates inheritance to be important in only 1% of neuroblastomas.

1.1.2 Incidence & Epidemiology.

Neuroblastoma is the most common malignant tumour of infancy and the most frequent extracranial solid tumour of childhood (Garvin *et al*, 1990). The disease accounts for 8-10% of all cancers within the first 14 years of life (Evans *et al*, 1987, Sawada *et al*, 1991), Hirayama (1981) noting a slightly higher incidence in Japan (9.8% malignant disorders of childhood) compared to the West (7.7% in USA (Young and Miller, 1975), 6.6% England (Jones, 1981). 80% of cases are detected within the first 5 years (Bigotti and Coli, 1990) and it is very rare in adults and indeed in the over 7's (Cropper, 1989) though Mackay *et al* (1976) diagnose 9 adult patients as having neuroblastoma. However, the percentage of childhood deaths attributable to neuroblastoma is much higher (15%) (Craft and Pearson, 1989). Weinblatt (1988) states that 1 in 39 infant mortalities are found to have neuroblastoma on *post mortem* examination, but that this may not always be the cause of death. Parkin *et al*, estimate incidence to be 1 in 6000 to 10000 births, whilst Scriver *et al* (1987) arrive at a figure of 1 in 10330. Christiansen *et al* (1990) and Sawada *et al* (1990) calculate frequencies of 8 and 8.2 per million under 15's respectively whilst Delattre *et al* (1991) estimate 13.5 per million per year. Grosfeld points out that the disease is marginally more common in boys. Muir *et al* (1990) support Miller's claims in 1989 and 1990 that neuroblastoma is less frequent in people of African descent, whilst this is disputed by Lucas and Fischer (1990). This difference may provide some important pointers to tumour biology (Miller, 1990, Muir *et al*, 1990) and to apparently higher incidence in Japanese studies.

To aid comparison of the data, Dr Parker constructed the following table at the Second International Symposium on Neuroblastoma Screening in Minnesota, 22^{nd}-24^{th} May 1991.

Area (time).	Incidence of Neuroblastoma/M Year	Mortality. Year
Quebec (77-86)	11.3	4.8
Denmark (70-80)	11.0	5.6
Great Delaware Valley (77-86)	10.6	5.0
Lyon	8.9	4-5
Brazil (80's)	7.7	6.2
U.K. (80's)	7.5	4.0
Sapporo (pre-screening)	13.6	10.8
Sapporo (post-screening)	14.1	4.1

Mortality is defined as number of neuroblastoma deaths per year/population size.

There is an association between parental participation in occupations that lead to increased exposure to electromagnetic fields (Spitz & Johnson, 1985, Wilkins & Hundley, 1990) though this is debated by Bunin *et al* (1990). No other significant association between parental occupation and neuroblastoma incidence is noted by these groups of authors.

Furthermore, White *et al* report an association between increased chance of neuroblastoma following *in vitro* fertilization and ovulation induction and Kobayashi *et al* (1991) make the same findings for ovulation induction alone. Toren *et al* (1992) reinforce the importance of extrinsic factors during embryogenesis in congenital disease.

1.1.3 Pathology.

The macroscopic appearance of a tumour varies with its position in the body, the degree of development and the extent of necrosis and haemorrhage. The tumour may be smooth or irregular, and generally becomes more nodular upon treatment. At first the mass is red/grey, vascular and friable. Although initially contained with in a capsule, tumours invade into surrounding tissue at an early stage, especially if found on the midline. Extension is generally along tissue planes and perineural pathways. Tumours vary widely in size from a few centimetres to structures of considerable volume. Haemorrhage and necrosis are often associated with larger

tumours, and this tends to lead to calcification. Major organs and vessels, the gut and ureters are often surrounded but rarely invaded by the cancer. This can lead to complications due to pressure upon and constriction of such bodies (Smith and Castleberry, 1990).

Microscopically neuroblastoma is difficult to differentiate from other "small blue round cell" tumours such as Ewing's sarcoma, acute lymphoblastic leukemia/lymphoma, Rhabdomyosarcoma and Wilm's tumour. The appearance of cells varies with degree of differentiation from uniform spreads to nests of very primitive cells. The presence of rosettes of cells around a pink centre, chromosome abnormalities such as deletions and double minutes (DM's) and dense neurosecretory core granules and microtubules in neural processes when viewed under electron microscopy give neuroblastomas a more distinctive appearance (Smith and Castleberry, 1990). Accurate diagnosis is now aided by immunoreactivity studies using a number of antibodies raised to different antigens (Wirnsberger *et al*, 1992, Roald & Brandtzaeg, 1991, Sugimoto *et al*, 1988). The value of chromosome aberrations in distinguishing these tumours is recognised by Sainati and colleagues in their 1992 paper.

1.1.4 Diagnosis.

Neuroblastoma has a wide range of symptoms, the subset of which are presented being related to the primary site, development and spread of the disease. The symptoms include pain, respiratory problems, swellings, masses under the skin, fever, weight loss, weakness, paralysis, blindness, watery diarrhoea, nausea, abdominal distension, stiffness and reluctance to walk. Older children may present with a feeling of general unwellness. The most common first symptom is a palpable tumour (Jereb, 1984, Smith and Castleberry, 1990, Evans, 1980). Some symptoms such as diarrhoea are related to vasoactive intestinal peptide production, whilst opsoclonus, darting eye movements (Carlsen, 1991, Smith and Castleberry, 1990),

and myoclonus (Pranzatelli & Balletti, 1992), jerks and ataxia of the trunk is often noted in patients and can provide an initial pointer to a diagnosis of neuroblastoma. Boney metastasis is associated with "bruising" around the orbit and some metastasized patients exhibit "blueberry muffin" marks under their skin (Smith and Castleberry, 1990, Evans, 1980). A minimum set of requirements for a diagnosis of neuroblastoma was proposed by Brodeur *et al*, 1988:

A diagnosis of neuroblastoma is established if:
Unequivocal pathologic diagnosis is made from tumour tissue by standard methods, including electron microscopy if necessary; or
Bone marrow contains unequivocal tumour cells (eg. syncytia) and urine contains increased urinary catecholamine metabolites (VMA and/or VMA >3 SD above the mean per mg creatinine for age).

This minimum could be modified to include more recently evaluated biological markers such as *N-myc* amplification, ferritin production and DNA ploidy. Prenatal diagnoses of neuroblastoma have been made on the basis of ultrasound scans. The neuroblastomal alterations in catecholamine metabolism of the foetus can have an adverse effect on the mother. Headache, sweating, paleness, tingling sensations in the toes and fingers and paroxysmal hypertension are all described as being associated with this syndrome and HVA and VMA level determination of the mothers urine and the amniotic fluid as well as examination of placental cells for signs of metastasis are recommended (Hosoda *et al*, 1992). Congenital cases can present with paraplegia, the outcome of these cases is frequently good, but neurological damage is often sustained (Munro *et al*, 1991).

Schummacher and colleagues note the raised incidence of rib anomalies, including bifurcation in children with neuroblastoma and malignancies in general when subjected to roentogenic analysis (Schummacher *et al*, 1992). The authors contend such features can act as pointers to disease since in neuroblastoma and 4

other paediatric cancers, the association is statistically significant (Schummacher *et al*, 1992). These diagnostic factors do not aid early clinical diagnosis and it is thought that 2/3 of a tumours life is subclinical (Abelson, 1987).

1.1.5 Staging of Neuroblastoma.

To allow accurate evaluation of clinical trials and patient monitoring, to provide prognostic information and to contribute information for the basis of appropriate therapeutic decisions, neuroblastoma has been divided into several stages. At least four staging systems have been widely considered, all of which have some incompatibilities, some being based upon the stage of disease at the time of diagnosis, for example that used by the Children's Cancer Study Group (CCSG) and others (Evans *et al*, 1971,) and some upon the resectability of the tumour, eg those used by St Jude's Children's Research Hospital and the Paediatric Oncology Group (Hayes *et al*, 1983, Nitschke *et al*, 1988) and systems based on the Tumour Node Metastasis (TNM) system proposed by the International Union Against Cancer (UICC) (Lippincott, 1983).

Although these systems all largely succeeded in distinguishing low stage good prognosis patients from those more advanced with poor outlook, comparison of individual cases is difficult. Points of disagreement include emphasis on the importance of tumour resectability, the prognostic significance of crossing the midline, the importance of lymph node involvement and the classification of stage 4 special (4S) cases (Brodeur *et al*, 1988). Most recently workers have been encouraged to use the International Staging System for Neuroblastoma (ISNN) proposed by Brodeur *et al* in 1988 (and the adaption of this by Nagahara *et al*, 1990) which attempt to incorporate these discrepancies. For addition comparison of staging systems, the reader is also referred to Evans *et al* (1990), Evans and D'Angio (1991), Carlsen *et al* (1986) and Halperin & Cox (1986), who favour the CCSG/Evans system, Hata *et al* who prefer the postsurgical version of the

UICC/TNM system. Fleming (1992), and Haase (1991) favour a blend of the two major divisions of staging systems (ISNN).

ISNN Staging.
Stage 1: Localized tumour confined to the area of origin; complete gross excision, with or without microscopic residual disease; identifiable ipsilateral and contralateral lymph nodes negative microscopically.
Stage 2A: Unilateral tumour with incomplete gross excision; identifiable ipsilateral and contralateral lymph nodes negative microscopically.
Stage 2B: Unilateral tumour with complete or incomplete gross excision; with positive ipsilateral regional lymph nodes; identifiable contralateral lymph nodes negative microscopically.
Stage 3: Tumour infiltrating across the midline with or without regional lymph node involvement; or, unilateral tumour with contralateral regional lymph node involvement.
Stage 4: Dissemination of tumour to distant lymph nodes, bone marrow, liver, and/or other organs (except as described in stage 4S).
Stage 4S: Localized primary tumour as defined for stage 1 or 2 with dissemination limited to liver, skin, and/or bone marrow.
Nagahara *et al* introduce the further prognostic subdivisions 4A and 4B with bone-cortex and bone marrow metastases respectively.

(ISNN staging will be used unless otherwise stated).

1.1.6 Management of Neuroblastoma.

1.1.6.1 Evaluation.

The minimal evaluation for clinical staging of neuroblastoma is described by Lippincott (1989) as being as follows: Complete blood count, (differential platelets), liver and kidney function studies, urinary catecholamine excretion study, bone marrow aspirate and diagnostic imaging.

Petrus *et al* summarised the current usage of radiological tests for studying suspected adrenal neoplasm in 1992 (Petrus *et al*, 1992). Here it is recommended that initial investigations should be performed using ultrasound and that NMR rather than CAT should provide more detailed information. This assessment is based upon the quality of information provided and the former techniques ability to function without administration of contrast enhancers or ionizing radiation, although GD-DTPA can be useful (Kornreich *et al*, 1991). NMR has the addition advantage of providing data on spinal canal extension without resorting to myelography, thus sparing the patient intrathecal injection (Petrus *et al*, 1992).

Moss and Sanders (1990) recommend monitoring of patients for relapse by immunological detection of neuroblasts in the patients blood. They commend this technique as being cost effective, non-distressing to the child and not requiring the use of ionising radiation. Detected relapse can be further evaluated with imaging studies.

1.1.6.2 Treatment.

The treatment of neuroblastoma has benefited disappointingly from the development of chemotherapy techniques (Craft and Pearson, 1989). Other paediatric solid tumours such as Wilms' tumour, nephroblastoma, teratocarcinoma and rhabdomyosarcoma have been found to respond well to this form of management, Wilms' tumour having a long term survival of 70-90% (Evans *et al*, 1992). The prognosis for older patients with advanced disease is still extremely poor (Smith and Castleberry, 1990, Sawaguchi *et al*, 1990, Ikeda *et al*, 1989). Despite this Thiery Philip identifies a subgroup of patients "curable" with "Megatherapy" (Philip *et al*, 1991, Philip, 1992). There are however problems in translating improved response to treatment into improved long term survival and aggressive treatment can have deleterious effects later in life (Craft and Pearson, 1989). The prognosis for less advanced cases is however somewhat better (Evans *et al*, 1987).

In general terms the strategy for treatment of neuroblastoma is surgical resection of as much of the primary tumour as possible, followed by chemotherapy to irradicate residual and metastatic disease (Tsuchida *et al*, 1992, Haase *et al*, 1989). For stage I tumours, surgery may prove sufficient (Pritchard and Kemshead, 1983). Whilst tumours are sensitive to ionizing radiation, administration of these techniques was generally superceded by chemotherapy except for pain control, diminishing problematic metastases and emergencies such as optic nerve compression (Pritchard and Kemshead, 1983, Smith and Castleberry, 1990). Radiotherapy is however being used again during autologous bone marrow rescue (August *et al*, 1984) to obviate complications of marrow toxicity when treating advanced cases with marrow involvement. Cure rate is however not improved on a long term basis by this technique (reviewed by Vossen in 1990). Silber and colleagues however report bone marrow therapy as the only treatment to alter the prognosis of advanced patients (Silber *et al*, 1991). Castleberry and co-workers argued in 1989 and 1991 that children older than 1 year with regional lymph node metastases have statistically significantly better response rates if chemotherapy is used in conjunction with radiotherapy. This is however debated by D'Angio *et al* (1991).

Chemotherapeutic agents seem most effective when used in multiple drug regimens which take advantage of drug synergism, differing mechanisms of cytotoxicity (esp cell cycle disruption), and vary side effects (Ikeda *et al*, 1989, Sawaguchi, *et al*, 1990). These protocols designed with reference to single agent approaches have achieved success in treating unresectable localized advanced disease, but there has been little improvement for over 30 years in the prognosis of older patients with more advanced disease (Vossen, 1990). Chemotherapy can be used to improve the operability of a large vascular, friable primary tumour, judged to be unresectable on a surgical "first look" by inducing shrinkage and a change to a fibrous less vascular nature (Smith and Castleberry, 1990).

Imashuku *et al* (1991) recommend the monitoring of initial response to chemotherapy by reference to biological markers such as catecholamine metabolite excretion and NSE levels, allowing tailoring of treatment to the individual. Novel treatments for advanced disease are actively sought in an attempt to improve the prognosis for this group of patients. One potentially promising technique, using radiolabelled I-MIBG is described by Sisson and colleagues, but benefits have only lasted a few months (Sisson *et al*, 1990, Pinkerton, 1990, Evans *et al*, 1992.). Yang *et al*, describe a technique for using high intensity focussed ultrasound to limit the growth of unresectable tumours, but this has not been thoroughly researched yet (Yang *et al*, 1992). Indicine N-oxide was tested in phase II trials with relapsed neuroblastoma patients, but to no effect (Miser *et al*, 1991). Evans and co-authors describe other novel treatments under development in 1992, as did Smith and Castleberry (1990).

Surgery is both diagnostic and therapeutic, the procedure can provide valuable information for the accurate diagnosis and staging of disease (esp. in illucidating lymph node involvement) as well as cure. The histologic nature of tumours is assessed from biopsy specimens, needle biopsy being used with increasing frequency (Roald & Brandtzaeg, 1991, Smith and Castleberry, 1990,). Hedborg *et al* (1992) whilst recomending this technique report problems in obtaining enough material for analysis and contaminmation of tumour cells with non cancerous tissue. Primary and metastatic disease can be operated upon and such procedures are classified as primary (exploration, prior to treatment), delayed primary (operation on primary tumour after initial therapy), secondary, tertiary, etc after primary surgery and/or treatment. Delayed and repeat operations allow for assessment of treatment and diagnosis or metastatic and recurrent disease without significantly influencing prognosis (Smith and Castleberry, 1990).

Primary tumours should be removed where ever possible (Tsuchida *et al*, 1992). Major organs, vessels and nerves should however not be compromised in

good prognosis cases in order to achieve removal of the mass. Resectability is judged in terms of mobility of the mass, position, relation to other important structures, friability, encapsulation, encasement of structures and vessel involvement (Smith and Castleberry, 1990). Surgical complications especially associated with neuroblastoma include uncontrolable haemorrhage, injury to renal vessels and neurological complaints. Younger children are more prone to complications, but are more able to overcome them (Azizkjan *et al*, 1985).

1.1.6.3 Risk Related Therapy.

The nature of treatment given is based upon a particular patients prognostic variables. Of these the most important are age and stage. Stage 1 and 2 tumours with a generally good prognosis are treated by solely by surgery (Stage 1 only, Jereb *et al*, 1984) and mild chemotherapy to ensure complete destruction a cancerous cells (Jereb *et al*, 1984, Smith and Castleberry, 1990). Stages 3 and especially 4 have a poorer prognosis, and require more aggressive chemotherapy following surgery (Ikeda, *et al*, 1989, Bigotti and Coli, 1990, Philip *et al*, 1991, Sawaguchi *et al*, 1990, Tsuchida *et al*, 1992). There is however, a subgroup, 4S (originally reported by D'Angio *et al*, 1970) of stage 4 disease which has a good prognosis and requires less (removal of primary tumour and mild chemotherapy, (Smith and Castleberry, 1990)) or no treatment (Evans *et al*, 1981). Haas *et al* dismissed surgery and advocated no treatment unless careful monitoring of disease warranted chemotherapy to preserve organ function. These authors preferred to hope for spontaneous 4S regression (Haas *et al*, 1988). Nitschke *et al* also refute the use of chemotherapy, claiming 100% success in the treatment of stage 4S disease without resort to cytotoxic drugs (Nitschke *et al*, 1983). Jereb and colleagues however warn that stage 4S disease is capable of progression to stage 4 with fatal outcome (1984). Paul *et al* propose that the dismal prognosis for stage 4 patients is somewhat less dire in children younger than 1 year if treated intensively with multiple chemotherapeutic agents (Jereb *et al*,

1984, Paul *et al*, 1991). Philip *et al* similarly identify a stage 4 subgroup responding better to therapy (Philip, 1991). Nakagawara *et al* advise aggressive therapy in all *N-myc* amplified patients regardless of other features (1988). Cervera *et al* (1990) point out the possibility of recurrent disease despite remission for many years and recommend rigourous follow up.

1.1.7 Regression.

Spontaneous regression of neuroblastoma is occasionally observed in young infants, but is extremely rare in older children (Carlsen, 1990). Neuroblastoma remission however accounts for around 17% of all human remissions from cancer (Everson & Cole, 1966). Dutch records show regression occurring in less than 2% of cases, but the actual incidence may be higher since some cases may have been recorded as malignant tumours, and some asymptomatic tumours may regress before diagnosis (Carlsen, 1992). Evans *et al* estimate regression in 8% of cases (Evans *et al*, 1976), whilst Pritchard and Kemshead agree with the Dutch estimate (Pritchard & Kemshead, 1983). Smith and Castleberry (1990) not that neuroblastoma has the highest remmission rate of all human cancers. All stages are capable of regression, with stage 2 and 4S comprising the majority (Evans *et al*, 1976). Both primary and metastatic lesions can regress (Evans, 1980).

Matsumura *et al* (1991) report a regressing stage 1 tumours that shrank in size and whose catecholamine metabolism gradually normalized. Over 18 months observation the tumour reduced by 90% in volume before surgical removal. No other treatment was given.

Knudson and Meadows proposed in 1980 that regressing stage 4S neuroblastoma was not in fact malignant since it lacked a second "hit" or genetic tumour activation. They furthermore suggest that the mutation is in the parent's germ line, newly arising in non-hereditary cases. It is proposed that stage 4S lesions arise from neural crest cells with a mutation affecting their normal developmental

pattern, and not as metastases of primary tumours (Knudson and Meadows, 1980, D'Angio, 1980,). The cells have the possibility to develop a malignant nature if a second mutation arises and this is taken as an argument against the administration of treatment with possible mutagenic side-effects (Knudson and Meadows, 1980). 4S lesions which eventually regress often do not respond to therapy (Evans *et al*, 1976). The lesion cells are thought to have arrested development, allowing cell proliferation. If no further mutagenic event occurs, the lesions cells should eventually complete benign differentiation to gangioneuromas or neurofibromas. The mechanism of tumour regression is still not ellucidated with any certainty (Haas *et al*, 1988).

1.1.8 Maturation.

Another favourable outcome for neuroblastoma patients is maturation of the tumour to benign ganglioneuroma (Dyke and Mulkey, 1967, Evans *et al*, 1976, Uchino *et al*, 1978, Garvin *et al*, 1984) and/or neurofibroma (Griffin and Bolande, 1969, Rangecroft *et al*, 1978). In these cases, malignant neuroblastoma cells differentiate to benign ganglia, either spontaneously or as a result of treatment (Evans, 1980). Maturation is a possible outcome for both localized and metastatic disease (Evans *et al*, 1976). Favourable biological markers (section 1.1.10) are associated with this rarely documented event (5 cases in 18 years) (Haas *et al*, 1988). Shimada *et al* reported in 1984 that patients with good prognosis showed signs of tumour maturation along and age dependant sequence. The recorded incidence of both spontaneous regression and maturation is perhaps somewhat lower than it might be due to the reluctance of pediatricians not to treat ill patients.

1.1.9 Prognosis for Neuroblastoma Patients.

In the early 1930's neuroblastoma was considered almost universaly fatal, aside from a few tumours which spontaneously regressed or matured. The application of surgery in the late 1920's and radiation therapy 10 years later increased overall survival rate to 20-30%. Chemotherapy has improved the prognosis for certain groups to greater than 80% survival (Carlsen, 1991). Prognosis for patients with advanced disease remains poor despite vigorous efforts, but complete remission rates and prolonged disease free survival have been improved (Evans *et al*, 1987). August *et al* recommend bone marrow therapy for improving the prognosis of advanced patients (1984).

1.1.10 Prognostic Factors in Neuroblastoma.

The evaluation of factors which relate to the eventual outcome and course of a disease is important in determining the most suitable treatment. By considering many such factors which are correlated with the prognosis, more accurate decisions can be made (Carlsen, 1991) and administration of treatment with undesirable side effects minimized (Evans *et al*, 1987). Correct treatment is also necessary to avoid complications of residual tumour resistance (Silber *et al*, 1991). Recognition of prognostic factors also aids comparison of different treatment modalities. The choice of system for diagnosis and staging may have an effect upon incidence, time of detection and prognosis (Carlsen, 1991).

1.1.10.1 Symptomology and Diagnosis.

The association of watery diarrhoea, and Dancing Eye Syndrome with neuroblastoma generally lead to an early diagnosis, improving prognosis. Incidentally discovered tumours generally have a good prognosis (Carlsen, 1990) as do ones detected by mass screening ((87.5% survival) Nishi, *et al* 1988). Conversely

symptoms associated with metastatic disease such as bone pain, rheumatic symptoms, preorbital ecchymoses and proptosis, malaise, irritability, anaemia and unexplainable fever indicate a poorer outlook (Carlsen, 1991).

1.1.10.2 General Prognostic Factors.

Age and stage are related to the outcome of a case (Breslow & McCann, 1971, Evans *et al*, 1987). Traditionally age at the time of diagnosis was considered to be the most important single prognostic factor, children under 1 year having the best prognosis (Kajanti & Holsti, 1986, Ninane, 1992). Furthermore Evans stage II, III and IV (Evans *et al* 1971), children under 1 year of age at time of diagnosis have a better prognosis than older children of the same stage (Jereb *et al* 1984, Philip, 1992). This indicates that the better prognosis for younger children is not because of a lower bias in the stage spectrum. These authors state that the ability of young patients' tumours to mature may be significant however, as a greater proportion of mature tumours (ganglioneuroblastomas) are found in older children. It is proposed that neuroblastic tumours progress along a maturational sequence with age and that only tumours overdeveloped for their age are truly malignant (Shimada *et al*, 1984, Cooper *et al*, 1991).

Evans stage (Evans *et al*, 1971) is an independent prognostic factor (Jereb *et al*, 1984, Evans *et al*, 1987). Patient treated at the Children's' Hospital of Philadelphia have the following 2 year survivals:

Evans Stage	I	II	III	IV	IVS
Survival(%)	100	82	42	30	100

Whilst the table above relates the general picture, it must be remembered that different staging systems will show different effects on prognosis. (It is therefore possible that different staging systems are more useful in evaluating different types of neuroblastoma). However, disease extent, the basis of several staging systems,

including Evans (Smith and Castleberry, 1990), is shown to be of great prognostic significance (Carlsen *et al*, 1986, Wilson and Draper, 1974, Breslow & McCann, 1971, Jaffe, 1976, Coldman *et al*, 1980), many authors attributing greatest import to this factor (Kajanti & Holsti, 1986, Delattre *et al*, 1991).

The site of the primary tumour is important in determining outcome. Retroperitioneal and especially adrenal neuroblastoma are associated with the poorest prognosis, whilst a mediastinal site indicates a better course of disease (Ninane, 1992).

In localised disease, lymph node involvement is indicative of poor prognosis (Hays *et al*, 1983, Ninane *et al*, 1982). The same has also be found to hold true in metastatic disease, and more surprisingly the prognosis of patients with distant lymph node metastasis is better than that of patients with regional involvement (Yamada *et al*, 1992).

The pattern of urinary catecholamine metabolite excretion is correlated to outcome, a high ratio of VMA to HVA production being favourable, indicating the maturity of the tumour (Laug *et al*, 1978). Sex and race are not of prognostic significance (Evans *et al*, 1987).

1.1.10.3 Tumour Markers with Prognostic Significance.

Ferritin is an iron binding proteinaceous component of normal and diseased serum (Hann *et al*, 1980a, Blatt *et al*, 1990). Increase in serum ferritin above 142ng/ml was shown by Hann *et al* (1980b) to be a indicator of poor prognosis. Prognostic value of ferritin levels is confirmed by Evans *et al*, 1987. The level of serum ferritin correlates approximately with tumour size (Carlsen, 1991). Tumour cells contain (Arioso *et al*, 1976) and secrete (Watanabe *et al*, 1979, Hann *et al*, 1984) ferritin. The work of Blatt and colleagues (1990) demonstrates that neuroblastoma cell synthesize ferritin *de novo* under regulation of iron levels. The role of the

protein in tumour biology is still unclear (Blatt *et al*, 1990) however, Blatt & Wharton (1992) report that ferritin stimulates mitosis in cultured neuroblastoma cell lines. The prognostic significance of ferritin levels is independent of age, stage, histology and etiology (Evans *et al*, 1987) and a 90% accurate model predicting outcome from ferritin levels, age, stage and histology was devised by Silber and colleagues (1991). Laboratory measurement of serum ferritin is routine and, coupled to age of accurate prognostic importance (Evans *et al*, 1987).

Serum neuron specific enolase (NSE) levels above 100mg/ml have been correlated with stage and dissemination of disease by Zelter and co-workers (Zelter *et al*, 1980, Zelter *et al*, 1983). NSE levels can be used to separate advanced patients into good and poor prognosis groups (Evans *et al*, 1987, Zelter *et al*, 1986).

In stage IVS (Evans *et al*, 1971) patients serum levels of lactose dehydrogenase correlate with prognosis and *N-myc* amplification (Nakagawara *et al*, 1990b).

1.1.10.4 Histopathologic Factors.

The Shimada classification (Shimada *et al*, 1984) divides patients into stroma-poor and stroma-rich subgroups on the basis of stromal development, organization and number of Schwann cells, stroma or "background" cells being a product of the tumour and also arising from the neural crest. The stroma poor group is further subdivided into favourable (84% survival) and unfavourable (4.5% survival groups) according to the patients age at diagnosis, tumour maturity and nuclear pathology (mitosis-karyorrhexis index (MKI)). Similarly, the stroma rich group is subdivided into well differentiated (100% survival), intermixed (92% survival) and nodular (18% survival) groups, solely on the basis of tumour morphology (Shimada *et al*, 1984). The authors make the interesting prediction that the tumours they classify as having good prognosis will eventually mature to benign masses, whilst poor prognosis tumours are essentially more aggressive and malignant in nature, requiring

more exhaustive management. In 1992 Shimada *et al* note a correlation between stroma poor undiferentiated tumours with a high MKI and amplification of the *N-myc* oncogene (Schwab *et al*, 1983). This they propose is because the DNA binding protein product of this gene can act upon Schwann cells, arresting development and neuroblast differentiation thus leading to a certain morphology. Carlsen (1991) extends the prognostic value of the Shimada classification by linking it to age.

Bigotti and Coli reported evidence that supported the validity of Shimada classification. These authors further add to the classification, reporting that in good prognosis cases, anti S-100 protein anti sera reacts with slender satellite cells in the stromal septa of neuroblasts. The use of NSE antisera is recommended to aid cell identification. Similarly Wirnsberger *et al* evaluated a panel of commercially available antibodies for diagnosis and gauging of tumour maturity (Wirnsberger *et al*, 1992).

1.1.10.5 Cellular Factors.

P-Glycoprotein is a conserved 170Kd plasma membrane protein with homology to bacterial active transport proteins. It is thought to be responsible for ATP dependent export of many chemotherapeutic drugs from the cell, thus causing multiple resistance. This protein is only detected using immunological methods in patients with non-localized aggressive disease, and is associated with poor prognosis. Pre-treatment levels of P-Glycoprotein levels correlate with the success of chemotherapeutic treatment in metastatic disease. It is thought treatment of patients with agents that block the action of this transport protein before chemotherapy may be beneficial (Chan *et al*, 1992). White however contends that the predictive value of P-Glycoprotein expression may be somewhat more complicated. The alkylating agent type drugs, most effective in the treatment of the neuroblastoma are not thought to be subject to P-Glycoprotein export and this author suggests that increased expression may be a marker for as yet undetected factors (White, 1992).

1.1.10.5.1 Genetic Abnormalities.

The study of genetic alterations is important in determining both prognosis and the underlying mechanisms of carcinogenesis.

Flow cytometric analysis has revealed a non random bimodal DNA index distribution. Approximately 1/3 of tumours have a near diploid genome, the remainder being hyperdiploid, most commonly near triploid. Tumour DNA content is a stable marker and does not vary with disease progression or maturation or therapy (Taylor *et al*, 1988). Hypodiploidy is rare in neuroblastoma (Delattre *et al*, 1991). Naito *et al* report a somewhat smaller proportion (around half) of aneuploid tumours but associate hyperdiploidy with a good prognosis-58.8% of patients older than 1 year surviving, compared to 28.6% of diploid tumour children of similar age. Furthermore, in the same study of patients with advanced disease, stage III and IV (Evans *et al*, 1971) 63.2% of aneuploid tumour patients survived, compared to 30.4% of children with diploid tumours. This is surprising in that in the majority of other tumours higher DNA content is associated with aggressive, behaviour and poor prognosis (Naito *et al*, 1991). 73.3% of these authors' patients who had aneuploid tumours survived, whilst 66.7% of the group with diploid lesions succumbed to disease. This is a statistically significant difference. DNA ploidy is an independent prognostic factor (Naito *et al*, 1991, Carlsen, 1991), possibly because aneuploid tumours have a better response to chemotherapy (Look *et al*, 1984). There is however, a marked association between diploidy and advanced disease, whilst aneuploid tumours are found at all stages of tumour development (Naito *et al*, 1991). Cohn *et al* report a statistically significant association between DNA diploidy and unfavourable histology and between ploidy and high proliferative activity (Cohn *et al*, 1990). Furthermore DNA content of tumours seems strongly correlated with age and stage (Carlsen, 1991). *N-myc* amplification is restricted to tumours with an unfavourable DNA content (Dominci *et al*, 1992), whilst Cohn *et al* found in 1990

that there was a non-statistically significant trend between *N-myc* amplification and DNA ploidy. The reason for increase in genetic material is unclear at present (Carlsen, 1991) and in hyperdiploid tumours a population of normal diploid cells is always found (Hayashi *et al*, 1986).

Cytogenetic signs of gene amplification such as homogeniously staining regions (HSR's) and extra chromosomal elements, double minutes (DM's) are associated with poor prognosis (Schwab *et al*, 1983, Franke *et al*, 1986). These structures develop during tumour evolution, largely due to amplification of the *N-myc* oncogene (Schwab *et al*, 1983) and surrounding sequences from 2p24 (Carlsen 1991). The number of copies of amplified sequence varies from 10's to thousands and usually consists of repeats of a few hundred Kb. HSR's are found on many different chromosomes (Franke *et al*, 1986). The elements are found in about 1/3 of all primary tumours and in most cell lines (Delattre *et al* 1991). The ability of a tumour to establish cell lines is itself a marker of poor prognosis (Evans *et al*, 1987). The features are associated only with advanced disease (Franke *et al*, 1986). These authors also report that whilst HSR's and DM's may both be present in the same tumour cell population, they are never found in the same metaphase. Brodeur *et al* point out in 1984 that more tumours may show signs of amplification when studied with molecular biology techniques giving a higher resolution that the 10^6-$5*10^6$ bp of light microscopy.

Aberrations of the short arm of chromosome 1 are associated with poor prognosis (Franke *et al*, 1986). At least 70% of human neuroblastomas display cytogenetically visible anomalies of this type. Analysis using molecular biology techniques shows the frequency to be nearer 90% (possibly 100%) in advanced cases (Weith *at al*, 1989). Allelic loss (loss of heterozygosity (LOH) (Carlsen, 1991)) is associated with these aberrations, the consensus deletion mapping to 1p36.1-2 (Weith *et al*, 1989). This 10 megabase segment (Weith *et al*, 1989) is thought to be

the location of the putative neuroblastoma tumour suppressor (Carlsen, 1991, Weith *et al* 1989, White *et al*, 1991). There is no correlation between putative suppressor loss and *N-myc* oncogene overexpression, suggesting different mechanisms of acquisition (Weith *et al*, 1989). This is however debated by Brodeur (1990) who's colleagues find strong association. LOH for chromosomes 14 and 13 may also be informative and provides evidence of neuroblastoma's genetic heterogeneity (Brodeur and Nakagawara, 1992).

Genomic amplification of the *N-myc* oncogene is closely related to the proliferation of neuroblastoma cells (Schwab *et al*, 1983). *N-myc* is part of a family of *myc* genes (DePinho *et al*, 1987). Only a patient's tumour cells are amplified implying somatic rather than germinal multiplication (Schwab *et al*, 1984). Closely regulated (unamplified) *N-myc* expression plays a role in human foetal brain development (Grady *et al*, 1987). Amplification is thought to occur after 1p deletion (Christiansen and Lampert, 1988) and is related to increased gene expression (Nisen *et al*, 1988) due to increases in 4kb mRNA transcript numbers (Seeger *et al*, 1985) though the link between overexpression and amplification is not inevitable (Schwab *et al*, 1984). The gene can be amplified from 3 to several hundred times (Brodeur *et al*, 1984) and copy number is temporally and spatially stable (Brodeur *et al*, 1987). Of neurological tissue, only primitive neuroblasts and not differentiated ganglia express *N-myc* RNA (Schwab *et al* 1984, Grady-Leopardi *et al*, 1986). Clinically this amplification is associated with poor prognosis, progressing Stage III and Stage IV (Evans *et al*, 1971) disease (Brodeur *et al*, 1984, Seeger *et al*, 1985, Nakagawara *et al*, 1987a). 65% of Stage III cases and 47.5% Stage IV are amplified for the oncogene, Stage II and particularly Stage I (Evans *et al*, 1971) tumours are however much less likely to show increased copy numbers (Brodeur *et al*, 1985). Nakagawara *et al* (1987b) extend the prognostic significance of *N-myc* amplification to Stage II and IVS (Evans *et al*, 1971) tumours, normally associated with a good prognosis, unless amplification is observed. Shimada *et al* have found that patients of any stage have a

worse outlook if their tumour is amplified when compared to similar non amplified cases (1992) and this argument is supported by Brodeur (1990). Tonini *et al* adopt report a less inevitable prognosis in 1990 however, and Cornaglia-Ferraris *et al* (1990) report that a highly tumourigenic cell line they studied had undetectable *N-myc* expression. Despite strong correlation with stage and primary site (Nakagawara *et al*, 1987a), amplification is however age independent (Brodeur *et al*, 1985). Although disputed by several authors, including Nakagawara *et al* (1987a) this suggests a strong role for *N-myc* in tumour biology, which few authors deny. Nerve growth factor receptor expression is inversely correlated to *N-myc* expression, and the ability to respond to this signal may play a role in determining outcome (Christiansen *et al*, 1990). Nakagawara and colleagues reported in 1990 (Nakagawara *et al*, 1990a,b) that even correcting for variables such as tumour volume, stage and prognosis, *N-myc* amplified tumours excreted more HVA and VMA and at a different ratio than their non-amplified counterparts. This links to Laug *et als'* 1978 correlation of tumour catecholamine metabolism immaturity and prognosis. *N-myc* gene is also amplified in retinoblastoma (Schwab *et al*, 1984, Lee *et al*, 1984) and astrocytoma (Garson *et al*, 1985) as well as Wilm's tumour and heptoblastoma, tumours not derived from the neuroectoderm (Nissen *et al*, 1986). The protein product of the oncogene is localised in the nucleus, so it is here that it is presumed to have its effect (Slamon *et al*, 1986, Ikegaki *et al*, 1986, Ikegaki *et al*, 1988) and would be expected to function in concert with a Max partner (Cole, 1991). In summary *N-myc* seems to have a key role in determining the aggressiveness of neuroblastoma tumours (Seeger *et al*, 1983), however, in some cases this has been shown not to be so (Seeger, *et al*, 1988, Cornaglia-Ferraris *et al*, 1990) and Cohn *et al* (1990) report that in some tumours a biologic factor other than *N-myc* plays a part in this determination. Recently Sansone and co-workers have found that *N-myc* amplification does not seem to have an effect on prognosis in older children though the sample they studied was small (Sansone *et al*, 1991). In stage IVS (Evans *et al*,

1971) tumour regression is independent of oncogene amplification and expression does not correlate with prognosis (Nakagawara *et al*, 1990b). Garvin and colleagues suggested in 1990 that *N-myc* expression may be a factor in tumourigenesis whilst amplification may be a later event, correlating with progression. Furthermore overexpression from a single copy *N-myc* gene is not a marker of poor prognosis (Brodeur, 1990).

Telomeres are the physical ends of eukaryotic chromosomes. Shortening of the G-rich repeated sequences forming these structures is associated with aging and a number of malignancies (Hiyama *et al*, 1992). The structures exist to prevent chromosomes fusion and degradation (Zaikan, 1989, Blackburn, 1991). Compared to average values, aggressive neuroblastomas with a poor prognosis show significant reduction in telomere length (Hiyama *et al*, 1992). It is also noted by these authors that similar observations can be made in stage IVS (Evans *et al*, 1971) cases which have favourable outcomes. This can not be adequately explained at present, though may relate to cell senescence before regression. It is reported in the same paper that a few older advanced patients had telomeres extended above the average length. This however could be an artefact of the study as there is a degree of individual variation in this characteristic, and the authors do not compare tumour to non lesion data on a personal basis.

1.1.10.6 Additional Factors.

Eckschlager concludes that increased peripheral lymphocyte count is associated with good prognosis in neuroblastoma patients. He however notes that lowered counts do not indicate poor prognosis. It is proposed that the lymphocyte count is raised due to the antigenic nature of the tumour. (Eckschlager, 1992).

1.1.10.7 Summary.

Studies of tumour biology have lead to the proposal of 2 models explaining neuroblastoma behaviour. Firstly all neuroblastoma could begin as benign disease with favourable prognosis and if untreated, evolve into more benign, aggressive disease with poor prognosis. Alternatively, there could be sub-populations of disease, the more favourable group being detected early and being more readily cured, the second, more aggressive with poor prognosis (Mauer, 1988, Brodeur, 1991). The former rarely if ever would progress to the latter, though Knudson thinks it theoretically possible (Woods *et al*, 1992). This would be consistent with the idea of single and double "hits" in the Knudson model of disease development although there is apparent scope for intermediates.

Clinical/Genetic Types of Neuroblastoma.

Feature	Type 1.	Type 2.	Type 3.
Age	<12mo	Any age	Any age
Stage	1,2,4S	3,4	Any stage
Ploidy	Hyper diploid	Near diploid	Near diploid
	Near tetraploid	Near tetraploid	Near tetraploid
LOH	No	No	Yes
DM/HSR	No	No	Yes
N-myc Amp.	No	No	Yes
Prognosis.	Good	Intermediate	Poor

The above table, adapted from Brodeur's 1991 paper and his submission to the Second International Symposium on Neuroblastoma Screening in Minnesota, May 1991 lists characteristics of the proposed subgroups. Similar findings are made by Hayashi *et al* (1988) and Woods *et al* (1992). It is debatable which is the more useful model, Shimada, Cooper and their colleagues having observed disease progression (Shimada *et al*, 1984, Cooper, *et al*, 1991) and Brodeur and co-workers being able to accurately predict prognosis by reference to subgroupings.

1.1.11 Screening for Neuroblastoma.

Neuroblastoma is associated with a distinctive urine chemical phenotype (Scriver *et al*, 1987). In 1957 Mason and co-workers in Newcastle first reported that a patient with neuroblastoma secreted adrenalin and noradrenaline in her urine (Mason *et al*, 1957). The presence of elevated levels of the catecholamine metabolites (Voorhess and Gardiner, 1960, 1961) HVA and VMA is now taken as diagnostic of the disease (Gitlow *et al*, 1960, 1970), 92% of patients excreting one or both compounds at raised levels (Tuchman *et al*, 1987b). Catecholamines are synthesized from tyrosine (Candito *et al*, 1992). The dopaminergic pathway which leads to HVA is often present in neuroblastoma cells, but lacks the enzyme dopamine-ß-hydroxylase, responsible for the conversion of dopamine to norepinephrine. Conversely cells rich in this enzyme have the capacity for generating norepinephrine and its metabolite VMA (Laug *et al*, 1978, Woods *et al*, 1992). Serum and urine catecholamine metabolite levels are strongly correlated (Dugaw *et al*, 1990). Biochemically immature neuroblastomas lacking dopamine-ß-hydroxylase are associated with a poor prognosis (Laug *et al*, 1978). Mastrangelo *et al* present evidence that both cell types could be present in the same tumour (1991). Neuroblastoma is the first paediatric cancer to be screened for (Hammond, 1988).

The general current methodology for screening is as follows: Mothers are provided with a neuroblastoma screening kit which explains the programme and contains an absorbent piece of filter paper. A sample of the child's urine is collected on the filter paper by the mother, air dried and returned for analysis. Random sample timing is thought to be adequate (Tuchman *et al*, 1985) though Weetman *et al* (1976) and Winkel and Slob (1973) report a diurnal variation in catecholamine metabolite excretion. At the screening centre the sample is eluted in aqueous solution (Seviour *et al*, 1988), checked for contamination by faeces or other compounds such as nappy creams and the phenolic acids extracted from it by differential solubility in organic solvent (Seviour *et al*, 1988, Lemieux *et al*, 1988).

This sample is derivitised if necessary (Dale *et al*, 1988) and screened for the presence of HVA and VMA with concentrations elevated above normal levels as determined by screening large numbers of healthy patients. Each group of workers has ascribed its own diagnostic cutoffs, usually 2-3 standard deviations above the mean excretion values sometimes with bias for age, sex and other factors (Cropper, 1989, Huddart & Mann, 1991 Henderson *et al*, 1992, Parker *et al*, 1992). Because the patients urine excretion pattern will vary the apparent concentration of compounds in the sample, HVA and VMA concentrations are expressed relative to creatinine concentration measured by the Jaffe reaction (Folin & Wu, 1947, Chasson *et al*, 1961, Kuroda *et al*, 1990). Creatinine is excreted at a roughly constant level throughout a 24 hour period and so provides a suitable standard (Tuchman *et al*, 1989). However, caution is advised when calculating urinary compound concentrations relative to creatinine from an untimed measurement (Di Ferrante & Lipscomb, 1970, Henderson *et al*, 1992). Positive samples are retested from a second paper or liquid sample before medical work up begins. Some screening techniques do not require extraction of phenolics from the resuspended aqueous urine solution (Kinoshita, 1988, Sawada 1988) whilst some require the use of fresh urine (Kodama & Nakata 1984). There is a strong correlation between HVA and VMA levels from dried filter paper and fresh urine samples, even when paper samples are stored for several days (Tuchman *et al*, 1987a).

1.1.11.1 Rationale for Screening.

> "The early detection of cancer, whether in children or adults has long been viewed as a worthwhile public health goal" (Prorok, 1992).

When neuroblastoma screening was initially discussed, it was held to be true that in neuroblastoma patient populations, age is strongly correlated to progression to a more malignant phenotype, suggesting that advanced patients have passed undetected through lower stage disease (with better prognosis). The 2 year survival

for patients under 1 year at the time of diagnosis is 75%, compared to 17% for patients whose disease is detected later, regardless of stage (Scriver *et al*, 1987). This would suggest early diagnosis is desirable. It is difficult to detect neuroblastoma clinically while in infancy or early stages due to lack of chraracteristic symptoms (Sawada *et al*, 1991, Berthold *et al*, 1991) and it was assumed that presymptomatic patients would have a better prognosis and require less treatment, with fewer side effects (Draper, 1988). However this author points out that some cases will be missed by a test (false negatives) and some detected cases will spontaneously regress, without the need for treatment that may be administered (Pastore *et al*, 1984), even "cautious" therapy being toxic (Carlsen, 1992). Furthermore it is assumed that the neuroblastoma that clinically manifests itself later in life is present during infancy to be detected. This is probably a safe supposition (Carlsen, 1988) but the tumour may be too small to produce detectable levels of catecholamine metabolites (Norman *et al*, 1987, Ishimoto *et al*, 1990) or initially quiescent (Weinblatt, 1988, Cropper, 1989). False positives cause excess parental anxiety and are equally undesirable (Sawada *et al*, 1991).

Aside from the medical and ethical reasons for screening, there is also an economic incentive. Whilst the cost of mass screening would be relatively small (Hillson *et al*, 1988, Sato *et al*, 1988, Nishi, *et al*, 1991, Berthold *et al*, 1991) the cost of suitable chemotherapy can be £400 per day per patient, up to £40000 for a full course despite the poor prognosis (Cropper, 1989). Woods & Tuchman report expenditure of $144256 on advanced patients and $24650 on early stage disease (1987). Cost of screening an entire population is roughly equal to treating the expected number of patients within it and there may be a small saving provided the test is reliable and accurate (Hillson *et al*, 1988, Nishi, *et al*, 1991, Berthold *et al*, 1991). Increased sensitivity improves cost benefit, although rescreening is performed at additional expense. (Nishi *et al*).

Thus there is an putative advantage in spending money on screening to detect early asymptomatic neuroblastomas in the hope of being able to cure a greater proportion of children and at lower cost.

1.1.11.2 History of Neuroblastoma Screening.

Large scale screening of infants for neuroblastoma was first instigated in the Kyoto Prefecture, Japan. Dr Sawada and colleagues employed the LaBrosse spot test method, based upon a purple colourmetric reaction of VMA with alkaline diazotized p-nitroaniline (LaBrosse, 1968). This method is only 75% accurate, missing tumours that do not secrete VMA, and prone to false positives due to reaction with other compounds in the patients urine (McKendrick and Edwards, 1965, LaBrosse, 1968). From 1972 the programme concentrated on 3 year old children, the only child detected dying from Evans Stage II progression, but attention was switched to 6 month old infants in 1974 in the hope of detecting more readily cured children. This proved more successful, 4 asymptomatic children being detected and cured after surgery in the 6 years up to 1979 (Sawada *et al*, 1991). This demonstrates the need for careful consideration of time of screening. Unlike phenylketonuria, cretinism and galactosemia neuroblastoma can not be detected at any age, as tumours may not be large enough to secrete sufficient catecholamine metabolites to detect (Norman *et al*, 1987, Ishimoto *et al*, 1990) as the number of false negatives in the Newcastle screening programme testifies. 30g is thought to be the borderline weight of a detectable tumour (Nishi *et al*, 1990b). This argues in favour of late testing as does early imbalance in catecholamine and creatinine metabolism (Tuchman *et al*, 1989), however the possibility of progression demands screening to be as soon as possible. Ideally each child should be screened at several stages during childhood, but this may be prohibitively expensive. Ishimoto *et al* (1990) report that screening at 6 months detects mainly triploid non *N-myc* amplified tumours (good prognosis) and that late occurring diploid and/or *N-myc*

amplified (poor prognosis) cases with immature catecholamine metabolism are more amenable to detection around the first birthday. This is supported by Woods *et al* (1992) and Kaneko *et al* (1988). Berthold *et al* argue in 1991 for screening between 10 and 12 months after birth whilst Ishimoto *et al* (1990) would prefer 18 months. Thus the efforts so far, typically screening at 6 months may only detect patients who could have been cured anyway and this seems to be reflected in the rise in incidence but no decrease in death rate (Weinblatt, 1988).

All recent mothers in the Kyoto prefecture were provided with a VMA screening kit during routine health checks and asked to return a urine soaked filter paper for analysis when their child reached 6-7 months. The spot test was adapted to ease assay and employed in Nagoya, Japan from 1977. Osaka started spot tests in 1980 (Sawada *et al*, 1991). Positive tests are confirmed (Takeda, 1989) and followed up by hospital visits, examination and tumour imaging (Sawada *et al*, 1991).

Mass screening started in Sapporo City in 1981, initially based upon spot testing for VMA and determination of HVA concentration by TLC, simultaneous detection being called for by McKendrick and Edwards (1965), Yoshiura *et al* (1987) and Nishi *et al* (1990a) due to the large number of non VMA secreting tumours (LaBrosse, 1968). HVA is a more useful diagnostic indicator as its excretion is not stress related (Cropper, 1989), but VMA is more sensitive at an early age (Nishi *et al*, 1990a, Tuchman & Woods, 1992). Candito *et al*, recommend additional consideration of methylated catecholamine metabolites (1992). Tuchman and Stoeckler (1988) note that conjugated HVA and VMA may have diagnostic importance in "borderline" cases with slightly abnormal urine composition. In 1984 the system was replaced by automated sampling and simultaneous VMA and HVA determination using HPLC and electrochemical detection (Takeda, 1989). Unlike the LaBrosse spot test, HPLC is not sensitive to false positive results due to dietary constituents, although certain drugs can alter the result (Sawada *et al*, 1992, Weetman *et al*, 1976) and therefore is not subject to Saito *et al's* recommendation

for a test diet when spot and dip testing (Saito *et al*, 1990). Despite initial problems of bacterial contamination in the wet paper samples leading to an apparently lower creatinine and VMA concentration (Mizuta, & Umisa, 1988), accuracy improved after drying of filter papers before return (Sato *et al*, 1988). Following Welfare Ministry advice, many Japanese self governing regions started to employ spot testing and TLC after 1985, and after 1988 financial support was extended to HPLC techniques (Takeda, 1989), 49 of 57 administrations using this technique in 1989 with 4 fold higher sensitivity and lower false positive rates, leading to less parental anxiety (Sawada *et al*, 1991).

In about 90% of the Japanese cases detected by screening, no clinical symptoms had been observed and tumour could not be detected during the first visit to hospital in 30%, despite screening evidence (Takeda, 1989). Patients typically range in age from 7-9 months when diagnosed as time is required for testing and retesting and due to late return of samples (Sawada *et al*, 1989). This is younger that the average age for clinical diagnosis 65% of children have stage I and II (Evans *et al*, 1971) tumours, whilst 10% have stage IVS disease (Evans *et al*, 1971), 11% of children are stage III (Evans *et al*, 1971) and 13% stage IV (Evans *et al*, 1971) (Tsunoda, 1987) and this supported by other studies (Sawada *et al*, 1991). The proportion of advanced stage disease is much the same in screened and clinically diagnosed groups (Gutjahr, 1988) and this argues against screening. Primary sites are much the same as for clinically diagnosed patients (Takeda, 1989). 5 year survival is 96% and 95% of Evans stage III and IV cases have responded well to treatment (Sawada *et al*, 1991).

Workers in Newcastle-upon-Tyne sponsored by the North of England Children's Cancer Research Fund and the Neuroblastoma Society initially evaluated HPLC for the purpose of neuroblastoma screening (Dale 1989). This however proved too slow for their requirements and results were difficult to interpret due to signals from other compounds present in urine. Similar contamination problems

(Matsumoto *et al*, 1985) were found with GCFID (Dale *et al*, 1988) though GCMS has yielded far better results. Screening takes place at 6 months of age (Seviour *et al*, 1988). Presenting their results at the Second International Symposium on Neuroblastoma Screening in Minneapolis, 22nd-24th May 1991, Drs Dale and Craft stated that from 20816 infants screened up to 1990, 2 patients had been correctly diagnosed along with 7 false positives. 3 neuroblastomas had been missed and were identified clinically. 1 case of congenital muscular dystrophy was also diagnosed , having elevated urinary catecholamine metabolite concentrations when compared to suppressed creatinine excretion

Workers in Quebec have used various thin layer chromatographic, chemical and spectrophotometric methods for neuroblastoma screening (Lemiuex *et al*, 1988). Screening began in 1971 and consists of a primary TLC screen followed by a secondary screen for accurate quantification of HVA and VMA levels in samples found to be positive on the first screen. A variety of secondary screens have been employed, GC-MS being used since 1989 (Tuchman *et al*, 1987a, Tuchman *et al*, 1991b) in collaboration with workers in Minneapolis who had previously run their own small pilot programme (Tuchman *et al*, 1989). Screening has been performed at various ages, 5, 14 and 21 days. The earliest screen gave poor compliance, the 14 day test high false positive incidence (Lemieux, 1988) and a 3 week screen is now employed followed by a 6 month check (Tuchman *et al*, 1991b). The TLC test has the benefit of being able to detect many organic acidurias (Lemieux *et al*, 1988), with the potential benefit of preventing death, mental retardation and developmental anomalies (Tuchman *et al*, 1991a) this advantage is extended to GC-MS by Tuchman *et al*, 1991a. The project is of especial interest since it provides a population based comparison between a screened and 4 similar but unscreened cohorts, thus allowing accurate analysis of the benefits of screening. This is a significant analytical advantage over the Japanese study.

The Canadian project has correctly detected 7 neuroblastomas and 2 additional cases of possible regression. Concurrently 12 additional patients have been diagnosed clinically, 6 prior to screening, 1 who missed testing and 5 false negatives due to non excreting tumours at time of testing. VMA seems to be a more sensitive marker in these early cases, being raised in all the secreting patients, whilst HVA was normal in 3. The tumours studied had biologic markers consistent with good prognosis (Tuchman *et al*, 1991b).

In 1989 Mellor *et al* succeeded in raising specific antibodies to VMA in sheep using derivitised antigen. This improved reproducibility and facility of assay over the technique of Yoshioka *et al* (1987) and previous workers. They considered their product to be the first basis of a possible practical immunological catecholamine metabolite screening test although Yokomori *et al* published the results of a pilot study, based upon the earlier work of their Japanese colleagues (1989), finding a strong correlation between immunological assay and HPLC techniques. This work was explained in greater technical detail by Kuroda *et al* (1990). These works report the increased speed of this methodology over physical techniques while stressing its accuracy. Most recently antibodies and immunological testing kits have become commercially available. These have been evaluated by a number of workers including Saito Wiedemann *et al* (1990) and McGill *et al* who reported their findings at the Second International Symposium on Neuroblastoma Screening in Minneapolis between 22nd and 24th May, 1991.

1.1.11.3 Compliance.

Unlike screening for diseases such as phenylketonuria, hypothyroidism and galactosemia, neuroblastoma screening can not be conducted before neonates are discharged from hospital after birth. This is because tumours may be too small to produce enough catecholamine metabolites to detect (Normal *et al*, 1987, Ishimoto

et al, 1990) and because catecholamine and creatinine metabolism seems to be initially prone to maternal influence (Tuchman *et al*, 1989). Samples thus have to be collected by health visitors (Newcastle Programme) or more commonly returned voluntarily by parents. Compliance with Japanese screening was 59% in 1985, rising to 79% in 1988 (Hanawa *et al*, 1990, Sawada *et al* (1990). As expected, return rates are good in Newcastle, 96% according to Cropper (1989) and Tuchman *et al* report 95% compliance in Canada (1990a). However Tuchman and Woods report only 15% return by a largely unprompted US population in their 1990 paper and correlate compliance with support and public awareness and education regarding screening.

1.1.11.4 Summary.

Due to its diagnostic urine phenotype, neuroblastoma provides a rare opportunity for the early detection of childhood cancer by mass screening (Hammond, 1988). The detection of phenolic acids has been shown to be amenable to mass screening by several groups using physical techniques (Sawada, *et al*, 1991, Dale, 1988, Tuchman, 1989). However, financial constraints argue for a cheaper primary screen followed by more accurate physical testing in positive cases (Tuchman & Woods, 1992).

One alternative strategy could be the use of ELISA techniques, another a biological test based upon the common soil prokaryote *Agrobacterium tumefaciens*. This bacterium is capable of detecting and responding to phenolic compounds with marked structural similarity to HVA and VMA as part of its normal biology (Melchers *et al*, 1989). The Guthrie test for PKU provides a precident for routine infant screening for disease by a bacterial method. Neuroblastoma screening and its ramifications screening will be evaluated in Chapter 6 (Discussion).

1.2 Agrobacterium.

1.2.1 The Genus *Agrobacterium.*

Taxonomically the genus *Agrobacterium* is a member of the family Rhizobiaceae and is closely related to the genus *Rhizobium* (Kersters and DeLey, 1984). Agrobacteria are a common component of the soil microflora (Melchers and Hooykaas, 1987) and are most commonly found in close proximity to plant roots, where their numbers can be up to a thousand times higher than in the nearby soil (New and Kerr, 1972). Microscopically they appear as short parallel sided rods with rounded ends. They are 0.6-1.0μm wide and 1.5-3.0μm long giving a modal equivalent spherical volume of around 0.8μm^3. There are 1-6 flagella, typically 4-5 times the length of the cell and these are arranged along the sides of the cell and in a polar tuft (Shaw *et al*, 1991). When stained, the bacteria are Gram negative (Melchers and Hooykaas, 1987).

The genus has traditionally been classified into four species, based upon pathogenic subdivisions (Melchers and Hookyaas, 1987). *Agrobacterium tumefaciens* causes crown gall disease in many species of monocotyledenous and dicotyledenous plants (Smith and Townsend, 1907) and the pathogenic nature of other members of the genus is detailed in the table below.

Species	Disesase	Reference
A.rhizogenes	'hairy root disease'	(Riker, 1930).
A. rubi	aerial cane gall	(Melchers and Hookyaas, 1987).
A. radiobacter	non-pathogenic	(Nester and Kosuge, 1981).

The pathogenic nature of each species is determined by the presence of either a tumour inducing (Ti) or root inducing (Ri) plasmid (Van Larebeke *et al*, 1974, Zaenen *et al*, 1974, Watson *et al*, 1975, Moore *et al*, 1979). These plasmids are capable of being transferred interspecifically (Kerr *et al*, 1977, Genetallo *et al*, 1977) rendering this classification controversial and subject to constant change. Van

Larabeke *et al* (1975) and Thomashow *et al* (1980) report that the pathogenic nature of each plasmid is unaltered by its host's background, implying that the only functional difference between the *Agrobacterium* species is the plasmid they carry.

Alternative classifications based upon metabolic, physiological, phage resistance and DNA hybridization studies have been proposed by Kerr and Panagopoulos (1977), and Kersters and DeLey (1982). These are perhaps taxonomically safer divisions, as being based upon chromasomally encoded characteristics they should be more stable. In accordance with common parlance "*Agrobacterium*" will be taken to mean *Agrobacterium tumefaciens* unless otherwise stated.

1.2.2 Crown Gall Disease.

Crown gall is a neoplastic over proliferation of plant cells caused by *Agrobacterium tumefaciens* (Smith and Townsend, 1907, Melchers and Hooykaas, 1987). It is of economic importance as it causes commercial losses in several crops including grapes, stone fruits and ornamental plants (Kerr and Panagopoulos, 1977, Sule, 1978, Bernearts & De Ley, 1963). It is almost geographically ubiquitous and is to be found in Africa, Asia, Australasia, Europe and the Americas (Elliot, 1951). The species can be isolated from both cultivated and non agricultural land (Binns and Thomashow, 1988). Although the disease itself was recognised in Classical times by the Greeks, the causative role of the bacterial pathogen was not established until 1907 (Smith and Townsend, 1907). This discovery has lead to intensive study of the field since it is fascinating in its own right and also a possible model system for processes underlying cell growth and animal tumours. The implications for plant transformation have also been addressed (Hoekema *et al*, 1983, An *et al*, 1985, Hooykaas and Schilperoort, 1992).

In 1942 it was demonstrated that crown gall tumours could be maintained and grown without the continued presence of inducing bacteria (White and Braun, 1942).

Whilst normal plant cells require cytokinin and auxin supplemented media for cultured growth, crown gall tissue grows rapidly without exogenous additions (White and Braun, 1942). Six years later Braun and Mandle proposed a "tumour inducing principle" whereby *Agrobacterium* modified or added to the plant cell such that it acquired a capacity for autonomous growth (Braun and Mandle, 1948).

Agrobacterium further modifies the plant cell such that it synthesizes novel sugar amino acid conjugates that would not normally be produced (reviewed Tempé and Goldman, 1982). These conjugates are termed opines, and the type of opine synthesized is determined not by the host plant cell, but by the strain of inciting bacteria (Petit *et al*, 1970). Secreted by the plant cell, opines can be utilised by rhizosphere Agrobacteria as a source of energy, carbon and nitrogen (Bomhoff *et al*, 1976, Montoya *et al*, 1977). Bomhoff *et al* (1976) demonstrated that only *Agrobacterium* strains carrying a Ti-plasmid capable of inciting the induction of a particular opine could metabolize that specific conjugate - other micro-organisms and *Agrobacterium* strains are unable to catabolise it (Melchers and Hooykaas, 1987) (although this is no longer held as universaly true (Binns and Thomashow, 1988). This modification of the plant cell to cause a proliferation of tissue secreting opines is thought to confer a selective advantage on the organism causing it (Schell *et al*, 1979) though it may not be particulary large (Ream, 1989).

It has been further demonstrated that large extrachromosomal elements, the 200Kb (approx) Ti Plasmids (Van Larabeke *et al*, 1974, Watson *et al*, 1975) are essential for tumourigenesis. By means of DNA hybridization experiments, it has been shown that a small discrete segment of the Ti plasmid is transferred and stably maintained in the plant cell (Chilton *et al*, 1977, 1980, Willmitzer *et al*, 1980). This small element, called the transfer or T DNA is responsible for the altered morphological and biochemical phenotype of crown gall cells. This is the only generally excepted example of genetic exchange between the prokaryotic and eukaryotic kingdoms (Winans *et al*, 1988). The proportion of virulent *Agrobacterium*

in the soil is however small. Even in areas where the incidence of crown gall is high, Ti plasmids are to be found in only 1-10% of the population (Binns and Thomashow, 1988).

1.2.3 Bacterial-Plant Interaction.

Plant wounding is a prerequisite of tumour formation (Binns and Thomashow, 1988). Wounded plants produce a variety of compounds to defend themselves against pathogenic attack (Stachel *et al*, 1985, De Clenne, 1988, Dharmatilake and Bauer, 1992, Song *et al*, 1991a). Amongst the spectrum of molecules exuded are sugars, phenolics and acid metabolites (Winans 1991, Mantis & Winans, 1992). The *Agrobacterium* - plant cell interaction is a two way chemical signalling process (Winans, 1992). Molecules released by the plant (Hawes *et al*, 1988) and wound conditions (Winans, 1991) act upon the bacterium to ellicit chemotaxis (Loake *et al*, 1988, Ashby *et al*, 1988,) and induction of *virulence* genes (Stachel *et al*, 1986a). These genes then act in concert to catalyse the transfer of the T DNA from the bacterium to the plant cell.

1.2.3.1 Chemotaxis.

Chemotaxis in bacteria involves the directed movement of motile cells in response to a chemical gradient of attractant or repellant (Adler, 1966). *Agrobacterium* is envisaged to be chemotactically attracted towards plants by wound exudates, where due to higher concentrations of biologically active molecules expression of the *virulence* genes is induced (Ashby *et al*, 1987, Loake *et al*, 1988). A similar scenario is proposed by Dharmatilake and Bauer (1992) who report that *Rhizobium meliloti*, a root nodule inducing organism is attracted towards target alfalfa roots by flavonoid root exudates. The *Agrobacterium* hypothesis is supported by the observation that for some exudate components, most notably phenolic lignin

precursors and breakdown products (esp. acetosyringone), the concentration threshold of positive chemotaxis is 1000 fold lower than for gene induction (Ashby *et al*, 1987, Stachel *et al*, 1985). Whilst Ashby *et al* used purified chemical attractants in their assays, Hawes *et al* (1988) were able to demonstrate positive *Agrobacterium* chemotaxis to as few as 300 excised living plant cells.

In 1989 Hawes and Smith demonstrated that in certain open matrix soil types non-chemotactic mutants of *Agrobacterium* were less virulent than their wild type counterparts. This dependence on soil type suggests that chemotaxis may be of greater significance in some environments than others. Loake *et al's* results (1988) suggest that sugars and amino acids may be the predominant root chemoattractants whilst attraction by phenolics may be important on a micrometre scale (Shaw *et al*, 1989, Shaw, 1991) an estimate in keeping with the calculations of Dharmatilake and Bauer (1992). Furthermore the work of Parke *et al* (1987) suggests that phenolic chemotaxis may only be of ecological importance in the most highly motile *Agrobacterium* strains. Phenolic chemotaxis is mediated by the interaction of a multifunctional chemoreceptor/effector system with more general chromosomal chemotaxis functions (Shaw, 1991) encoded by as many as 60 genes (MacNab, 1987a,b).

Wounding is possibly of further importance in making the plant susceptible to infection. Following wounding cell division takes place to repair damage. The consequent local increase in DNA replication is possibly important in allowing integration of bacterial DNA (Binns and Thomashow, 1988). Furthermore, once a tumour has been incited and opines are being produced, these compounds induce the conjugative transfer of the Ti plasmid amongst rhizosphere Agrobacteria, ensuring they are capable of benefiting from the available energy, carbon and nitrogen source (Binns and Thomashow, 1988).

1.2.3.2 Attachment of Agrobacterium to Plant Cells.

Having arrived at a suitable specific site for infection, Agrobacteria attach themselves to the plant. This step is essential for virulence (Lippincott and Lippincott, 1969). At first individuals congregate to form polar chains, but this is followed by a massive aggregation at the plant surface (Matthysse *et al*, 1981). Cellulose fibrils help to maintain the aggregation and attachment to the host (Matthysse, 1983, Matthysse *et al*, 1981) but only one virulent *Agrobacterium* interacts with each wound site to induce tumour formation (Lippincott and Lippincott, 1969). The cellulose fibrils are synthesized by the bacterium (Matthysse *et al*, 1981). Ti plasmid presence is a prerequisite for bacterial attachment (Matthysse *et al*, 1978). Bacterial aggregation by chemotaxis and cell division may provide enough bacteria secreting lytic enzymes to enable digestion of the plant cell wall (Matthysse *et al*, 1981), aiding T DNA transfer.

Binding involves both specific plant cell and bacterial receptors (Binns and Thomashow, 1988). The plant saturable bacterial receptor appears to be a cell wall component (Binns and Thomashow, 1988), possibly a protein (Gurlitz *et al*, 1987). The bacterial recognition factor is probably a lipopolysaccharide (Matthysse *et al*, 1978, Whately *et al*, 1976) although this is not incontrovertible (Binns and Thomashow, 1988). Three bacterial genetic loci are also involved, *chvA*, *chvB* (Douglas *et al*, 1986) and *pscA* (Cangelosi *et al*, 1987) and are thought to facilitate ß-1,2-D-glucan production and export (Binns and Thomashow, 1988) aiding tight binding (Ream, 1989).

1.2.3.3 Structure of the Ti-Plasmid.

The 2 best studied types of Ti plasmid, encoding the production and catabolism of the opines octopine (*eg* pTiAch5 and pTiA6) or nopaline (*eg* pTiC58 and pTiT37). Whilst there are specific difference in the organisation of these elements, in general terms, Ti plasmids have 5 functional regions, namely: The T

region and functions for *virulence*, conjugative transfer, replication and opine catabolism & uptake (Melchers and Hooykaas, 1987). Octopine plasmids tend to have single T DNA elements whereas several mobile elements are common in nopaline encoding systems (Zambryski, 1992).

The T region, which is bounded by almost identical 25bp direct repeats is transferred to the host plant cell (see figure 1.2.3.3.A, reproduced by kind permission of Mark Levesley). This region contains the T DNA which, on expression in the plant, encodes functions for auxin and cytokinin production and opine synthesis/export (Melchers and Hooykaas, 1987). Any DNA bounded by the *cis* acting 25bp repeats will be exported from the bacteria cell to the plant nucleus and expressed (Stachel and Zambryski, 1986b, Zambryski, 1992), and this phenomenon has been widely exploited for plant transformation (Hoekema *et al*, 1983, An *et al*, 1985, Hooykaas and Schilperoort, 1992).

The *virulence* or *vir* regulon is essential for the catalysis of the transfer process. Acting in concert the products of these genes work in *trans* but are not themselves transferred (Hille *et al*, 1982, Klee *et al*, 1982, Hoekema *et al*, 1983, Melchers and Hooykaas, 1987). There are 8 *vir* operons named A-H in clockwise direction towards the T region, together comprising approximately 30Kb (Rogowsky *et al*, 1990). Several of these genes are poly-cistronic and each gene in a cistron is designated numerically along similar lines.

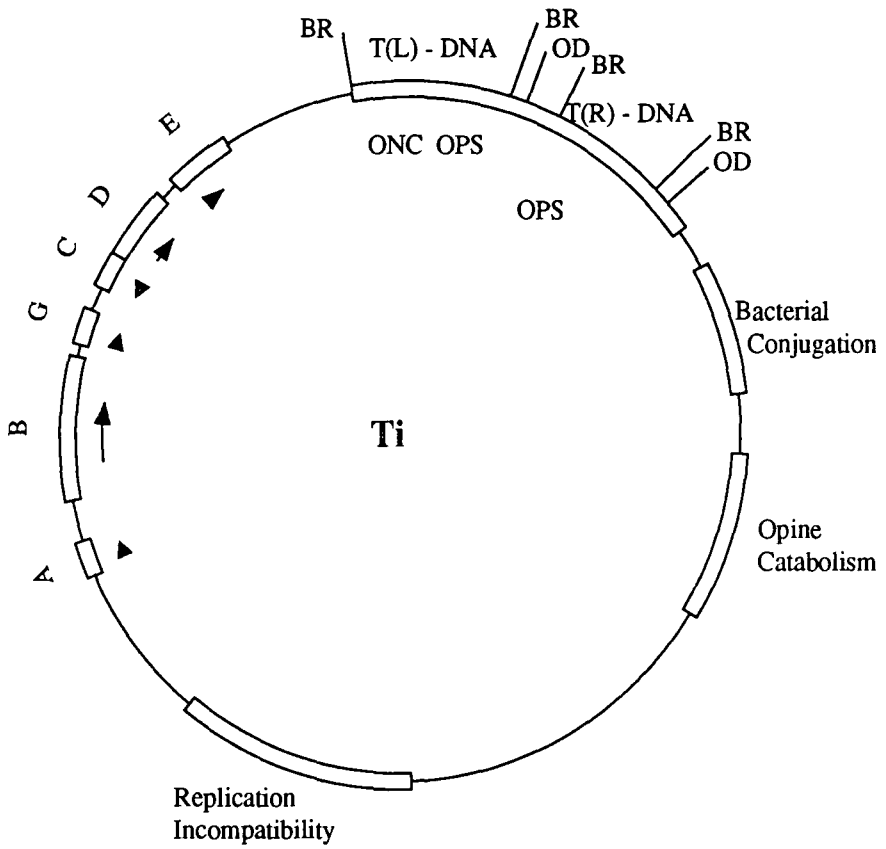


Figure 1.2.3.3.A - Ti plasmid (octopine type) showing location, size and polarity of the *vir* loci trasncripts, the location and general functions of the T-DNA and border elements and the approximate locations encoding plasmid replication, bacterial comjugation and opine catabolism. BR = Border Repeat, OD = Overdrive sequence, ONC = Oncogenes, OPS = Opine synthesis genes.

Cistron	No. Genes	Functions.
A	1	Signal transduction - response to pH, sugars, phenolics.
B	11	Transmembrane pore proteins /transport functions
C	2	Overdrive functions ?
D	4	T strand production specific DNA binding/pilot protein
E	3	Non specific single stranded DNA binding.
F		Not known
G	1	Two component regulator system effector and chemotaxis mediator
H Formerly *pinF*	2	Cytochrome P-450 monooxygenases

(Adapted from Melchers and Hooykaas (1987), Rogowsky *et al* (1990) and other sources).

Expression of the *vir* genes is closely regulated (Stachel *et al*, 1986a). Transcription of *virA*, *B*, *C*, *D*, *E*, *F* and *H* is under the control of the *virA/G* system (Stachel and Zambryski, 1986a). This system is part of a family of bacterial genes known as two component regulators (Winans *et al*, 1986, Leroux *et al*, 1986). *virA* acts a signal transducer responding to pH and temperature (Melchers *et al*, 1989), sugars (in conjunction with the periplasmic *chvE* sugar binding protein (Cangelosi *et al*, 1990)) and phenolics (Stachel *et al*, 1985, 1986a, Stachel and Zambryski, 1986a). Under inducing conditions, autophoshorylated *virA* transfers phosphate from histidine 474 (Huang *et al*, 1990, Jin *et al*, 1990b) to aspartate 52 on *virG* (Jin *et al*, 1990a,b). Phosphorylated effector acts as a sequence specific DNA binding protein and transcriptional activator (Jin *et al*, 1990c, Pazour and Das, 1990a). Inducible *virulence* genes all have a consensus *cis* acting '*vir*-box' regulatory element in their promotors (Das *et al*, 1986,). *virG* expression is autoregulated although subject to phosphate starvation and pH regulation (Mantis and Winans, 1992, Winans *et al*, 1986). Since the 'pool size' of *virG* is thought to be the limiting factor on induction

(Mantis and Winans, 1992) it is envisaged that a non induced *Agrobacterium* has residual levels of *virA* and low levels of *virG*, if prevailing conditions alter however, the effector pool size increases and as a result more receptor is produced, conferring greater ability to induce *vir* expression if necessary.

Ti plasmid genes also encode for the metabolism of opines (Bomhoff *et al*, 1976) and their specific uptake (Klapwijk *et al*, 1977). The plasmid also has functions for plasmid replication and incompatibility. There are 3 incompatibility groups *inc*Rh-1 to *inc*Rh-3 and entry inhibition operates though conjugative transfer is possible (Melchers and Hooykaas, 1987).

1.2.3.4 Generation of a T DNA Copy.

As a result of *vir* gene expression, the products of these coding regions act upon the T DNA of the Ti plasmid to produce a DNA element capable of being transferred to its target. The initial step is a endonucleolytic cleavage between the 3rd and 4th bases of the 25bp Ti plasmid direct repeats. These nicks, formed only in the bottom strand, once detected are used as the initiation and termination sites for the liberation of the bottom strand of the T DNA between them. This DNA, designated the T strand is displaced in right to left direction leaving a 3' end at the right border which acts as a primer for resynthesis of the second plasmid DNA strand (Albright *et al*, 1987, Stachel, *et al*, 1986b, Wang *et al*, 1987, Zambryski *et al*, 1992).

The protein products of *virD1* and *virD2* have been shown to be required for T strand production (Stachel *et al, 1987*, Yanofsky *et al*, 1986). As mutations in these genes cause loss of nicking ability and T strand synthesis these proteins are implicated as having site specific endonuclease activity (Zambryski, 1992). *In vitro* studies support this conclusion and also demonstrate topisomerase activity for VirD1, suggesting that prior to nicking by VirD2 the DNA is specifically relaxed by

VirD1 (Zambryski, 1992, Ghai and Das, 1989). VirD2 is clearly a multifunctional molecule and will be further discussed below. Only the N terminal 50% of the protein is required for 25 bp repeat recognition and binding (De Vos and Zambryski, 1989, Yanofsky *et al*, 1986).

In octopine Ti plasmids border nicking is thought to be enhanced by "overdrive" (Peralta *et al*, 1986) functions (Toro *et al*, 1988, 1989, Zambryski, 1992). The effect of overdrive, a 24bp sequence (Peralta *et al*, 1986) active either up or downstream of the border repeats (Van Haaren *et al*, 1987) is thought to only be important when VirD1 and VirD2 would otherwise be limiting (De Vos and Zambryski, 1989). VirC1 has been shown to interact with this sequence (Toro *et al*, 1989), though how the subsequent increase in nicking activity (Toro *et al*, 1988) is mediated is not clear. The function of the second protein product of the *virC* operon is even more uncertain.

T strand production would be expected to require helicase, polymerase and unwindase functions. These are thought to be provided by constitutive bacterial enzymes or induced under conditions preceding *virulence* gene expression (Zambryski, 1992). These enzymes are not *Agrobacterium* specific since T strands can be produced by *E.coli* harbouring T DNA substrate and *virD1* and *virD2* functions (De Vos and Zambryski, 1989).

1.2.3.5 Formation of a T Strand/Protein Complex.

During its transit from the bacterial cytoplasm through the bacterial and plant membranes and cell walls, the plant cytoplasm and nuclear membrane the T strand must withstand degradation by nucleases. This is effected by the formation of a T strand/protein complex which both protects and guides the invading genetic material (Howard and Citovsky, 1990).

VirE2 is a sequence aspecific single stranded DNA binding protein (Christie *et al*, 1988, Citovsky *et al*, 1988, Das, 1988, Gietl *et al*, 1987), which binds co-

operatively to render single stranded DNA nuclease resistant (Citovsky *et al*, 1989, Sen *et al*, 1989). This molecule is the most highly expressed *vir* inducible product and accounts for 0.05% total cell protein (Citovsky *et al*, 1988), sufficient to totally cover and protect the entire T strand (Zambryski, 1992). A further function of VirE2 may be to ensure that the linear T strand maintains an unfolded morphology aiding transfer (Zambryski, 1992).

In addition to VirE2, VirD2 is also tightly bound to the T complex. Localised at the 5' end of the T strand by possible covalent bonding (Herrera-Estrella *et al*, 1988) this protein is thought to provide targeting information guiding the T complex to specific bacterial and plant structures (Zambryski, 1992).

1.2.3.6 Movement of the Complex From the Bacterium Towards the Plant Nucleus.

This is a multi step process involving the transfer of the T complex through the bacterial membranes and cell wall, the plant cell wall and membranes, the plant cytoplasm and eventually the plant nuclear envelope. It is clear that the T complex itself is insufficient to provide the functions and a number of bacterial and plant encoded functions are also required.

The 11 open reading frames of the *virB* locus specify *trans*-bacterial-membrane and membrane associated proteins thought to create a pore or channel structure. This conclusion is based on the presence of membrane targeting leader sequences and considerable hydrophobic domains in these proteins. The pore is thought to adopt an "I" shape in the membrane with a hollow channel in the downward stoke and the crossways strokes lying parallel to and in association with the membranes (see figure 1.2.3.6.A).

As yet little is known about the uptake of the T complex by the plant cell though the role of bacterial enzymes in lysing cell wall components has already been mentioned. Targeting of the complex towards the nucleus has however been investigated in some detail and is most likely to involve specific uptake through

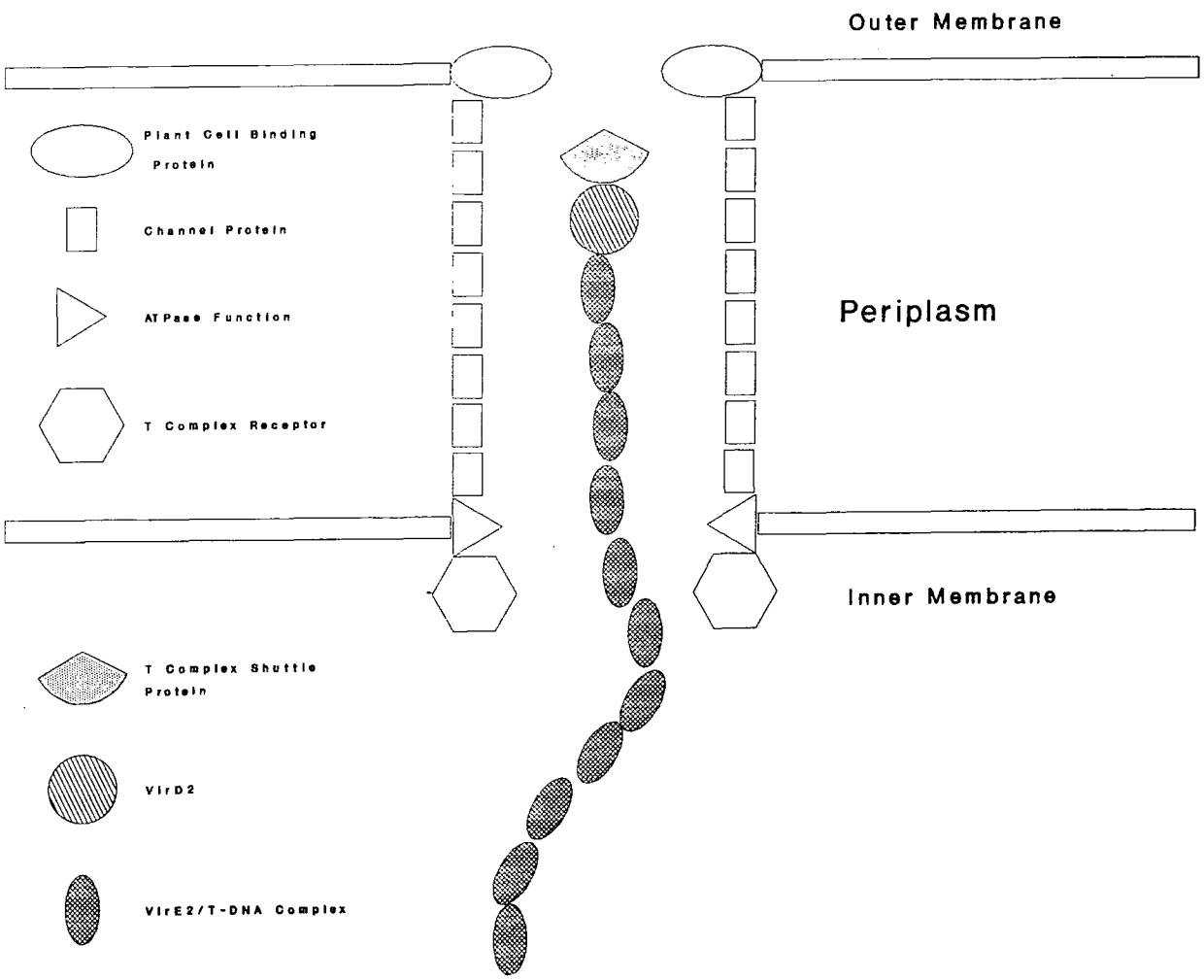
nuclear pores (Zambryski, 1992). VirD2, the protein covalently bound at the 5' end of the T strand contains a nuclear localisation signal functional in eukaryotic cells. This sequence of amino acids is found at the extreme C terminus of the protein and shows wide variation between Ti plasmid subgroups (Zambryski, 1992). Nuclear pores are thought to be 9-10nm in diameter (Gerace & Burke, 1988) and 60nm long (Reichelt *et al*, 1990), more than large enough to accommodate the 2nm wide T complex (Zambryski, 1992).

The calculated length of the T complex (3600nm) and its proposed molecular weight of $5 * 10^7$ Daltons suggest however that the VirD2 nuclear localisation signal alone would be insufficient to deliver the complex to the nucleus (Zambryski, 1992). The VirE2 single stranded DNA binding protein has also been demonstrated to have similar though somewhat weaker targeting properties (Hirooka *et al*, 1987, Winans *et al*, 1987, Zambryski, 1992). Zambryski (1992) envisages that VirD2 ensures that the 5' end of the T complex is presented to the pore to aid transport, similar to threading a piece of cotton through a needle. Transport through the pore is then facilitated by the targeting information of VirE2 and its ability to keep the T strand straight.

1.2.3.7 Integration of Invading DNA into Host Genome.

In general T DNA integration can occur on any of the hosts plants chromosomes (Ambros *et al*, 1986, Chyi *et al*, 1986) and transcriptionally active regions are preferred (Herman *et al*, 1990, Koncz *et al*, 1989), presumably as their unravelled structure eases access. In general large rearrangements of the host genome are not induced by T DNA insertion, though small deletions of up to 80bp are often found at the target. In addition "filler" DNA stretches up to 33bp in length are frequently associated with the T DNA/plant chromosome junctions. This filler is composed of sequence homologous to plant DNA found close to the junction however there is no target sequence preference, other than for AT rich regions

Hypothetical T-Complex and Transport Channel.



(Gheysen *et al*, 1987, Zambryski, 1988). 5-10 base regions of homology between the T DNA ends and plant genetic material may however be influential in insertion (Matsumoto *et al*, 1990, Zambryski, 1992) by promoting homologous recombination, an idea supported by the work of Venkateswarlu and Nazar (1991) who used this principle to promote T DNA integration in the chloroplastic genome of tobacco plants. The inserted T DNA endpoints are usually within the first few bases of the left and right 25bp borders (Gheysen *et al*, 1991, Wang *et al*, 1987) and the high accuracy of 5' end insertion suggests a role for the bound VirD2 protein, perhaps in initiating DNA repair (Zambryski, 1992).

Single insertions of T DNA per cell are common, but the mean number is 3 (Zambryski, 1988). Copy numbers as high as 20-50 per cell have been observed (Barton *et al*, 1983). Multiple copies can be found in clusters or separate locations (Zambryski, 1989). Although several models have been proposed (Gheysen *et al*, 1987) and its similarity to bacterial conjugation and viral infection noted (Stachel and Zambryski, 1986b, Zambryski, 1992, Howard and Citovsky, 1990), precise details of T DNA insertion into the host genome are unclear. Zambryski (1988, 1989) and Gheysen *et al* (1987) however summarise the known facts as follows: Having gained access to the plant nucleus, a protein at the 5' of the T complex interacts with a nicked sequence in the plant DNA. Following Attachment of the 2 DNA's at this site, the local torsional strain on the plant DNA is relieved by the production of a nick on the other strand opposite to the target site. The T DNA is then ligated into the plant chromosome at the insertion site and its homologous strand copied by plant encoded enzymes. The staggered nicks in the plant DNA are replicated and repaired as a consequence of which, the repeated and filler DNA sequences are generated at the ends of the inserted DNA. Occasionally such large scale manipulation of the plant genome can cause errors in replication and repair of DNA, leading to the observed direct and inverted repeats of the invading material. Binns and Thomashow (1988) note the lack of requirement for bacterial enzymes in

catalysing these processes and discount the possibility of a transposition like insertion event.

1.2.3.8 T DNA Expression.

T DNA encoded genes fall into 2 groups: those concerned with oncogenicity and those encoding opine related functions. Genes *tms1* (a.k.a. *gene1*, *iaaM*), *tms2* (*gene2*, *iaaH*) and *tmr* (*gene4*, *ipt*) code for the synthesis of auxin and cytokinin, potent plant growth regulators, responsible for the uncontrolled over-production of crown gall cells leading to a tumour (Binns and Thomashow, 1988). *tms1* is responsible for the catalysis of the conversion tryptophan to indole-3-acetamide (Thomashow *et al*, 1986, Van Onckelen *et al*, 1984) whilst *tms2* promotes the conversion of this to indole-3-acetic acid (Inzé *et al*, 1984, Schröder *et al*, 1984, Thomashow *et al*, 1984), the primary plant auxin (Binns and Thomashow, 1988). *tmr* encodes the production of the cytokinin iso-pentenyladenosine 5'-monophosphate from dimethylallyl-pyrophosphate and 5'-AMP (Akiyoshi *et al*, 1984, Barry *et al*, 1984, Buchmann *et al*, 1985).

Opine related genes fall into 2 subgroups: those related to opine biosynthesis and those concerned with export of the conjugates from the plant cell. On octopine plasmids, gene *ocs* (a.k.a. *gene3*) encodes octopine synthase, which catalyses the condensation of pyruvate and arginine, similarly on nopaline plasmids a gene encodes the condensation of arginine and α-ketoglutarate (Kemp, 1982, Tempé and Goldman, 1982). *ons* (*gene6a*) encodes a protein responsible for transport of octopine across the plant membranes, ensuring its availability to rhizosphere Agrobacteria (Messens *et al*, 1985). It should be noted that the octopine plasmid also has functions leading to the production of the opines mannopine and agropine and that nopaline type plasmids encode enzymes responsible for the catalysis of agrocinopine. There are at least 20 opine produced as a result of Agrobacterial

action (Ream, 1989). There are also additional transcribed regions of Ti plasmids whose functions are unknown (Binns and Thomashow, 1988).

1.3 The Basis for an Agrobacterial Neuroblastoma Screening Test.

1.3.1 vir Gene Regulation.

The induction of pathogenesis related genes is a recurring theme in microbiology (Mekalanos, 1992, Winans *et al*, 1989). The stimulation of a system such as that involved in *Agrobacterium* / plant infection must be a considerable metabolic drain. It would clearly be a selective advantage to a cell to express these genes only under appropriate circumstances, such as when in proximity of a wounded plant cell. *Agrobacterium* has developed a chemosensory ability that under these conditions acts to induce the transfer of genetic material to the host plant cell.

As already mentioned, the expression of the majority of the *vir* genes is regulated by a the VirA/G protein pair (Stachel and Zambryski, 1986a). Together, these proteins are part of a family known as two component regulators (see section 5.2), capable of sensing and responding to environmental stimuli. *virA/G* show strong homology to this conserved group of proteins (Stachel *et al*, 1986, Leroux *et al*, 1986). Other members of this family include *NtrB/C*, *PhoR/B* and *EnvZ/OmpR* (Albright *et al*, 1989).

VirA is a 92KDa transmembrane protein (Winans *et al*, 1989) and forms the sensor part of the 2 component system, VirG is a 30Kda cytoplasmic transcriptional regulator (Winans *et al*, 1986) These proteins are additionally required for the mediation of phenolic chemotaxis (Shaw *et al*, 1988). According to hydropathy predictions and molecular biology studies, VirA has 2 trans-cytoplasmic membrane regions (Winans *et al*, 1989) joined by a 221 residue periplasmic convoluted loop (Melchers *et al*, 1989), thought to be involved in pH, temperature and sugar sensing (Melchers *et al*, 1989, Cangelosi *et al*, 1990). In addition there is 550 residue

(Melchers *et al*, 1989) cytoplasmic domain with carboxy terminal protein kinase activity (Winans *et al*, 1989). Phenolic sensing is thought to be associated with the second transmembrane region (Melchers *et al*, 1989) although more recent evidence suggests reasons to doubt this as will be discussed later. The carboxy terminus of VirA can be autophosphorylated at histidine residue 474 (Huang *at al*, 1990, Jin *et al*, 1990b) and when in receipt of a suitable signal can transfer this phosphate to aspartate 52 of VirG (Jin *et al*, 1990a,b). Intramolecular signalling within the VirA molecule is thought to be brought about by conformational changes induced by ligand binding (Ames and Parkinson, 1988). Phosphorylation of VirG by Vir A renders the effector capable of sequence specific DNA binding, recognising the *vir*-box (Jin *et al*, 1990c, Pazour and Das, 1990a, Powell *et al*, 1989). The *vir*-box is a conserved regulatory element found upstream of *vir* genes. Binding of phosphorylated VirG through its carboxy terminal domain to the *vir* box (Powell and Kado, 1990) induces transcription of the coding region and consequently expression of the gene (Winans *et al*, 1986). In addition VirA interacts through its periplasmic domain with the protein ChvE (Cangelosi *et al*, 1990). ChvE shows strong homology to *E.coli* periplasmic sugar binding proteins (Cangelosi *et al* 1990) and is implicated in induction and chemotactic responses to sugars.

1.3.1.1 vir Inducing Stimuli.

There are essentially 3 influences which lead to *vir* gene induction, namely: phenolic compounds, sugars and acidic pH. Of these, greatest research emphasis has been placed upon phenolic compounds such as acetosyringone. This is perhaps a false bias.

Stachel *et al* (1985) isolated 2 monocylic aromatic hydrocarbons with *vir* inducing properties from tobacco roots and cells. These phenolics, acetosyringone and hydroxyacetosyringone have been the basis of a great deal of fruitful research. However, these are only found in a few species of plant and not always in

appreciable quantities (Spencer and Towers, 1989). There are additional compounds with *vir* inducing activity with equal or greater potency (Spencer *et al*, 1990, Delay and Delmotte, 1990, Delmotte *et al*, 1991, Song *et al*, 1991b). By studying compounds with similar structures to acetosyringone, Bolton *et al* (1986), noted the structural requirements for efficient *vir* inducing activity as follows: the presence of a substituted aromatic ring, with 1' polar, 4' hydroxyl and 3' methoxyl groups. For maximal efficiency a 5' methoxyl residue was also required. This work was continued in 1988 by Spencer and Towers who found conditions for induction including: guaiacyl or syringyl substitution on a benzene ring, with certain exceptions, a carbonyl group on a substituent *para* to the hydroxyl group. 5' hydroxylation was found to decrease effectiveness although aromatic rings could be linked to give far more complex inducing structures.

Cangelosi *et al* (1990) and Shimoda *et al* (1990) report that specific monosaccharides such as glucose, galactose, fucose and 6-deoxy-D-glucose may play a synergistic role in increasing phenolic induction as well as being inducers in their own right. There is no requirement for metabolism of the carbohydrate in this interaction (Shimoda *et al*, 1990). All inducing sugars are aldoses and none ketoses. Song *et al* (1991a) discuss one non metabolically active synergistic monosaccharide, inositol as relieving some unknown inhibition to phenolic induction.

The third environmental condition for *vir* induction is acidic pH. Induction is dependent upon acidic pH with an optimum of pH 5.25 and does not occur under neutral conditions (Stachel *et al*, 1986a, Rogowsky *et al*, 1987). It is striking that the three requirements for *vir* gene induction are met by the conditions to be found in a plant wound site. As already mentioned, phenolics will be present as they are produced as an inducible defense response by the plant and are lignin precursors and breakdown products and so likely to be found at sites of cell wall growth and repair. Furthermore monosaccharides will be common components of plant cytoplasm and sap, likely to be lost when damage occurs on a cellular or macroscopic scale. Wound

pH is likely to be acidic due to lysis of damaged vacuoles which are generally acidic and spillage of sap, also of lower than neutral pH (Winans, 1991).

1.3.2 Agrobacterium and Catecholamine Metabolites.

Comparison of the 'ball and stick' (figures 1.3.2.A) molecular models for AS, ASOH, HVA and VMA shows distinct structural similarities between the catecholamine metabolites taken as diagnostic of neuroblastoma and the potent *vir* gene inducer. These models and the 3 dimensional space filling models (figures 1.3.2.B) were generated by the procedure described in section 2.19.

Furthermore the catecholamine metabolites HVA and VMA fulfil many of the structural requirements (Bolton *et al*, 1986, Spencer and Towers, 1988) for interaction with the *Agrobacterium* 2 component regulatory system. HVA and VMA are both substituted phenolic compounds. A polar group is found in *ortho* position to the 4' hydroxyl group. Both compounds have 3' methoxyl groups, and although they lack similar functional substitution at the 5' position, neither is hydroxylated at this site. HVA and VMA were thus thought to be candidate *vir* inducers and chemoattractants. Based upon this premise, this project aimed to research the feasibility and nature of a possible neuroblastoma screening test based upon the responses of the bacterium *Agrobacterium tumefaciens* to the catecholamine metabolites, HVA and VMA which are taken as diagnostic of the disease when found at elevated levels in a patients urine.

Figure 1.3.2.A.

AS ASOH

HVA VMA

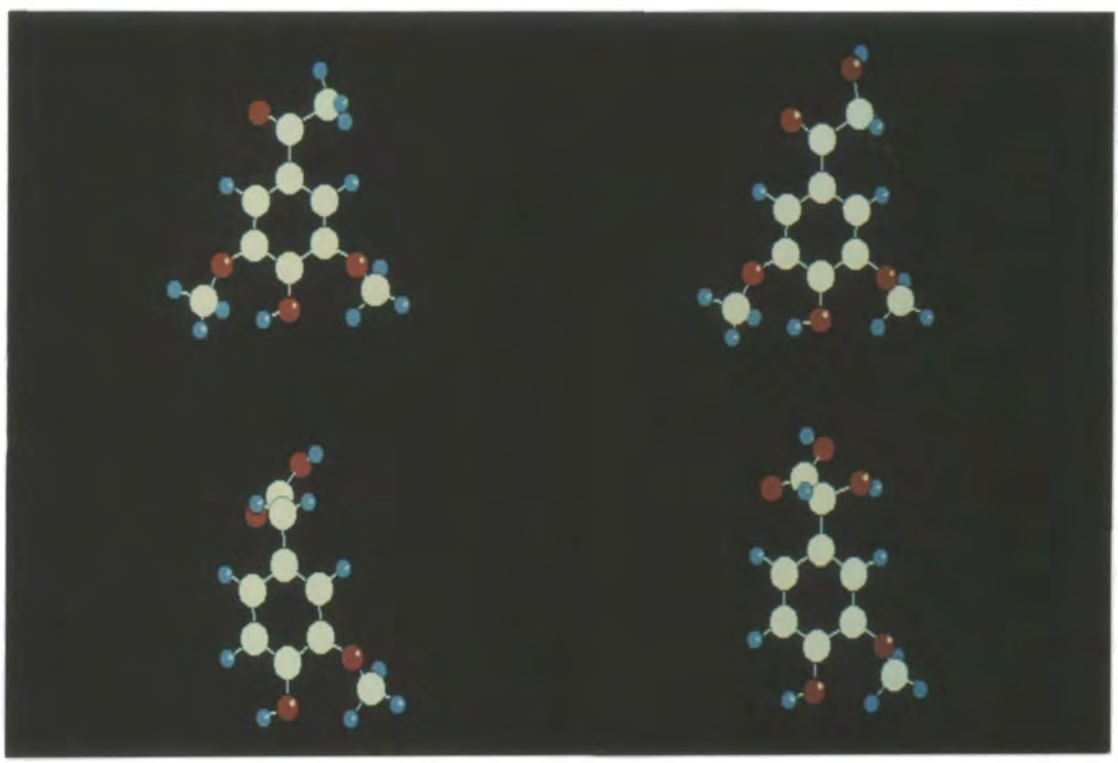
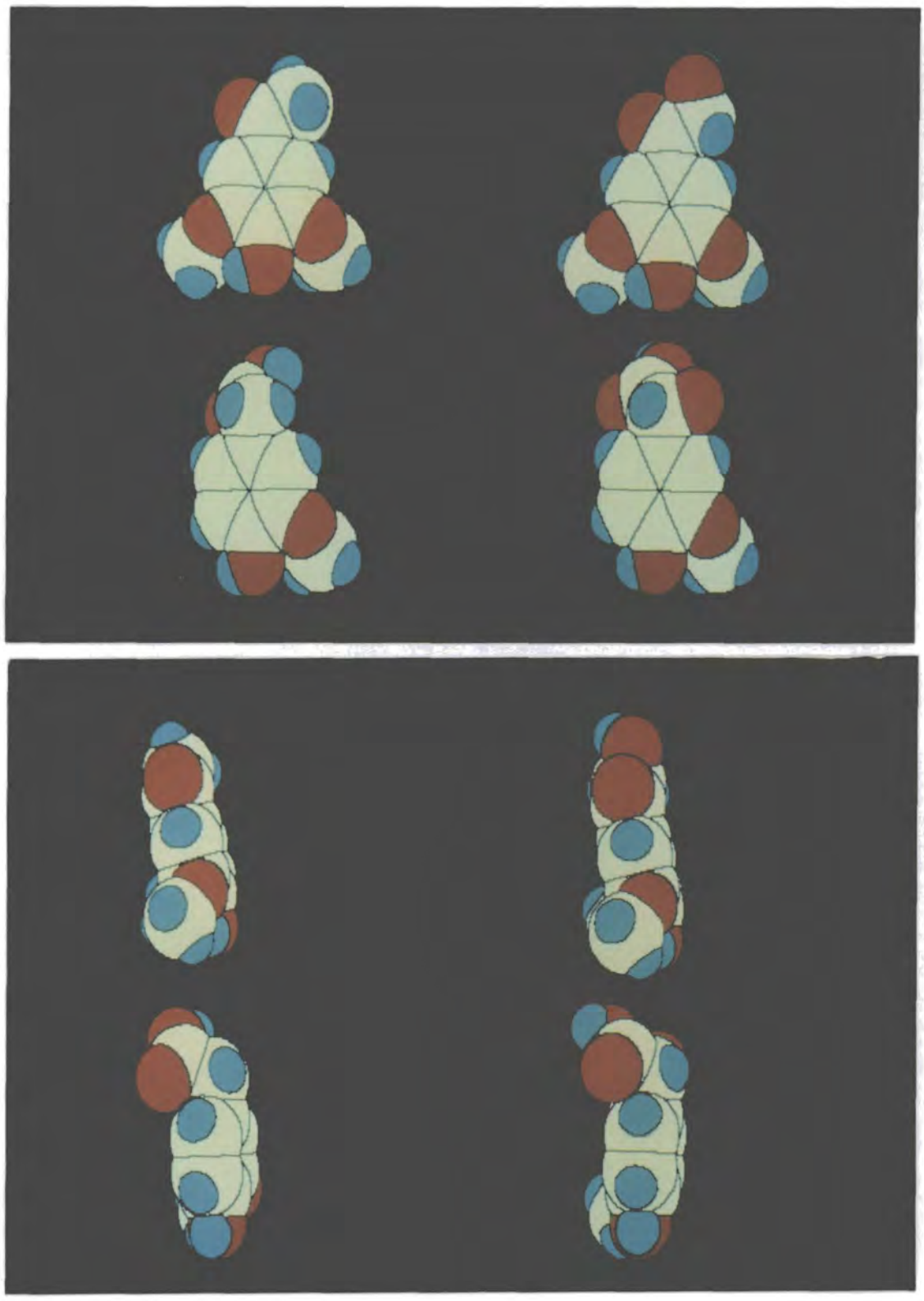


Figure 1.3.2.B.

AS ASOH

HVA VMA



2. Materials and Methods.

2.1 Materials.

Except for the following, all chemicals and biological reagents were purchased from Sigma Chemical Company Ltd or BDH Ltd:

Restriction endonucleases, DNA modifying enzymes, X-gal and IPTG were from Northumbria Biologicals Ltd and Boehringer Mannheim.

Agarose and low melting point agarose were from Bethesda Research Laboratories (UK) Ltd.

Bacteriological agar and yeast extract were from Oxoid Ltd.

Typticase peptone was from Becton Dickinson Microbiology Systems, Cockeysville, USA.

Sodium chloride was from Reidel de Haen.

The digoxigenin DNA labelling and detection kit was from Boehringer Mannheim.

The FLASH chemiluminescent DNA labelling and detection kit and accompanying membranes were from Stratagene.

Electroporation cuvettes and Bradford Reagent were from Bio-Rad Laboratories, Herts, UK.

LAB M nutrient broth, nutrient agar and nitrocellulose filters were from Amersham International plc.

Qiagen columns were from Diagen.

Petri dishes and Nuclepore polycarbonate membranes and blood dilution vials were from Sterilin.

The Coulter Multisizer II and Isoton II were from Coulter Electronics Ltd.

Microtitre plates were from Falcon or Nunc.

Phage plates were from Nunc.

2.2 Bacterial Strains.

The strains of bacteria used in this study are described in the following tables:

2.2.1 Escherichia coli.

Strain	Characteristics	Reference.
BM2KNaI^R.		
C2110	*pol*A, *rha*, *his*, Nal^R.	(Prince & Borlam, 1985).
DH5α	F$_-$, *end*A1, hsdR17,*supE*44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, ▲(*lac*ZYA-*arg*F), U169,Φ80, d*lac*Z▲M15.	(BRL).
HB101	F$^-$, *hsd*S20(r$^-$,m$^-$), *rec*A113, *pro*A2, *leu*-6, *thi*-1, *rps* L20, (Str^R), *ar1*-14, gal K2, *lac* Y1, *xyl*-5, *mtl1*-1, *sup* E44.	(BRL).
W3110	*thy*$^-$, F$^-$, *thy* A36, lambda$^-$	(Bachmann, 1972).

Nal, nalidixic acid; Str, streptomycin, ^{R} resistant.
BRL, Bethesda Research Laboratories.

2.2.2 Agrobacterium tumefaciens.

Strain	Characteristics	Reference.
A348	W.t. strain pTiA6NC Ti-plasmid	(Stachel *et al*, 1986).
C58	W.t. strain pAtC58 Ti-plasmid	(van Larebeke *et al*, 1974).
C58C^{1}Rif	Rif^R, Ti-plasmid cured.	(van Larebeke *et al*, 1974).
C58C^{1}EryCm	Ery^R, Cm^R, Ti-plasmid cured.	(van Larebeke *et al*, 1974).
GMI9023	Rif^R, Strp^R, Ti-plasmid cured.	(Rosenberg *et al*, 1984).
A1030	Km^R, *virA::Tn5*, pAtC58 Ti-plasmid	(Garfinkel & Nester, 1980).

Cm, chloramphenicol; Ery, erythromycin; Km, kanamycin; Rif, rifampicin; Str, streptomycin, ^{R} resistant.

2.3 Plasmids.

The plasmids used in this study are described in the following tables:

Plasmid	Characteristics	Reference.
pVK 219	Km^R, *virAB*, Inc P	(Knauf & Nester, 1982).
pVK 225	Km^R, *virGCDE*, Inc P	(Knauf & Nester, 1982).
pVK 257	Km^R, *virABGC*, Inc P	(Knauf & Nester, 1982).
pSG 687	Km^R, Sp^R, *virG*D52N, Inc W	(Jin *et al*, 1990a).
pSG 689	Km^R, Sp^R, *virG*, Inc W	(Jin *et al*, 1990a).
pTB108	Cb^R, Tc^R, *virA*, Inc W	(Jin *et al*, 1990b).
pRS0824	Cb^R, Tc^R, *virA*H474Q, Inc W	(Jin *et al*, 1990b).
pIB50	Km^R, *virB::lacZ*, *virE::cat*, IncP	(Ankenbauer *et al*, 1991).
pIB100	Cb^R, *virA,G*, Inc W	(Ankenbauer *et al*, 1991).
pIB415	Cb^R, Tc^R, *virA*▲63/240, *virB::lacZ*, IncP	(Cangelosi *et al*, 1990).
V21	*virB* promotor in pUCD derivative	(Lilley).
pIJ3100	$Strp^R$, InQ, promotorless *cat*	(Osbourn *et al*, 1987).
pRN3	Sp^R, $Strp^R$, Su^R, Tc^R, Hg^R, Co-integrate forming.	
pRK2013	Km^R, Helper plasmid	(An *et al*, 1985).
pSM30	Km^R, Cb^R, *virB::lacZ*	(Stachel *et al*, 1986).
pDUB1003▲31	Nm^R, Gm^R, Nopaline Ti-plasmid	Shaw *et al*, 1984
pUCD1187	Km^R, *virB::lux*, *ori*pSa, *ori*pBR322	(Rogowsky *et al*, 1987).
pUCD1194	Km^R, *virE::lux*, *ori*pSa, *ori*pBR322	(Rogowsky *et al*, 1987).

Plasmid	Characteristics	Reference.
RPI-85	Amp^R, Tc^R, Hg^R, IncP	(Stanisich & Bennett, 1976).
211MxCb	Cb^R, *virA* Tn3HoHo Insert	Stachel *et al*, 1985
226MxCb	Cb^R, *virA* Tn3HoHo Insert	Stachel *et al*, 1985
19MxCb	Cb^R, *virG* Tn3HoHo Insert	Stachel *et al*, 1985

Amp, ampicillin; Cb, carbenicillin; Gm, gentamycin; Hg; mercury ion; Km, kanamycin; Nm, neomycin; Sp, spectinomycin; Strp, streptomycin; Su, succinic acid; Tc, tetracyclin; ^{R} resistant.

The generous gift of numerous plasmids from Professor E.W.Nester is gratefully acknowledged. Similarly C.J.Lilley is thanked for the donation of V21. The donation of pIJ3100 was also much appreciated.

2.4 Methods.

Sterile reagents and aseptic technique were employed as appropriaate.

2.5 Media and Growth Conditions.

Liquid cultures of bacteria were routinely grown in Luria-Bertani medium, L-broth (Sambrook *et al*, 1989) or Lab M nutrient broth number 2. Liquid cultures were agitated during growth unless otherwise stated. Solid media consisted of L-agar (Sambrook *et al*, 1989) or Lab M nutrient agar unless alternately indicated. Culture density and growth phase were estimated where necessary by determining $O.D._{600nm}$ using a Phillips PU8700 Series UV/Visible Spectrophotometer, 10^8 cells/ml giving a reading of approximately 1. For long term preservation strains were stored at -80°C in sterile 40% glcerol.

L-broth.	Tryptone 10g/L Yeast Extract 5g/L NaCl 5g/L	**L-agar.**	As L-broth but including 10g agar
Lab M Broth.	Lab M broth 25g/L		
Lab M Agar.	Lab M agar 28g/L		

Swarm plates (for selection of motile bacteria) consisted of L-broth, supplemented with 0.17% agar.
The media were autoclaved after mixing.

Agrobacterium tumefaciens was grown at 27°C, *Escherichia coli* at 37°C.

Minimal Media (Min A) consisted of : (Miller, 1972)	100ml 5*Min A salts 0.5 ml 1M $MgSO_4$ ddH_2O to 500ml
Min A + glucose consisted of Min A supplemented with 10ml/L 20% glucose (Miller, 1972).	
5*Min A salts: (Miller, 1972)	52.5g K_2HPO_4 22.5g KH_2PO_4 1.0g $(NH_4)_2SO_4$ 2.5g Na Citrate ddH_2O to 1l
Media were prepared by adding sterile constituents to autoclaved water.	

YEP (Ebert *et al*, 1987).	10g 10g 5g ddH_2O	Yeast Extract Peptone NaCl to 1l
Media was adjusted to pH 4.5 and autoclaved after mixing.		

Lactose Agar (for the *Agrobacterium* plate test) : (Bernaerts & De Ley, 1963). Extract	10g 1g 20g ddH_2O	Lactose Yeast Agar to 1l.
Media was autoclaved after mixing.		

M9 Media. (Miller, 1972).	10g 768ml	Agar ddH_2O
Autoclaved prior to the addition of :	200ml 2ml 100μl 20ml	5*M9 salts 1M $MgSO_4$ 1M $CaCl_2$ 1mg/ml thiamine

5*M9 salts. (Miller, 1972).	30g	Na_2HPO_4
	15g	KH_2PO_4
	2.5g	NaCl
	5g	NH_4Cl
	ddH_2O	to 1l

Induction Media.

(Rogowsky *et al*, 1987): Murashige Minimal Organics Media, 12.5mM potassium phosphate, antibiotics as appropriate.

(Vernade *et al*, 1988): Dissolve 12.5g sorbitol and 2.5g sucrose in 400ml ddH_2O. Autoclave. Add 100ml 5*salts. pH to 5.6.

(Winans *et al*, 1988): 1*AB salts, 2.5mM phosphate, 3% sucrose, 20mM Mes.

5*Salts (Vernade *et al*, 1988).

2.5g	$(NH_4)_2 SO_4$
1.25g	sodium citrate
2.5g	KNO_3
4.4g	KH_2PO_4
0.8g	Mes

Dissolve in ddH_2O, add 6ml 10% $MgSO_4$, 2.5ml 1% thiamine, make up to 500ml, pH to 5.45.

AB Salts (PNAS, 71, 3672-3676).

3g/l	K_2HPO_4S
1g/l	NaH_2PO_4
1g/l	NH_4Cl
0.3g/l	$MgSO_4.7H_2O$
0.15g/l	KCl
0.01g/l	$CaCl_2$
2.5mg/l	$FeSO_4.7H_2O$
0.5%	glucose

Octopine Assay Plates (to confirm presence of Ti-plasmid in *Agrobacterium tumefaciens).*

760ml	2% Agar
200ml	5*N0,0 Salts
2.8ml	1M $MgSO_4$
4ml	22mM $CaCl_2$
10ml	20% Glucose
20ml	5mg/ml Octopine

Components autoclaved separately and mixed when cool.

5*N0,0 Salts (Miller, 1972).

K_2HPO_4	51.3g
KH_2PO_4	61.75g
NaCl	0.75g
ddH_2O	to 1l

Adjusted to pH7.5 with 10N NaOH and autoclaved.

2.5.1 Antibiotics.

When antibiotic selection was required, stock solutions were prepared, filter sterilized and the appropriate volume added to cooled media as detailed in the following table (Leemans *et al*, 1981, Sambrook *et al*, 1989):

Antibiotic	**[Stock] (mg/ml)**	**Solvent**	**[Final] (μg/ml)**
Ampicillin	25	H_2O	50
Carbenicillin	50	H_2O	100
Chloramphenicol	12.5	EtOH	50(A.t.)/25(E.c.)*
Erythromycin	50	EtOH	100
Gentamycin	12.5	H_2O	100(A.t.)/25(E.c.)*
Kanamycin	12.5	H_2O	25(A.t.)/50(E.c.)*
Neomycin	25	H_2O	100
Rifampicin	50	DMSO	100
Spectinomycin	50	H_2O	100(A.t.)/50(E.c.)*
Streptomycin	20	H_2O	300(A.t.)/50(E.c.)*
Tetracyclin	12.5	EtOH/H_2O 1:1	10(A.t.)/15(E.c.)*

* When two figures are shown different final concentration were used for *Agrobacterium tumefaciens* (A.t.) and *Escherichia coli* (E.c.)

2.6 Isolation of DNA.

2.6.1 Plasmid Mini-Prep.

Small amounts of plasmid DNA were routinely prepared by the following method, essentially as described by Sambrook *et al*, 1989.

Solution 1: Sterile 1% Glucose, 10mM EDTA, 25mM Tris-HCL, pH 8.0.

Solution 2: 0.2N NaOH, 1% SDS.

Solution 3: Sterile 11.5ml Glacial acetic acid, 28.5ml ddH_2O, 60ml 5M Potassium acetate, pH 4.8.

TE Buffer: Sterile 10mM Tris-HCL, 1mM EDTA, pH 8.0.

Phenol/Chloroform: 25 volumes twice TE saturated phenol, 24 volumes chloroform, 1 volume iso-amyl alchohol.

DNAse Free Pancreatic RNase: Pancreatic RNase was dissolved to a concentration of 10mg/ml in 10mM Tris-HCl, pH7.5, 15mM NaCl and boiled for 15 minutes in a water bath. The mixture was then allowed to cool slowly before use.

A single colony was grown overnight in 5ml L-broth with appropriate antibiotic selection. 1.5ml of culture were transferred to a sterile Eppendorf tube and spun for 1 minute in an MSE Microcentaur microfuge. The supernatant was removed and the pellet lightly dried. The cells were resuspended in 100μl ice cold solution 1 using a Fisons vortex mixer. The contents of the tube were left to stand for 5 minutes at RTP. 200μl of freshly prepared solution 2 were added and mixed in by repeated inversion. The bacterial suspension was cooled on ice for 5 minutes to allow cell lysis, before the addition of 150μl solution 3. After vortexing the mixture was kept on ice for 5 minutes and then centrifuged for 5 minutes to remove cellular debris. 400μl of the resulting supernatant were transferred to a fresh Eppendorf and an equal volume of phenol/chloroform added. The contents of the tube were vortexed before centrifugation at 12000rpm for 2 minutes in an MSE Microcentaur.

The aqueous phase of the preparation was transferred to a fresh tube and mixed with 2 volumes of 100% ethanol at room temperature. After vortexing the tube was incubated at RTP for 2 minutes before centrifugation for 5 minutes to pellet plasmid DNA. The supernatant was poured off and contaminating salt dissolved away by the addition of 1ml 70% ethanol. A further 2 minutes centrifugation in an MSE Microcentaur were followed by removal of the ethanol and the DNA dried under vacuum. The preparation was taken up in 50μl TE supplemented with RNase to a final concentration of 20μg/ml.

2.6.2 Plasmid Midi-Prep (Alkaline Lysis/Glass Fines Method).

NaI solution: 90.8g NaI and 2.0g Na_2SO_3 were dissolved in 100ml ddH_2O and autoclaved.

This method was used when more DNA was required. A 5ml culture was grown overnight with appropriate plasmid selection. 1ml of this was used to inoculate 50ml of fresh L-broth supplemented with antibiotic for growth overnight. Cells were harvested by spinning for 10 minutes at 4000g in an MSE 18 High Speed centrifuge. The supernatant was discarded and the bacterial pellet drained. Cells were resuspended by pipetting in 3.0ml ice cold solution 1. After 10 minutes incubation at RTP, 6.0ml fresh solution 2 were mixed in by inversion. Cell lysis was visually confirmed by the increased viscosity of the preparation after 10 minutes incubation on ice. 4.5ml of solution 3 were added and vortexed in before chilling the preparation for 15 minutes on ice. The supernatant resulting from centrifugation at 7000g, 4°C for 15 minutes was removed to a fresh tube and 5.0ml phenol chloroform mixed in. The mixture was spun at 6000g, 4°C for 15 minutes and the resulting aqueous phase pipetted to a fresh tube. Two volumes of isopropanol were vortexed in and the contents of the tube left for 5 minutes at RTP. DNA was pelleted by centrifugation at 6000g for 10 minutes, the resulting supernatant being discarded. The DNA pellet was resuspended in 200μl TE and transferred to an Eppendorf tube

containing 1.0ml NaI solution. 30μl of silica fines suspended in TE were mixed in and the tube left for 10 minutes at RTP. The fines were pelleted by centrifugation in an MSE Microcentaur, washed by resuspension in 1ml 70% ethanol, pelleted again and taken up in 100μl TE. After incubation at 37°C for 10 minutes the mixture was centrifuged in an MSE Microcentaur for 15 seconds and the DNA recovered in the supernatant. For higher yields the 37°C incubation and subsequent steps were repeated.

2.6.3 Plasmid Maxi-Prep (Caesium chloride/Ethidium bromide Method).

STE: sterile 0.1M NaCl, 10mM Tris-HCl, 1mM EDTA (Sambrook *et al*, 1989).

This method yeilds high amounts (>1mg) of high purity DNA. A 5ml overnight culture grown with appropriate selection was used to inoculate 500ml liquid media supplemented with antibiotics. Cells grown overnight were harvested by centrifugation at 4000g for 10 minutes in an MSE 18. The bacterial pellet was washed in 20ml STE and spun at 4000g, 4°C for 10 minutes. Cells were resuspended by pipetting in 10ml solution 1 supplemented with lysozyme to a final concentration of 5mg/ml. The mixture was split into 2 fresh Oakridge tubes before incubation at RTP for 10 minutes. 20ml of solution 2 were added and the tubes allowed to stand for a further 20 minutes. 15ml of solution 3 were shaken in and the tubes chilled on ice for 10 minutes. After a 55000g, 4°C spin for 20 minutes in a Du Pont Instruments Sorvall RC5B Refrigerated Superspeed Centrifuge, the supernatant was transferred by pipette to 30ml Corex tubes and 0.6 volumes of isopropanol added. The contents of the tubes were mixed and allowed to stand at RTP for 15 minutes. The DNA was recovered as a pellet after a 30 minute, 20000g spin. Phenol chloroform extraction (see section 2.9.1) was performed until the solution cleared. The mixture was centrifuged at 15000g, 4°C for 10 minutes and the DNA ethanol precipitated

(section 2.9.2). A 70% ethanol wash was followed by centrifugation and vacuum drying.

The DNA was combined and taken up in 4ml sterile ddH_2O in a Universal tube. 4g high purity CsCl were dissolved in the DNA solution and 5ml of the resulting mixture transferred to a fresh Universal. 400μl of 10mg/ml ethidium bromide solution were added and the mixture transferred to a 5ml Beckman Quick Seal tube using a needle and syringe. The tube was balanced and heat sealed before centrifugation overnight at 250000g, 15°C in a DuPont Instruments Sorvall OTD 65B Ultracentrifuge, equipped with a VTi50 rotor.

The following morning, the tube was carefully removed from the rotor and viewed under UV light. Two fluorescent bands of DNA corresponding to open circular plasmid and contaminating chromosomal DNA (upper band) and supercoiled plasmid (lower band) were seen. A hole was made in the top of the tube to allow entry of air, and the lower band collected in a syringe with a wide bore needle passing through the wall of the vessel. The DNA was transferred to an Eppendorf tube and 1 volume of butan-2-ol saturated in sterile ddH_2O and CsCl added. After mixing the top layer was removed, and the procedure repeated until no ethidium bromide remained. 300μl aliquots of the DNA solution were placed in Eppendorfs and 600μl sterile 0.45M sodium acetate plus 540μl isopropanol added. The mixture was stored at -20°C for 1 hour and subsequently centrifuged for 5 minutes in an MSE Microcentaur. The DNA was 70% ethanol washed, pelleted, vacuum dried and resuspended in TE.

2.6.4 Isolation of Plasmid DNA By Anion Exchange Chromatography.

In the later stages of research the use of Qiagen columns replaced the above midi and maxi prep protocols as the method of choice due to increased speed and facility.

Buffer P1: 100μg/ml lyophilized RNase A, 50mM Tris-HCl, 100mM EDTA, pH8.0

Buffer P2: 200mM NaOH, 1% SDS.

Buffer P3: 2.55M KAc, pH 4.8

Buffer QBT: 750mM NaCl, 50mM MOPS, 15% EtOH, 0.15% Triton X-100, pH 7.0

Buffer QC: 1.0M NaCl, 50mM MOPS, 15% EtOH, pH 7.0

Buffer QF: 1.25M NaCl, 50mM MOPS, 15% EtOH, pH 7.0

The protocols for the two scales of preparation are essentially the same. The figures in the text refer to the midi size prep (100ml culture, 100μg average yield), with maxi prep (500ml culture, 500μg average yeild) variations being shown in brackets.

100ml (500ml) of L-broth with appropriate selection were inoculated from a 5ml stationary phase culture and the cells grown overnight. Bacteria were harvested by centrifugation at 4000g for 10 minutes in a MSE 18. The pellet was resuspended in 4ml (10ml) buffer P1. One volume of buffer P2 was gently mixed in and the tube incubated at room temperature for 5 minutes to allow cell lysis. Following addition of 4ml (10ml) buffer P3 the mixture was vortexed and spun at 15000g, 4°C for 30 minutes. The resultant supernatant was removed and subjected to an identical second spin. A Qiagen tip-100 (tip-500) was equilibrated by passing 3ml (10ml) buffer QBT down it. The supernatant from the spin was passed down the column to allow plasmid DNA to bind to the anion exchange resin. Contaminating, protein, RNA and chromosomal DNA were washed away with 10ml (30ml) buffer QC. The plasmid DNA was eluted at high salt concentration by the addition of buffer QF, (5 or 15ml as appropriate). 0.7 volumes of isopropanol at RT were added to precipitate

the DNA which was recovered by centrifugation at 15000g for 30 minutes. The sample was washed with 70% ethanol, air dried and resuspended in TE.

2.7 Introduction of DNA into *Agrobacterium tumefaciens.*

2.7.1 Conjugation.

2.7.1.1 Triparental Mating (Ditta et al, 1980).

This method allows mobilization of a plasmid from *E.coli* to *Agrobacterium*, through conjugal *pili* formed by the helper strain HB101 (pRK 2013). Plasmid transfer is independent. Drawbacks include low efficiency and length of time taken to complete the protocol.

5ml cultures of the helper, donor and recipient strains were grown to late log phase with antibiotic selection. The strains were then mixed in the ratio of 8 parts recipient, 1 part helper, 1 part donor and allowed to stand for 30 minutes. The bacteria were then incubated at 28°C overnight on a 13mm nitrocellulose disc which had been placed on a non selective L-agar plate. The bacteria were harvested from the filter in 10ml 10mM $MgSO_4$, pelleted in an MSE 18 centrifuge and resuspended in 100μl L-broth and before plating out on appropriate selection to allow growth of transconjugants. Donor, helper and recipient strains were plated out on the same selection as controls. Plasmid transfer was confirmed by restriction analysis of plasmid DNA.

2.7.1.2 Two Step Mating.

STAGE 1.

5ml cultures of BM2KNaI^R(pRN3) and the *E.coli* donor strain were grown overnight without antibiotics. The 2 cultures were mixed and 0.1ml of the mixture spread on an L-agar plate. As a control 0.1ml of donor and recipient strains were also plated out. The plates were incubated at 37°C for 4 hours. The cells were scraped off from the surface of the agar in 3ml 10^{-2}M $MgSO_4$. RN3 transconjugants

were selected by plating out 10^{-4}, 10^{-5} and 10^{-6} fold dilutions on appropriate selection. Controls were plated out on selection for transconjugants at 1 and 10^{-1} fold dilutions to ensure they did not grow. A few transconjugant colonies were checked for growth on unselected markers.

STAGE 2.

Overnight cultures of the final recipient strain and the recombinant strain were grown without antibiotics. The cultures were mixed in a 3:1 ratio as above and 0.1ml aliquots spread on to L-agar to be grown overnight at 28°C. 0.1ml aliquots of donor and recipient were also incubated under the same conditions. The cells were recovered in 3ml $MgSO_4$ as in stage 1 and plated out on appropriate selection to allow growth of recombinants. Control plates of donor and recipient cells on the selection for recombinants were also set up. Putative recombinants were screened on secondary selection and plasmid DNA extracted for analysis.

2.7.1.3 Plate-Mate Conjugation.

This simple method allowed the transfer of plasmids between competent *E.coli* strains and *Agrobacterium*, in addition it was used to transfer plasmids between *E.coli* strains. 5ml cultures of the donor and recipient strains were grown overnight. 100μl of each were mixed in a pool on a non selective L-agar plates and allowed to grow overnight (28°C, *E.coli/Agrobacterium*, 37°C *E.coli/E.coli*). Loopfulls of transconjugants were harvested and streaked out on selection. As controls, the inability of donor and recipient to grow on the selection were confirmed. Plasmid content was confirmed by restriction analysis.

2.7.2 Transformation.

2.7.2.1 Freeze Thaw (Höfgen & Willmitzer, 1988).

An overnight culture of *Agrobacterium tumefaciens* was diluted in 200ml L-broth and grown until mid log. phase. Cells were harvested by spinning at 4000g for 10 minutes in an MSE 18 centrifuge at 4°C. The pellet was washed in 10ml precooled TE buffer and resuspended in 10ml L-broth. 0.5ml aliquots were rapidly frozen in liquid nitrogen and stored in Eppendorfs at -70°C until required.

Stored cells were thawed on ice prior to transformation. Five hundred to 1000ng of DNA in TE buffer were added. Cells were incubated on ice for 5 minutes, in liquid nitrogen for 5 minutes and at 37°C for 5 minutes. This was repeated. After dilution with 1ml L-broth, cells were shaken at 28°C for several hours before being plated on appropriate selection and incubated at 28°C for 2 days. Single colonies were chosen for plasmid content analysis. Non transformed cells were grown on plasmid selection as a control.

2.7.2.2 Electroporation.

2.7.2.2.1 After Mattanovich et al (1989).

A 300ml L-broth culture with antibiotic selection was inoculated and grown to an $O.D._{600nm}$ of 0.5. Bacteria were chilled on ice and spun down at 4000g, 4°C for 10 minutes in an MSE 18 High Speed Centrifuge. The cells were resuspended in 10ml 1mM HEPES, pH7.0. Two further HEPES washes were followed by a 10ml 10% glycerol and 3ml 10% glycerol wash. 200μl aliquots of cells were rapidly frozen in liquid nitrogen and stored at -80°C until required.

Prior to transformation, cells were thawed on ice and mixed with 200ng plasmid DNA. 40μl of material were placed in a 0.2cm electrode gap electroporation cuvette and subjected to a 2.5KV pulse dissipated through 25μF of capacitance using a Biorad Gene Pulser. Cells were immediately diluted with 1ml L-broth and

shaken for one hour at 28°C. Transformants and suitable controls were plated out on selective L-agar and plasmid presence verified by restriction analysis.

2.7.2.2.2 After Nagel et al (1990).

This protocol had the highest efficiency and greatest ease of use of the published *Agrobacterium* transformation protocols employed.

250ml of cells were grown with plasmid selection to an absorbance of 0.5-1.0 at 600 nanometres. Cells were harvested by centrifugation in an MSE 18 at 4000g for 10 minutes and resuspended in 250ml ice cold 1mM HEPES/KOH buffer, pH7.0. Cells were sequentially pelleted at 4°C and resupended in 125ml HEPES buffer, 100ml 10% glycerol and 750μl 10% glycerol, all solutions being ice cold. 100μl aliquots of competent cells were frozen in liquid nitrogen and stored at -80°C.

Electroporation was conducted by mixing 40μl of cells with 1μl miniprep DNA dissolved in TE. The mixture was transferred to a 2mm electrode gap Biorad cuvette and exposed to a 12.5KV/cm electrical pulse at 200Ω and 25μF in Gene Pulser apparatus. Transformed cells were grown in L-Broth for several generations to allow expression of anti-biotic resistance genes and plated out on selection. Plasmid content was demonstrated by restriction pattern analysis and control selective plates were inoculated with un-transformed cells.

2.7.2.2.3 After Wen-jun & Forde (1989).

100ml agrobacterial cultures were grown to an $O.D._{600nm}$ of 0.6. Cells were cooled on ice, pelleted in an MSE 18 centrifuge at 4000g, 4°C for 10 minutes and resuspended in 1 volume ice cold 10% glycerol. Further washes followed, using 0.5, 0.02 and 0.02 culture volumes of cold 10% glycerol. Competent cell storage was as above.

Electroporation conditions were 12.5KV/cm, 600Ω, 25μF, 0.2cm cuvette. Cells were grown for several hours in L-broth before plating out on selection. Appropriate control were performed and transformation success confirmed.

2.7.2.2.4 After Wirth et al (1989).

Transformation buffer: sterile 300mM Sucrose, 7mM sodium phosphate, 1mM $MgSO_4$, pH 7.4.

Bacteria were grown in liquid media with selection to an $O.D._{600nm}$ of 0.3. Pelleting in an MSE 18, 4000g for 10 minutes was followed by washing in 1 volume transformation buffer. Cells were resuspended to 10^9 c.f.u. per ml. in transformation buffer and mixed with 200ng DNA per ml. After 30 minutes on ice 800μl of mixture were transferred to a cooled 0.4cm electroporation cuvette and subjected to a 2.5KV pulse using 25μF capacitance. The cells were kept on ice for 20 minutes and then allowed to grow for several generations before exposure to selection on solid plates. Plasmid presence was verified in putative transformants. Untreated host was verified as unable to survive plasmid selection.

2.8 Transformation of *Escherichia coli.*

2.8.1 Rubidium Chloride Method (Kushner).

A 10ml culture of *E.coli* was grown to an $O.D._{680nm}$ of 0.4-0.5. 1.4ml of culture were transferred to an Eppendorf tube and cells pelleted for 30 seconds in a MSE Microcentaur. The supernatant was discarded and the cells gently resuspended in 0.5ml fresh 10mM MOPS, 10mM RbCl, pH 7.0. The cells were pelleted for 15 seconds as before and resuspended in 0.5ml 100mM MOPS, 10mM RbCl, 50mM $CaCl_2$, pH 6.5. After standing on ice for 90 minutes, the cells were centrifuged for 10 seconds in an MSE Microcentaur and resuspended in 150μl 100mM MOPS, 10mM RbCl, 50mM $CaCl_2$, pH 6.5. 3μl of DMSO were added followed by 1-250ng DNA in

TE buffer. The mixture was held on ice for 1 hour. The contents of the tube were mixed and then heat shocked at 55°C for 30 seconds in a waterbath before being transferred to an ice bucket for 1 minute. 1ml of prewarmed L-broth was added and mixed in by inversion. The cells were incubated at 37°C for one hour without antibiotics. The suspension was mixed and the cells plated out on appropriate selection. Control reactions without DNA added were also set up. The plates were incubated at 37°C overnight.

2.8.2 Electroporation after Wirth *et al*, 1989.

As for *Agrobacterium*, see section 2.7.2.2.4.

2.9 DNA Manipulations.

All essentially as described by Sambrook *et al*, 1989.

2.9.1 Phenol/Chloroform Extraction.

Protein was removed from DNA samples due to differential solubility in organic solvent. DNA solutions were mixed with an equal volume of TE saturated redistilled phenol. The phases were mixed by vortexing and then reseparated by centrifugation for 3 minutes in an MSE Microcentaur. The upper aqueous layer was removed and the DNA similarly extracted with an equal volume of TE saturated phenol:chloroform:iso-amyl alcohol (25:24:1).

2.9.2 Ethanol Precipitation.

DNA was recovered from aqueous solution with high efficiency by precipitation in alcohol. One tenth volume of 3M sodium acetate, pH4.8 and 2 volumes of ethanol were added to the DNA solution. The sample was vortexed and allowed to stand for a few minutes before chilling at -80°C for at least 45 minutes. Centrifugation at greater than 12000g for 10 minutes yielded a DNA pellet. Salt was

removed from the sample due to its higher water solubility during a 70% ethanol wash. After 5 further minutes of centrifugation the DNA was dried under vacuum and resuspended in a suitable volume of TE.

2.9.3 Restriction Digests.

TBE: per litre: 121.1g Tris-HCl, 51.33g boric acid, 3,72g EDTA.

6*Stop Dye: 0.25% w/v Bromophenol blue, 0.25% w/v xylene cynol, 40% sucrose, 1* TBE.

500ng of plasmid DNA were typically digested with 5 units of the desired restriction enzyme. The reaction was buffered with the recommended concentration of restriction enzyme buffer (as supplied with the endonuclease). Sufficient sterile distiled water to make the reaction volume up to 10μl was added, the reaction mixed and incubated at the optimum temperature (usually 37°C) for 1-2 hours. Digestion was stopped by the addition of 0.2 volumes of stop dye.

2.9.4 Agarose Gel Electrophoresis.

2.9.4.1 Lambda-PstI Size Markers.

50μg of phage *lambda* DNA were digested for 4 hours using 100 units of *Pst* I in 1ml of 1**Pst* I restriction buffer (as supplied with the enzyme). This yielded fragments of the following sizes (Kb):

14, 11, 5(triple), 2.8, 2.5(double), 2.19, 1.99, 1.7, 1.16, 1.09, 0.8, 0.52, 0.47, 0.45, 0.34.

50*TAE: per litre: 242g Tris, 57.1ml glacial acetic acid, 0.05M EDTA, pH8.0

Unless otherwise stated 0.7% agarose gels were used, providing efficient separation of DNA between 0.8 - 10 Kb.

2.9.4.2 Mini-Gels.

Mini-gels were used for routine checks of plasmid presence in strains and for estimating DNA concentration. 500ml of 1* TBE were prepared and supplemented with 10 μl 10mgml^{-1} ethidium bromide. 40ml of agarose solution in 1*TBE were made up for a small mini-gel, 70ml for a large one. The suspension was placed in an Electrolux Mealmaker microwave oven at power setting 9 for 2 minutes. The agarose solution was cooled and poured into a Pharmacia GNA 100 minigel former, air bubbles were removed and a comb placed in the gel before it was allowed to set. The gel and the former were placed in the electrophoresis tank and covered with 1* TBE buffer. Samples were loaded into the gel wells. Phage *lambda Pst* I digest was run as a size marker. A p.d. (30-100V, 50-100 mA) was applied along the length of the gel to allow adequate fragment separation. The gel was examined under a U.V.P. Incorporated U.V. transilluminator and photographed through a red filter with a Polaroid RP4 Land camera using Polaroid 667 Professional film.

2.9.4.3 Maxi-Gels.

Maxi-gels were run when a high quality record was required, when fragment isolation was being performed or when a large volume of solution was being electrophoresed.

A glass plate was cleaned with ethanol and allowed to dry. A gel former was sealed to the glass using Dow Corning High Vacuum Grease, and a suitable comb placed in position. Sufficient molten agarose solution in 1*TBE (with ethidium bromide added as above) to fill the mold was prepared. The solution was allowed to cool after being microwaved and then poured into the former. Air bubbles were removed, and once the gel had set it was placed in an electrophoresis tank. One times TBE containing 10μl 10mg/ml ethidium bromide was placed in the tank, covering the gel. The comb was removed and the samples loaded. Size markers were run alongside the samples. Fragments were separated and photographed as above.

2.9.5 Fragment Isolation.

This method allows the purification of a DNA fragment suitable for cloning into a vector. The required band was cut from the gel with a scalpel while visualised under UV light. The band was trisected and transferred to a small Eppendorf with a hole in the base and a packing of glass wool. The tube was stored at -80°C for 30 minutes. The tube was placed inside a large Eppendorf and centrifuged in an MSE Microcentaur for 10 minutes. The volume of elutant was made up to 100μl. Phenol/Chloroform extraction and ethanol precipitation were performed, followed by a 70% ethanol wash, pelleting of DNA and resuspension in 5μl of TE buffer.

2.9.6 DNA Concentration Estimation.

Prior to cloning attempts DNA concentration was estimated to allow usage of correct ratios of vector to fragment (1:3 moles of termini).

2.9.6.1 U.V. Fluorescence Method.

Five, 2.5, 2.0, 1.5, 1.0, 0.5, 0.25 0.1 and 0.05 ng/μl solutions of phage *lambda* DNA were prepared in TE. 2μl of each of these solutions were mixed with equal volumes of 2μg/ml ethidium bromide and placed as discrete spots on a piece of Nescofilm. 2μl of a 10, 50 and 100 fold dilutions of the DNA sample were treated in the same way. The nescofilm was photographed using Polaroid 667 Professional film and a Polaroid RP4 Land Camera equipped with a red filter. Illumination was from a UV transilluminator, UVP Inc. The DNA concentration was estimated by seeing which spot of known concentration most closely corresponded to the brightness of the unknown sample.

2.9.6.2 Spectrophotometric Method.

Appropriate dilutions in TE were made of the DNA sample (typically 1:50 or 1:100). Absorbance of the solution was measured at 260 and 280nm using a Beckman Instruments DU7500 spectrophotometer. Pure DNA has a $O.D._{260/280nm}$ ratio of 1.8 in solution, protein and RNA contamination altering this. An $O.D._{260nm}$ of 1.0 corresponds to a double stranded DNA concentration of 50μg/ml

2.9.7 Alkaline Phosphatase Treatment.

Vector DNA was treated with alkaline phosphatase to limit self ligation. Restrictions of vector DNA were set up as usual and 1 unit calf intestinal phosphatase per 10μl was added for the last 30 minutes of the digest. 4.8ml of NTA per 11μl were added and the tube stored at 70°C for 15 minutes to denature the enzymes. The DNA was phenol/chloroform extracted and then ethanol precipitated. Following 70% ethanol washing and centrifugation in an MSE Microcentaur, the DNA was dried under vacuum and resuspended in TE.

2.9.8 DNA Ligation.

In a total volume of 20μl, adjusted with ddH_2O, 2μl ligation buffer, 4 units of T4 DNA ligase and the fragment solutions were incubated overnight at 15°C and used to transform *E.coli.*

2.9.9 Colony Hybridization.

This technique, adapted from Sambook *et al*, 1989, is used to confirm the presence of a desired DNA sequence or fragment in a bacterial host. Protocols using digoxigenin labelled probes are safer to perform than older protocols using radiolabelled material, but sufficiently sensitive for this purpose.

2.9.9.1 Transferring Small Numbers of Colonies to Nitrocellulose Filters.

A grided nitrocellulose filter was placed on a selective agar plate. Using sterile wooden toothpicks, individual colonies were transferred onto the filter and a master selective agar plate, marked with a similar grid. Each colony was placed in an identical position on both plates. Colonies were streaked as diagonal lines, 2-3mm apart. As positive and negative controls, colonies with and without the desired sequence were included on the grid. The cells were grown until the streaks were approximately 1mm wide. The filter was marked in 3 places to allow its orientation to the determined relative to the master plate at a later time. The master plate was sealed with Nescofilm at kept at 4°C until required.

2.9.9.2 In Situ Colony Lysis and Biding of DNA to Filters.

Denaturing Solution: 0.5M NaOH, 1.5M NaCl.

Neutralizing Solution: 1.5M NaCl, 0.5M Tris-HCl, pH 7.4

20*SSC: Dissolve 175.3g NaCl and 88.2g sodium citrate in 800ml H_2O. Adjust to pH 7.0 with NaOH. Make up to 1l. Autoclave.

The bottoms of 4 petri dishes were fitted with circles of Whatman 3MM paper. Each paper circle was saturated with one of the following: 10% SDS, denaturing solution, neutralising solution, 2*SSC. Excess liquid was poured off. Using blunt ended forceps, the nitrocellulose filter was transferred from the agar plate to the SDS soaked paper. The filter was placed with the colonies on the upper side, SDS soaking through the membrane to limit the diffusion of DNA during the following steps, resulting in a sharper eventual signal. After 3 minutes exposure to SDS, the filter was lightly dried and transferred to the denaturing solution dish for 5 minutes. Similar 5 minute incubations with neutralising solution and 2*SSC were performed. The filter was then allowed to dry fully at RT before being sandwiched

between 2 dry sheets of 3MM paper and glass. DNA was fixed to the membrane by baking in a vacuum oven for 1.5 hours at 80°C.

2.9.9.3 Preparation of DNA Probes.

Following the guide-lines of the Boehringer and Mannheim Nonradioactive DNA Labelling and Detection Kit, target DNA was labelled with digoxigenin labelled dUTP. The random priming technique of Feinberg and Vogelstein, 1983 was employed.

Between 10ng and 3μg of linearized target DNA was prepared by restriction digest, agarose gel electrophoresis, fragment isolation, phenol/chloroform extraction and ethanol precipitation. Double stranded plasmid was denatured at 95°C for 10 minutes followed by rapid chilling. 2μl of a concentrated random hexanucleotide mixture, 2μl of dNTP labelling mix (1mM dATP, 1mM dCTP, 1mM dGTP, 0.65mM dTTP, 0.35mM Dig-UTP pH6.5) and sufficient ddH_2O to make the reaction volume up to 19 μl were added. 1μl of labelling grade 2 units/μl Klenow enzyme were introduced and the mixture incubated at 37°C for at least 1 hour. The labelling reaction was stopped by the introduction of 2μl 0.2M EDTA, pH8.0. Labelled DNA was precipitated with 2μl 4M LiCl and 60μl ethanol prechilled to -20°C. After at least 30 minutes at -80°C, labelled DNA was pelleted in a MSE Microcentaur, washed in 70% ethanol and taken up in TE.

2.9.9.4 Gel Filtration of Probes.

To remove unincorporated label from the probe, a 1ml disposable syringe was filled with Sephadex G50 resuspended in TE. Glass wool was used to pack the base of the syringe to prevent the liquid flowing through. The column was placed in a plastic test tube, with the syringe finger grips preventing it from falling into the tube. A short 1800rpm spin in a Wifug Bench Top centrifuge forced excess liquid through the column. The syringe was refilled with Sephadex G50 suspension and

respun. This procedure was repeated 4 times. Fresh aliquots of TE buffer were spun through the column 4 times to wash it and the matrix dried by a 5 minute spin. The sample was added to the top of the column and spun through for 5 minutes, the purified probe being collected in an Eppendorf at the syringe lock.

2.9.9.5 Hybridization of Probes.

Hybridization solution: 5*SSC, 1% w/v Boehringer Mannheim blocking reagent, 0.1% w/v lauroylsarcosine, 0.2% w/v SDS.

Nitrocellulose membranes with the DNA of interest bound to them were presoaked in water and then 20*SSC. Filters were prehybridized in a sealed plastic bag at 65°C for longer than 1 hour with 20ml Hybridization solution per 100cm^2 of nitrocellulose. Prehyrbridization solution was replaced with 2.5ml/100cm^2 filter of hybridization solution containing the freshly boiled labelled probe. The probe was hybridized to the bacterial DNA overnight at 65°C. Prior to signal detection, the unbound labelled probe was washed away by 2 incubations with each of the following: 50ml 2*SSC, 0.1% SDS, RT, 5 minutes, 0.1*SSC, 0.1% SDS, 15 minutes, 65°C. The membranes were allowed to dry.

2.9.9.6 Immuno-Detection of Labelled Sequences.

Buffer 1: 100mM Tris-HCl, 150mM NaCl, pH 7.0.

Buffer 2: 0.5% w/v Boehringer Mannheim blocking reagent in buffer 1.

Buffer 3: 100mM Tris-HCl, 100mM EDTA, 50mM $MgCl_2$, pH 9.5.

Buffer 4: 10mM Tris-HCl, 1mM EDTA, pH8.0.

Colour Solution: 45μl 75mg/ml nitroblue tetrazolium salt in 70% DMF, 35μl 50mg/ml 5-bromo-4-chloro-3-indoyl phosphate toluidinium salt in DMF, 10ml buffer 3.

Filters were washed for 1 minute in buffer 1 then for 30 minutes in 100ml/cm^2 nitrocellulose buffer 2. This was followed by a brief wash in buffer 1. Sheep polyclonal anti-digoxigenin antibody Fab fragment conjugated to alkaline phosphatase was diluted to an activity of 150mU/ml in 20/cm^2 ml buffer 1 and incubated with the filters. Unbound antibody conjugate was removed by washing twice for 15 minutes in 100ml/cm^2 buffer 1. The membranes were allowed to equilibrate with 20ml/cm^2 buffer 3 for 2 minutes. The filters were exposed to 10ml/cm^2 of colour solution in the dark until purple precipitate started to appear. The reaction was stopped by the addition of 50ml/cm^2 buffer 4. All reactions except the colour development were performed with shaking at room temperature . The position of colouration on the filters was correlated with the position of colonies on the master plate to ascertain which bacteria were carrying the sequence of interest.

2.9.9.7 Chemiluminescent Detection of Labelled Sequences.

A theoretically more sensitive protocol using the Stratagene FLASH detection system was tested. The protocol was essentially the same as for the digoxigenin labelled probe, with the exception of the signal detection step. The bound alkaline phophatase conjugate was incubated with a substrate that produced light upon cleavage. This emission was detected using photographic film. Whilst promising, the technique was not routinely employed due to problems in reducing strong background signals.

2.12.2 GUS Assays (Jefferson, 1987).

GUS Extraction Buffer: 50mM Na_2PO_4, 10mM 2-mercaptoethanol,10mM EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton-X.

Assay Buffer: 1mM 4-methyl umbelliferyl beta-D-glucuronide in extraction buffer.

Stop Buffer: 0.2M Na_2CO_3.

C58C1Rif (pVK 257) cells harbouring a *virB::GUS* fusion provided by Dr C.Garret were induced with varying concentrations of phenolic compounds, see previous section. Positive and negative controls were also as above, strains cured for the GUS plasmid were used to determine the background cleavage of substrate. 1.5ml of cells were resuspended in 0.5ml extraction buffer and frozen in liquid nitrogen. The tubes were thawed at 37°C for 10 minutes, then transferred to the fridge for 2 hours. 0.5ml aliquots of assay buffer were incubated concurrently at 37°C to pre-warm. 15μl of extract were mixed into the prewarmed assay buffer. After 4 minutes incubation, 0.1ml of assay mixture was removed and added to 0.9ml stop buffer. Substrate cleavage was estimated visually by examination of U.V. fluorescent product (MU).

2.12.3 ß-Galactosidase Assays (Miller, 1972).

Induction Media: Murashige Minimal Organics Media, 12.5mM potassium phosphate, antibiotics as appropriate (Rogowsky *et al*, 1987).

Z-buffer: 0.06M $Na_2HPO_4.7H_2O$, 0.04M $NaH_2PO_4.H_2O$, 0.01M KCl, 1mM $MgSO_4.7H_2O$, 0.05M ß-mercaptoethanol. pH7.0

Overnight cultures of A348 (pSM30) were grown overnight with kanamycin selection. 1ml aliquots of cells were added to 10ml induction media, putative inducers added at the desired concentration and the cells allowed to incubate at 27°C for exactly 12 hours. The contents of the tubes were chilled on ice for 30 minutes to prevent further growth or induction before the addition of 0.5ml of cells

to 0.5ml Z-buffer. 8μl chloroform and 4μl 0.1% SDS were added to each of the tubes and mixed in by vortex. 50μl extract was removed and stored at -80°C with 10% glycerol for protein determination at a later stage (see section 2.10). 0.19ml prewarmed 4mg/ml ONPG (excess to maintain reaction linearity) in 0.1M phosphate buffer, pH7.0 was mixed in and substrate cleavage allowed to proceed at 28°C for 440 minutes in the dark. 0.575ml of reaction mixture were then added to 0.25ml 1M Na_2CO_3, stopping the reaction and the sample spun down in a MSE Microcentaur for 10 minutes to clarify the solution of protein and cell debris. Optical density at 420nm was determined using a Phillips PU8700 Series UV/Visible Spectrophotometer, allowing the quantification of yellow product formation. Protein determination was performed, and specific ß-galactosidase activity of the extract determined in terms of μmoles *o*-nitrohenol/min/mg protein with reference to an *o*-nitrophenol $O.D._{420nm}$ vs concentration calibration curve. Experiments were performed in duplicate.

2.13 Chemotaxis Assays.

2.13.1 Swarm Experiments.

Chemotaxis Medium: 0.1mM EDTA, 10mM KH_2PO_4, pH7.0 (Adler, 1973).

Initially swarm plates (Parkinson, 1976) comprised of chemotaxis medium supplemented with 0.17% agar and the desired concentration of filter sterilised attractant. Equal numbers of cells were inoculated into the centre of the plate and allowed to grow for 2 days. After incubation the diameters of the resulting swarms were measured. Treatments that produced large swarms were thought to be attractive to the bacteria since they metabolize the substance where they are growing and then move outwards towards the edge of the plate in response to a concentration gradient of unmetabolised material. Experiments were performed in triplicate.

2.13.2 Blind Well Assays.

Isoton II: 7.9g/l NaCl, 1.9g/l Na_2HPO_4, 0.4g/l EDTA, disodium salt, 0.4g/l KCl, 0.2g/l NaH_2PO_4, 0.3g/lNaF

Motile populations of the strains to be tested were selected by 3 sequential rounds of swarming on selective rich media swarm plates. Bacteria from the outermost edge of the colony were used to inoculate the subsequent swarm plate. Following selection of motile bacteria plasmid presence was confirmed by restiction analysis of mini-prep DNA and culture purity was checked microscopically using a Nikon Optiphot microscope equipped with phase contrast optics and biochemically by the *Agrobacterium* plate test.

Liquid cultures of the cells were grown overnight in 5ml aliquots of Min A + glucose contained in McCartney bottles. The bacteria were spun down in a Wifuge 500E centrifuge at 1000g for 10 minutes and washed 3 times in equal volumes of chemotaxis medium. A cotton bud was used to grease the assay chamber of a Blind Well assembly (see figure 2.13.2.A adapted with permission from C.H. Shaw) with a very small amount of Dow Corning High Vacuum Grease. 200μl of the culture was placed in the chamber and a 13mm diameter, 8μm pore size Nuclepore polycarbonate membrane introduced above it. The assembly's cap was screwed in and 480μl of attractant solution placed in the attractant chamber. Parafilm was stretched over the top of the assembly, excluding air bubbles, the chambers inverted and air flicked to the top of the assay chamber. After 2 hours inverted incubation, the number of cells passing through the membrane in response to the attractant was enumerated by diluting 200μl of the fluid from the attractant chamber with 20ml Isoton II and counting with a Coulter Electronics Multisizer II.

Experiments were performed in triplicate and the average of 2 counts taken for each assembly. Initially results were expressed in terms of net number of cells attracted/ml (ie each experimental average number of cells counted - average control value).

Attractant solutions were carefully prepared so as to a maintain a constant and low concentration of solvent in all assays. Stock solutions of phenolic were prepared in decimal decrements from 10^{-1} to 10^{-5}M using 1:1 ethyl acetate and methanol. Attractant solutions were prepared for assays by dilution of these stocks with 1000 volumes of chemotaxis medium. Control solutions consisted of chemotaxis medium with an equal concentration of solvent to that used in assay solutions, but no phenolic compound.

2.14 Mutagenesis Protocols.

2.14.1 Ultra-Violet Light Mutagenesis (Miller, 1972).

To establish suitable conditions for mutagenesis, since none had been published, a killing curve was constructed. A single colony of A348 (pSM30) was grown to stationary phase overnight in 5ml selective L-broth. The cells were spun down in a Wifug 500E bench top centrifuge at 1000g for 10 minutes. The bacteria were resuspended in an 10ml of sterile ddH_2O and chilled to prevent further division. Henceforth the procedure was conducted in the absence of direct illumination to minimize photoreactivation of DNA (Iosson, 1982). The cells were transferred to a 9cm radius petri dish and a sterile 4.5cm magnetic stirrer bar added. The dish was placed on a Gallenkamp Magnetic Stirrer Hotplate 400 and illuminated with a Camlab CAMAG Universal U.V. Lampe 254nm U.V. light source. At various times following the commencement of irradiation, 0.1ml samples were withdrawn from the dish and diluted. These samples were then plated out on L-agar at varying dilutions and incubated for 2 days at 28°C. From these plates, the 95% killing dose was calculated under these conditions.

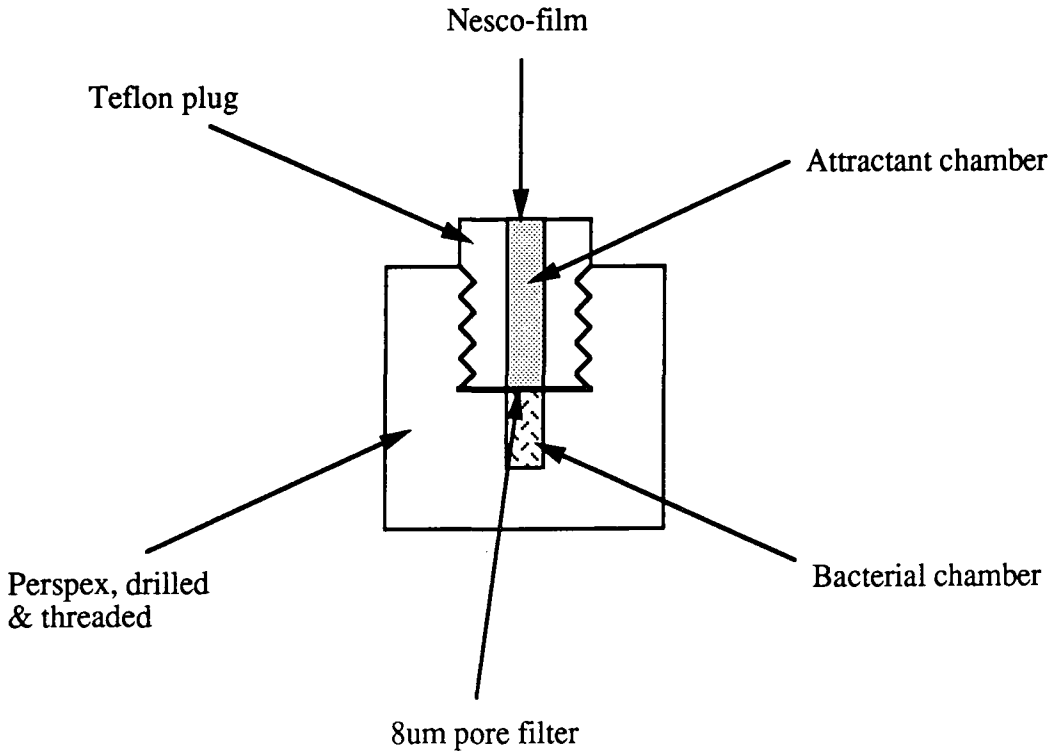


Figure 2.13.2.A: The Blindwell Assay

2.14.1.1 Screening for Potential HVA and VMA Sensitive Agrobacterium Mutants.

Colonies were exposed to the 95% killing dose of U.V. radiation and plated out at 10^{-2}, 10^{-3} and 10^{-4} fold dilutions on 9cm diameter plates of L-agar with following additions:

$1.4*10^{-5}$M VMA
$2.4*10^{-5}$M HVA
0.04mg/ml X-GAL
25μg/ml Kanamycin.

The plates were incubated for 2 days at 28°C and then 4 hours at room temperature. Representative colonies then streaked out under similar conditions, but without the addition of catecholamine to characterise constitutive mutants.

2.14.2 Hydroxylamine, *in vitro* (Ankenbaur et al, 1991, Humphreys et al 1976).

Hydroxylamine solution: 1M hydroxylamine hydrochloride, 1mM EDTA adjusted to pH6.0 with NaOH (Humphreys *et al*, 1976).

Qiagen purified pIB50 plasmid DNA was mutagenized as follows. DNA was added to 5 volumes of 0.1M sodium phosphate buffer pH6, supplemented with 1mM EDTA, and 4 volumes of hydroxylamine solution. Typically the reaction was conducted in a total volume of 200μl with 5μg DNA. The mixture was chilled on ice for 45 minutes followed by 2 hours incubation at 68°C. Following this, the mutagen was extensively dialysed (Sambrook *et al*, 1989) against 3 2l volumes of sterile 10mM $CaCl_2$ at 4°C, ethanol precipitated and used to transform GMI9023(pIB50) after Nagel *et al*, 1990. Electroporated cells were plated out to select for catecholamine sensitive mutants.

2.14.3 Hydroxylamine, *in vivo* (Miller, 1972).

C58CEryCm (pIB50) was grown overnight in 5ml L-broth with kanamycin selection. The cells were spun down at 6000g for 10 minutes in an MSE 18 High Speed Centrifuge and washed and resuspended in 4ml 1M $NH_2OH.HCl$ previously adjusted to pH6.0 with 1N NaOH. After 30 minutes incubation at 28°C, the bacteria

were spun down again, washed and resuspended in L-broth before plating out for selection of HVA and VMA sensitive mutants.

2.14.4 EMS mutagenesis (Cangelosi et al, 1991).

AB Salts: (Chilton *et al*, 1974.) **Solution A:** 3g/l K_2HPO_4, 1g/l NaH_2PO_4 **Solution B:** 1g/l NH_4Cl, 0.3g/l $MgSO_4.7H_2O$, 0.15g/l KCl, 0.01g/l $CaCl_2$, 2.5mg/l $FeSO_4.7H_2O$ 0.2% glucose
Autoclave 2 parts separately and mix when cool.

C58C1EryCm (pIB50) was grown overnight in minimal AB selective medium. The culture was diluted in 50ml fresh medium to an $O.D._{600nm}$ of approximately 0.25. Incubation continued for a further few hours until the culture achieved an $O.D._{600nm}$ of 0.35. 4ml aliquots were transferred to sterile Universal tubes and EMS added to a final concentration of 4% w/v. The mixture was incubated at 28°C for a further 90 minutes before centrifugation at 6000g for 10 minutes in an MSE 18 High Speed Centrifuge. The bacteria were washed twice in TE buffer before resuspension in 4ml AB media. After 2 hours incubation cells were plated out to select catecholamine sensitive mutants.

2.14.5 NG mutagenesis (Pazour et al, 1991).

ABMes: 1*AB salts, 0.2% glucose, 25mM phosphate, 50mM Mes, pH5.5.

A 50ml ABMes culture supplemented with 6.5mg NG was inoculated with C58C1EryCm (pIB50) to an $O.D._{600nm}$ of 0.22. Cells were grown for 75 minutes at 30°C, Mutagenesis was stopped by centrifugation at 6000g, for 10 minutes in an MSE 18 High Speed Centrifuge and washing in one volume of Min A + glucose, pH7.0. Cells were resupended in 1ml L-broth and plated out to select for catecholamine sensitive mutants.

2.14.6 Nitrous Acid Mutagenesis. (Miller, 1972).

0.1M Acetate Buffer, pH4.6:
Solution A: 11.5ml conc. glacial acetic acid, made up to 1l in ddH_2O
Solution B: 27.29g NaAc.3H_2O in per l
Add 25ml A to 24.5ml B and 50ml H_2O, adjust pH and autoclave.

Nitrous Acid: Immediately before use dissolve sodium nitrite to a final conc. of 0.05M in 0.1M acetate buffer, pH4.6, pH critical.

A 5ml culture of C58C1Ery Cm (pIB50) was grown overnight with kanamycin selection. The cells were spun down in an MSE 18 High Speed Centrifuge at 6000g for 10 minutes and washed in an equal volume of 0.1M acetate buffer, pH4.6. The bacteria were centrifuged again and resuspended in 0.3ml nitrous acid. After 10 minutes incubation at 27°C, 5ml 1*Min A (pH7.0) was added to stop the reaction. Cells were plated out to select catecholamine sensitive mutants.

2.15 The *Agrobacterium* Plate Test (Bernaerts & De Ley, 1963).

Benedicts Solution:
Solution A: Dissolve with heating 173g sodium citrate and 100g anhydrous sodium carbonate in 600 ml water.
Solution B: 17.3g $CuSO_4$ in 100ml water.
Slowly add B to A with constant stirring. Cool and make up to 1 litre.

Bacteria to be tested were grown for 2 days on Lactose media at 28°C. Benedicts solution was poured onto the surface of the agar and allowed to stand for at least 10 minutes. The presence of yellow rings of Cu_2O around streaked out colonies was taken as diagnostic of *Agrobacterium* species, since from 80 strains from various genera tested by Bernaerts & De Ley, only *A.tumefaciens* and *A. radiobacter* had the ability to produce 3-keto-lactose, a reducing sugar.

2.16 Carbon Source Assays.

As necessary, the ability of bacteria to utilise certain carbon sources for growth was determined. This was generally done by using a minimal media supplemented with the compound of interest.

2.16.1 Octopine Assays.

The ability of *Agrobacterium* to metabolize opines is dependent on the presence of a Ti-plasmid (Bomhoff *et al*, 1976). This method was thus used to confirm tumour inducing plasmid presence.

The *Agrobacterium* strain of interest was grown on octopine agar. Positive controls with a Ti-plasmid present and negative control cured strains were also streaked out. Growth of the experimental strain after 2 days incubation was taken to confirm Ti-plasmid presence.

2.16.2 Inositol Assays.

The ability of bacteria to metabolize inositol was checked by incubation for 2 days on Min A supplemented with 1% agar and 200mM inositol.

2.16.3 Phenolic Assays.

Similarly the ability to metabolize HVA and VMA was determined using Min A supplemented with 1% agar and 10^{-2}M to 10^{-9}M phenolic.

2.17 Effects of Urine on *Agrobacterium tumefaciens.*

2.17.1 Bacterial Growth Assay.

Samples of adult urine were collected and filter sterilized. 5ml aliquots were introduced into sterile Universal bottles and inoculated with 1 ml of C58C^{1}Rif. Controls were set up with no bacteria added to ensure no growth of organisms

occurred without inoculation and a C58C1Rif culture was grown in L-broth for comparison. After overnight incubation, the culture density was assessed using a Coulter Electronics Multisizer II. To ensure freedom from contamination the resulting population of bacteria was checked using the *Agrobacterium* plate test (Bernaerts & De Ley, 1963) furthermore non-innoculated filter sterilised samples were incubated under the above conditions to ensure no organisms grew. The experiment was repeated several times with different urine samples to determine the overall effect.

2.17.2 *vir* Induction Assay.

C58C1Rif (pVK257, pUCD1187) cultures were set up as described above. Positive controls of urine and L-broth supplemented with 10^{-4}M AS were included. An equal amount of solvent was added to experimental bottles. As a negative control bacteria were grown in L-broth + solvent. After overnight incubation cell density was determined as above and 1ml of bacteria added to 15ml induction media (Rogowsky *et al*, 1987). Photon production was measured using an IBM PC linked Packard 2000 CA Tricarb Liquid Scintillation Analyser. Photon production per cell per minute was compared for the different treatments. This experiment was also repeated several times.

2.18 The Synthesis of Bromo-Acetosyringone.

With the assistance of Dr David O'Hagan, Department of Chemistry, Durham University, the protocol of Miksche, 1973 was followed.

Equimolar amounts of Br_2(l) (1.6ml, 31m moles) and AS (6g, 31m moles) were mixed with 50ml chloroform in a bulb fitted with a condenser and drier. The exothermic reaction between these reagents was allowed to run to completion (assayed by TLC) over a 40 minute period. Excess bromine was removed by partitioning in saturated aqueous sodium thiosulphate. The aqueous layer of this

mixture was separated in a funnel and discarded. Product was washed in ethyl acetate and then water. Anhydrous $MgSO_4$ was added to remove the water and then filtered off under vacuum. Solvent was boiled off using a rotary evaporator. The brominated sample was crystallized on ice and washed with 70% ethanol, giving a light brown solid. To purify the material it was taken up in a minimum volume of pre-warmed 100% ethanol, filtered and re-crystallized over a period of several hours. The product was harvested by vacuum filteration and desiccated overnight with P_2O_5. The sample's melting point was determined and a N.M.R. 250nm proton spectrum performed using a Bruker instrument.

2.18.1 Assaying Reaction Completeness.

Small quantities of AS and reaction mixture were strongly diluted in ethyl acetate and spotted onto a foil backed TLC plate. The chromatogram was run in a mixture of chloroform and ethyl acetate (approximately 3:1) and viewed under U.V. light.

2.19 Generation of Molecular Models.

'Ball and stick' and 3 dimensional space filling molecular models were generated by the application of the requisite algorithms of the IBM PC2 based program 'Desktop Molecular Modeller', version 1.2 (Oxford University Press Electronic Publications), to fully energy minimised structures of AS, ASOH, HVA and VMA. The resultant images were photographed from the computer display and are shown in figures 1.3.2.A-B.

3. Virulence Gene Induction by Catecholamine Metabolites.

3.1 Introduction.

As already mentioned (section 1.3) one of the main reasearch aims was to investigate the effect of the catecholamine metabolites HVA and VMA on *Agrobacterium*. It was initially proposed that the use of a reporter gene construct, induced by the presence of these compounds would be the most amenable method for implementing a bacterial screening test.

3.2 Reporter Genes.

Reporter gene fusions are a particularly useful tool for studying gene expression, allowing relatively simple estimation of promoter activity. The coding region is removed from the gene to be analysed and the promoter of interest fused to the coding region of a gene whose expression is easily quantifable. Ideally, expression of the reporter gene should not normally take place in the cell, nor should it be deleterious to normal cell behaviour. The fusion gene is re-inserted into the organism and its expression under the control of its novel promoter assessed. Fusions are of two types, with much variation within the types. Transcriptional fusions are defined as fusions in which all protein coding sequence is derived from the reporter, whereas translational fusions produce a polypeptide as a result of coding information from the reporter and the controlling gene (Jefferson, 1987). The reporter gene systems used in this study were as follows:

3.2.1 Lac Z:

The coding region of the *E. coli* ß-galactosidase gene was fused to various *vir* promoters. When expressed this gene has the ability to degrade the colourless dye X-GAL to a vivid blue product. Activity is then easily monitored visually or by

colourimetry. Additionally the colourless substrate ONPG can be cleaved to yellow *o*-nitrophenol and galactose (Miller, 1972).

3.2.2 Lux:

In this system a self contained luciferase cassette produces visible light which can be detected visually, by film or in a scintillation counter, depending on the degree of sensitivity required (Shaw and Kado, 1986, Rogowsky *et al*, 1987). This method has the advantage that no external substrate is required.

3.2.3 GUS:

The expression of ß-glucuronidase under the control of the subject promoter can be assayed via a number of spectrophotometric, colourimetric and fluorimetric means, depending on the substrate chosen. Common substrate's include 4-methyl umbelliferyl ß-D-glucuronide (4-MUG), a non-fluorescent compound which, when enzymatically cleaved produces a fluorescent product. This is highly desirable since a high signal to noise ratio is available in the assay, which is also relatively easy to perform. Sensitivity is reported as being 2 to 4 orders of magnitude higher than for spectrophotometric and colourimetric assays. A colourless substrate for colourimetric assay, X-GLUC is also available which cleaves to a blue product (Jefferson, 1987).

Qualitative assays of gene expression can be made quantitative by reference to calibration curves.

3.3 Stock Phenolic Solutions.

Initially solutions of AS, HVA and VMA were maintained in 75% methanol. However addition of large amounts of methanol (even though almost saturated with phenolic) to bacterial cultures in order to achieve the final concentrations of phenolics required, proved most deleterious to bacterial growth. Ethyl acetate was then tested as a solvent. However, although allowing higher phenolic stock concentrations this was still not a sufficiently good solvent. Following the suggestion

of Dr D. O'Hagan (University of Durham, Department of Chemistry) a 1 : 1 mixture of ethyl acetate and methanol was used. This provided excellent results, with 1M stock solutions of phenolics being readily achievable. The resultant decrease in volume of solvent addition required to produce high final attractant concentrations greatly aided culture maintenance.

3.4 vir Gene Expression Studies.

It had been hypothesized that HVA and VMA would interact with the *Agrobacterium virA/G* system to induce *vir* gene expression. This hypothesis was tested using a number of reporter gene constructs as there is no readily applicable direct assay for the expression of these genes on an individual basis. *virB* and *virE* promotors were chosen for the majority of these studies as they regulate the most highly inducible *vir* genes (Rogowsky *et al*, 1987).

3.4.1 lux.

Plasmid miniprep pUCD1187 DNA (*virB::lux*) was introduced into strain C58C^{1}Rif (pVK257) by the freeze/thaw method of (Höfgen & Willmitzer, 1988). Similarly strain C58C^{1}Rif (pVK257, pUCD1194) (*virE::lux*) was also constructed. Strain C58C^{1}Rif (pVK257) was chosen as the background since this readily allowed selection of transformants and carried *virA* and *G* functions on the cosmid clone. Plasmid content was confirmed by restriction analysis of plasmid mini-prep DNA. *lux* inducibility was tested by exposing test strains to 10^{-4}M AS as described in Materials and Methods. Fold induction was calculated as a ratio of this figure to the negative control. pUCD1187 gave a 8.5 fold increase in photon emission whilst pUCD1194 gave a 7.23 fold induction when exposed to 10^{-4}M AS (concentration chosen for strong induction qualities). The *virB::lux* fusion was thus selected for future work due to its higher inducibility.

Figure 3.4.1.A shows the results of *vir* gene expression assays (see section 2.12.1) following exposure of C58C^{1}Rif (pVK257, pUCD1187) to a range of concentrations of AS, HVA and VMA between 10^{-9} and 10^{-4}M. This range of concentrations was employed since it was reasoned that *Agrobacterium* was not likely to respond to unnatural ligands at lower concentrations than the threshold of response to AS (10^{-9}M) and because it was practically difficult to acheive concentrations higher than 10^{-4}M without adversely affecting the bacterial cells. *vir* gene expression is strongly induced by 10^{-4}M AS, comparing well to the results of Rogowsky *et al* (1987). Other acetosyringone concentrations do not illicit light production greater than the solvent control. No significant *vir* gene induction above the control level was detected on exposure to HVA and VMA, indicating that these compounds do not act as *vir* inducers within the range of concentrations tested. 10^{-4}M AS was chosen as a positive control for future experiments.

To assay for possible synergism or repression of induction, the test strains were exposed to the same range of concentrations of HVA and VMA supplemented with 10^{-4}M AS. (see figure 3.4.1.B). However, once again no significant variation was noted between the experimental and control treatments, indicating an inability of these compounds to interact with the bacterial induction system. The photon production is slightly lower than in the previous experiment, despite the inclusion of 10^{-4}M AS presumably because of the greater volume of solvent introduced into the bacterial cultures. In an attempt to improve *vir* induction by HVA and VMA, the assay protocol was modified such that the conditions for induction were optimised. A number of "induction media" have been devised by various groups for this purpose, see section 2.5, (Rogowsky *et al*, 1987, Vernade *et al*, 1988, Winans *et al*, 1988). 5ml aliquots of cells were grown up overnight and the cells spun down in an MSE18 High Speed centrifuge at 7000g for 10 minutes. The bacterial pellet was resuspended in an equal volume of the induction media supplemented with phenolics as desired. Appropriate controls were included and after overnight shaking at 27°C

Reporter Gene Expression in C58C1 Rif(pVK257, pUCD1187).

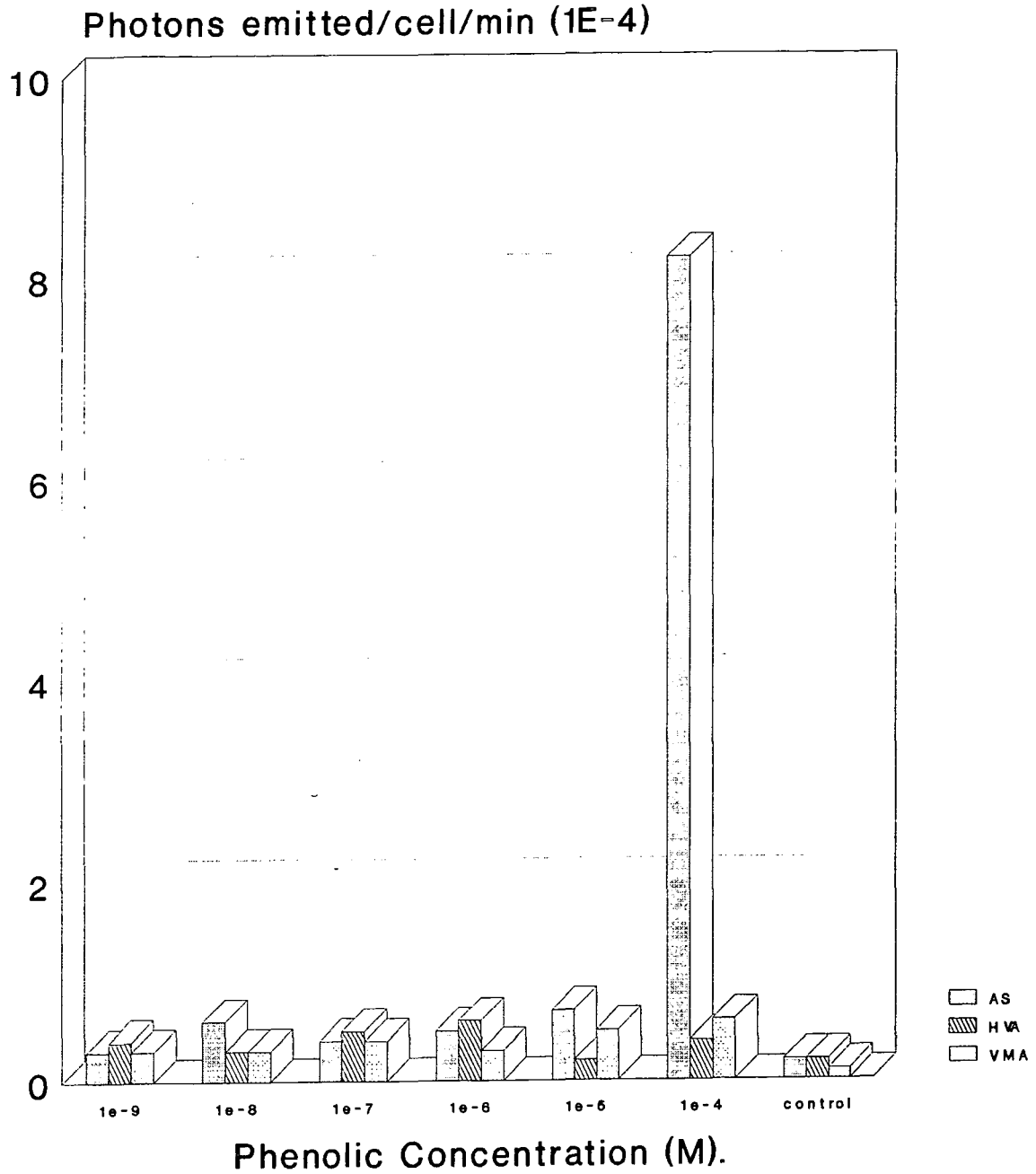


Figure 3.4.1.A

measurements made, allowing calculation of light emission as before. Experiments were performed in duplicate. Data from these experiments are presented in figure 3.4.1.C and indicate the medium devised by Rogowsky *et al* (1987) produces the highest levels of reporter activity. These results compare well to those of the previous experiments and indicate that the use of the modified protocol leads to slightly higher levels of induction.

Concurrently with these experiments the effect of these phenolic compounds on bacterial growth was studied by plotting the stationary phase culture densities of the different treatments as determined during the experiments. This data is presented in figure 3.4.1.D and illustrates the toxic nature of HVA and VMA to *Agrobacterium* cells, as increasing phenolic concentrations are more and more deleterious to cell growth. Repression of induction or competition between catecholamine metabolites and acetosyringone for a phenolic receptor site is discounted as there is no alteration of effect with concentration.

3.4.2 GUS.

A *virB::GUS* fusion in a C58C^{1}Rif (pVK257) background was kindly provided by C.S. Garrett. To test the strain and assay procedure, 5ml aliquots of cells were grown overnight in L-broth supplemented with 10^{-4}M AS or an equal volume of solvent. The cultures were assayed for cleavage of 4-MUG as in section 2.12.2 with various reaction times being employed. Longer reaction (60min) durations produced higher substrate cleavage leading to increased fluorescence, but shorter reaction periods (2min) gave a much lower background (control) signal (see figure 3.4.2.A). As result of these experiments a 4 minute period was decided upon for all future experiments.

Reporter Gene Expression in C58C1Rif (pVK257, pUCD1187).

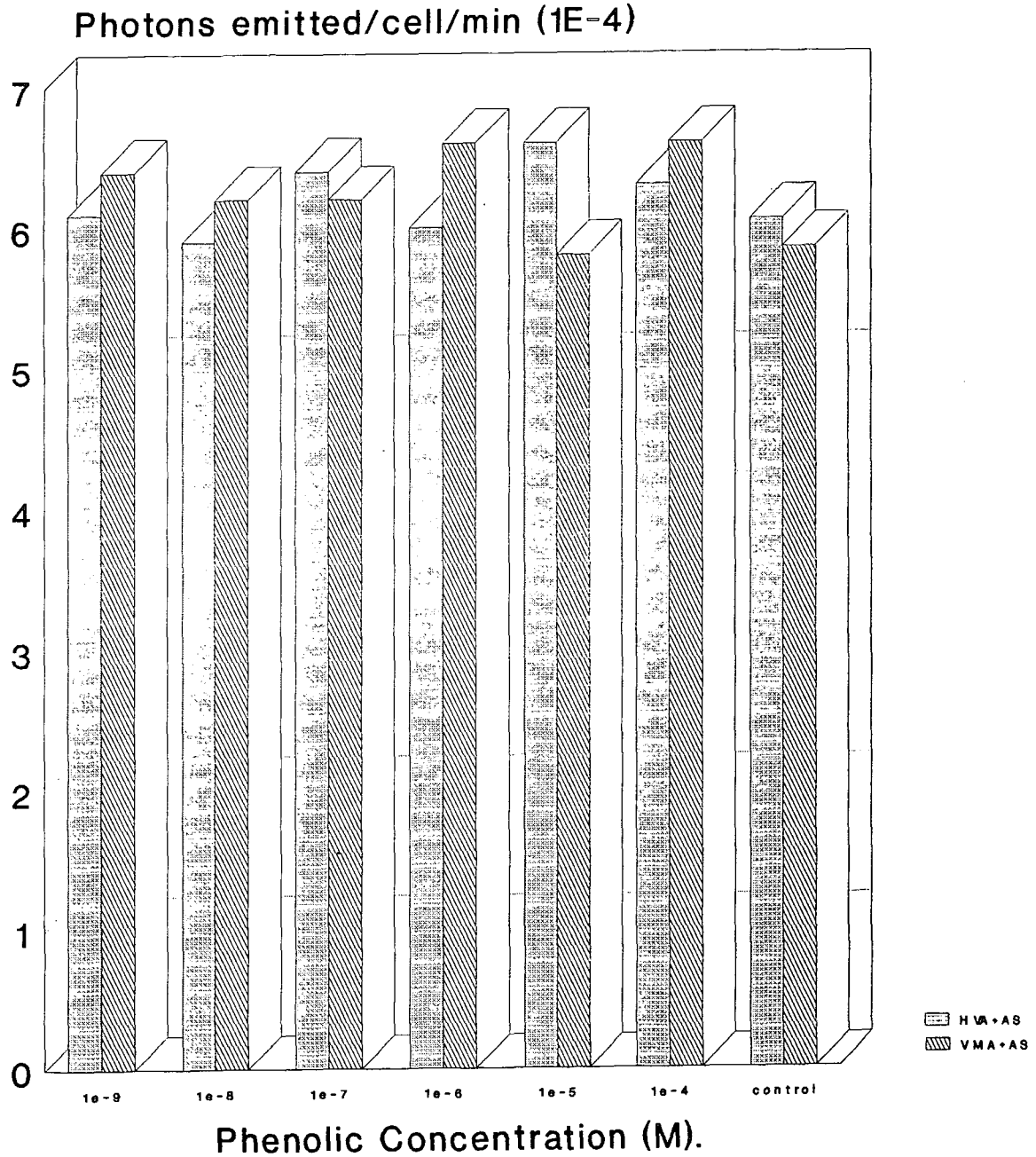


Figure 3.4.1.B

Reporter Gene Expression
in C58C1Rif (pVK257, pUCD1187).

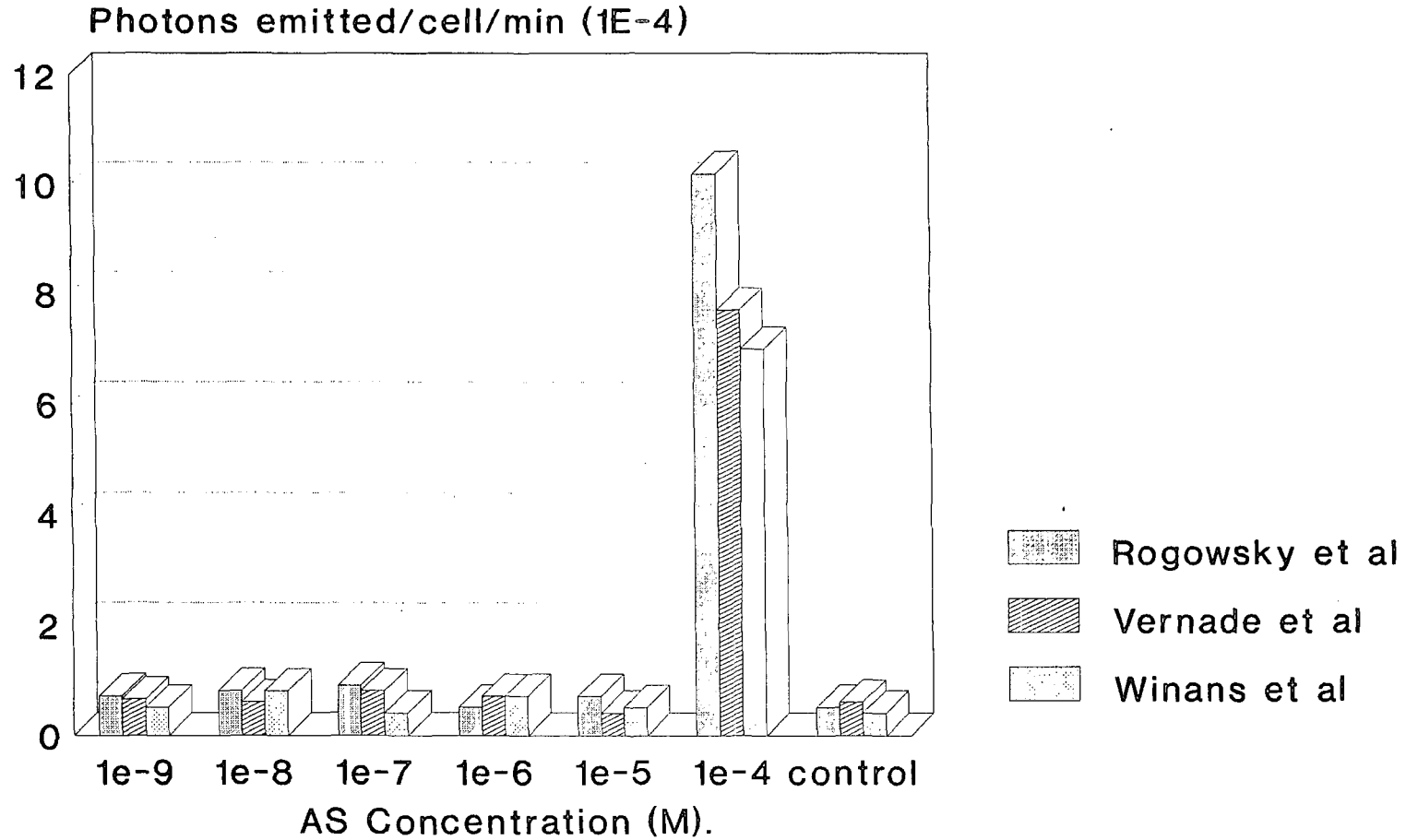


Figure 3.4.1.C

Stationary Phase Culture Density.

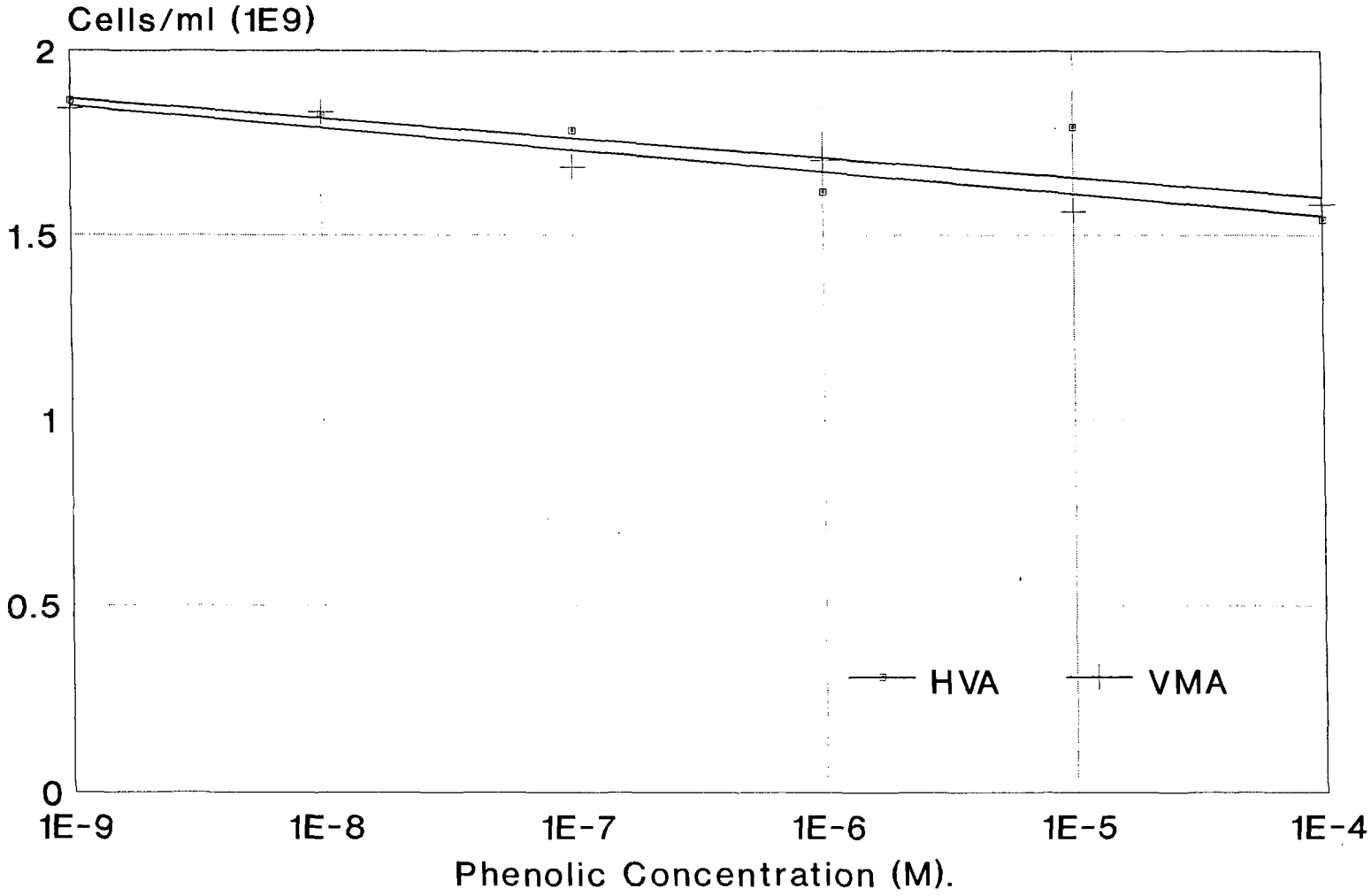


Figure 3.4.1.D

Figure 3.4.2.A.

60 minute incubations.
+ AS -AS
2 minute Incubations.

Figure 3.4.2.B.

10^{-4}M ..... 10^{-9}M
AS
HVA
VMA

When performing more detailed experiments the assay procedure was modified as follows. 200ml selective L-broth cultures of the GUS strain and C58C^{1}Rif (pVK257) were grown overnight. The cells were spun down at 10000g for 10 minutes in an MSE 18 High Speed Centrifuge and resuspended in an equal volume of induction media (Rogowsky *et al*, 1987). 5ml aliquots of cell suspension were incubated overnight at 27°C with the desired concentrations of phenolics. Controls with an equal amount of solvent but no phenolic were included. C58C^{1}Rif (pVK257) provided a cured strain control with no inducible GUS activity. This allowed comparison of any reporter induction against the cellular background level. When assayed visually, no significant background activity was detected upon exposure to solvent controls or inducing levels (10^{-4}M) of AS. Moreover, the range of HVA and VMA concentrations used did not lead to significantlty higher fluorescence than noted in the solvent control. This suggests these compounds did not interact with *Agrobacterium* to induce the *vir* promotors (see figure 3.4.2.B). The acetosyringone positive controls shown in this figure indicate the inducibility of the GUS fussion under assay conditions.

Since the GUS system is reported as being a highly sensitive measure of gene expression (Jefferson, 1987) it was decided to try and improve upon this qualitative assay. Possible errors were thought to come from the visual estimation of enzyme activity and from differences in the extraction procedure including possible toxicity of phenolics to the cells. To overcome these problems and make the assay quantitative, fluorescence was measured in a Baird Atomic Fluoripoint Spectrofluorimeter set to 365nm excitation and 455nm emission, blanked against the solvent control preparation. Induced substrate cleavage was calculated relative to a calibration curve of cleavage product (4 MU) dissolved in stop buffer. To control for differences in efficiency of extraction of specific activity, the total protein concentration in all GUS extracts was estimated as in section 2.10. This allowed the calculation of *vir* gene induction in terms of MU production/μM/μg total

protein/min. The results of this experiment, shown in figure 3.4.2.C, net activity being calculated by the subtraction of solvent control activity from experimental figures. These results illustrate that acetosyringone is a strong inducer at 10^{-4}M concentration, other AS concentrations are less potent. The range of HVA and VMA concentrations tested do not lead to significant *vir* induction above that of the control. Once again, to detect possible synergism or repression of induction by catecholamine metabolites, assays were performed against a range of HVA and VMA concentrations with and without supplementary 10^{-4}M AS. This graph shows that within the sensitivity of the assay employed the concentrations of HVA and VMA used do not interact with the *Agrobacterium vir* gene induction regulatory elements to cause any response when compared to the solvent controls (see figure 3.4.2.D). For each catecholamine concentration an appropriate solvent control was included, allowing calculation of net activity as before. The lack of detected synergysm or competition between the catecholamine metabolites and acetosyringone is comparable to the *lux* experiment, as are the slightly lower overall results due to the increased volume of solvent.

3.4.3 lacZ.

The plasmid pSM30 encodes a *virB::lacZ* fusion. The *lacZ* gene encodes an *E.coli* ß-galactosidase enzyme. Miller (1972) describes an ultra-sensitive assay for ß-galactosidase. With long incubation times this protocol is accurate enough to detect differences in enzyme activity caused by as few as 10's of enzyme molecules (M.D. Watson, personal communication).

Assays were performed as in section 2.12.3. C58 was used as a cured strain control, having no inducible *lacZ* contruct but providing an estimate of background uninduced ß-galactosidase activity. A348 (pSM30) was incubated with the same concentration of solvent as in experimental assays but no phenolic as a negative control for induction by assay conditions.A similar negative control was set up with

ß-Glucuronidase Activity Assays.

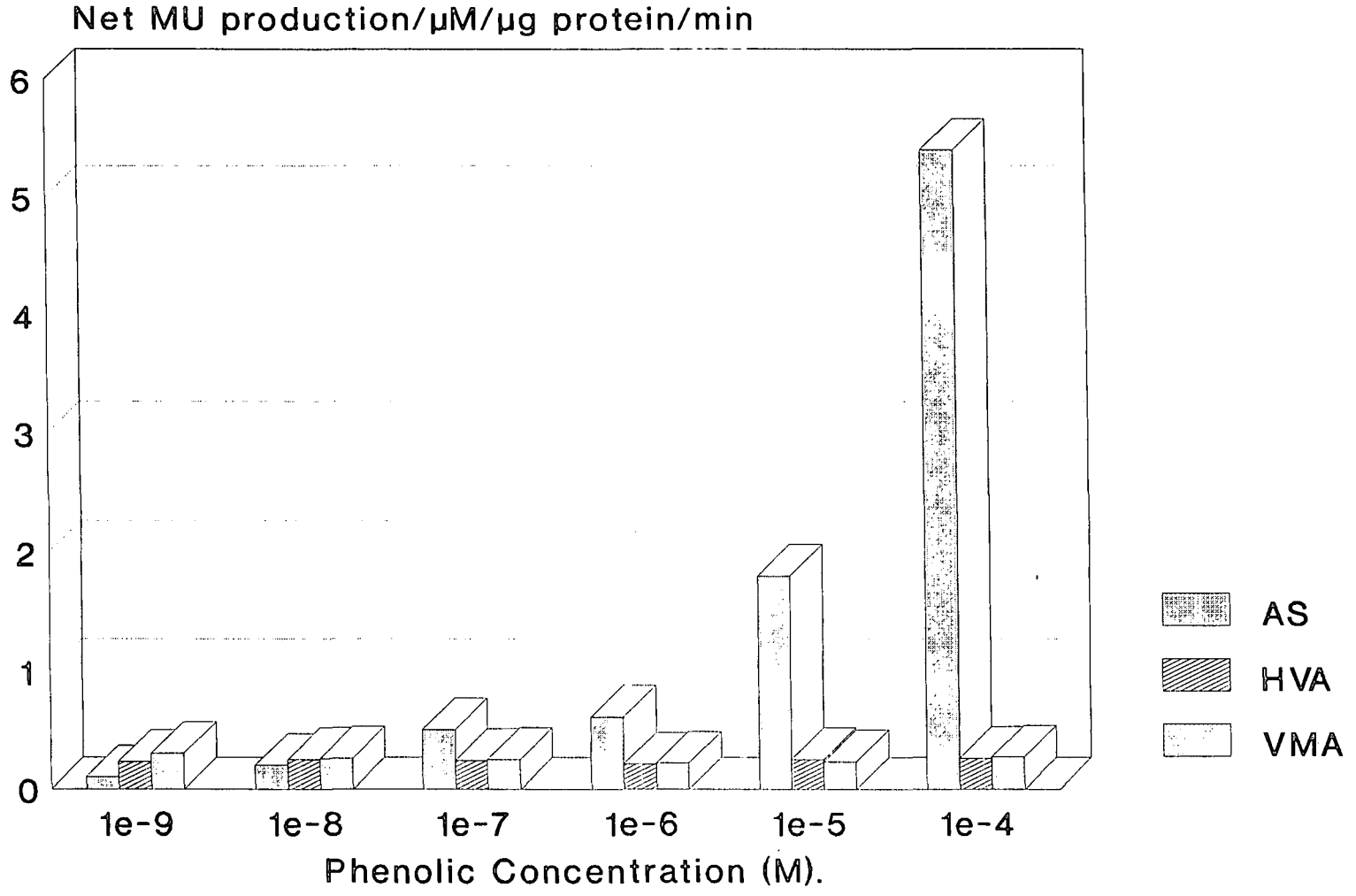


Figure 3.4.2.C

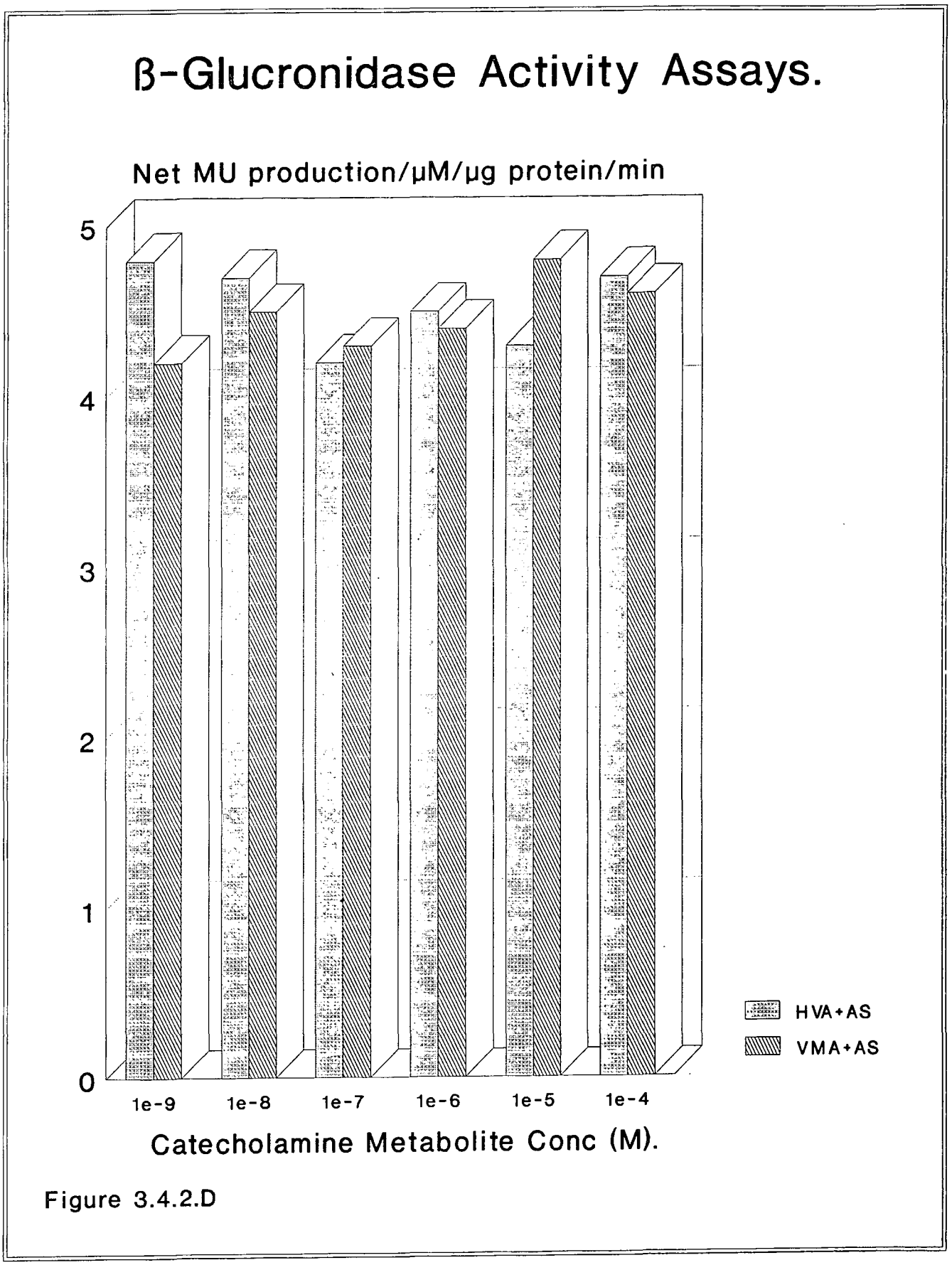

β-Glucronidase Activity Assays.
Net MU production/µM/µg protein/min
5
4
3
2
1
0
1e-9
1e-8
1e-7
1e-6
1e-5
1e-4
Catecholamine Metabolite Conc (M).
HVA+AS
VMA+AS

Figure 3.4.2.D

the cured strain C58. As positive controls the cured strain and A348 (pSM30) were incubated with inducing concentrations of acetosyringone (10^{-4}M). The results of these assays are presented in figure 3.4.3.A and indicate that treatment with catecholamine metabolites does not cause an induction response above that of the solvent control (bar A). In comparison with the positive control (bar D) substrate cleavage in experimental assays is small, but somewhat higher than that of the cured strain (bars B+C). This small but detectable cleavge is probably due to a low background level of reporter expression by the non induced *vir* promotor since it does not appear to vary significantly with either concentration or the phenolic used.

3.4.4 Expression Studies in Urine.

One of the objections to the proposal of an Agrobacterial screening test for neuroblastoma is that compounds present in urine other than HVA or VMA would be likely to induce *vir* gene expression. Candidate molecules would include phenolics and sugars. A further possibility would be that compounds which suppress *vir* induction would alter test reliability. To test these arguments the experiments described in section 2.17.2 were performed using the *lux* reporter gene system for ease of assay. The results presented in figure 3.4.4.A show that the procedure provided an aseptic sample and that *Agrobacterium* is capable of growing almost as well in urine as in rich media. Furthermore urine appears not to contain compounds which induce *vir* gene expression although conditions may repress induction by acetosyringone (figure 3.4.4.B), this may simply be a pH effect however (*vir* induction pH optimum 5.25). The organisms recovered at the end of the experiments were confirmed to be *Agrobacterium* by the plate test.

Reporter Gene Expression
in A348 (pSM30).

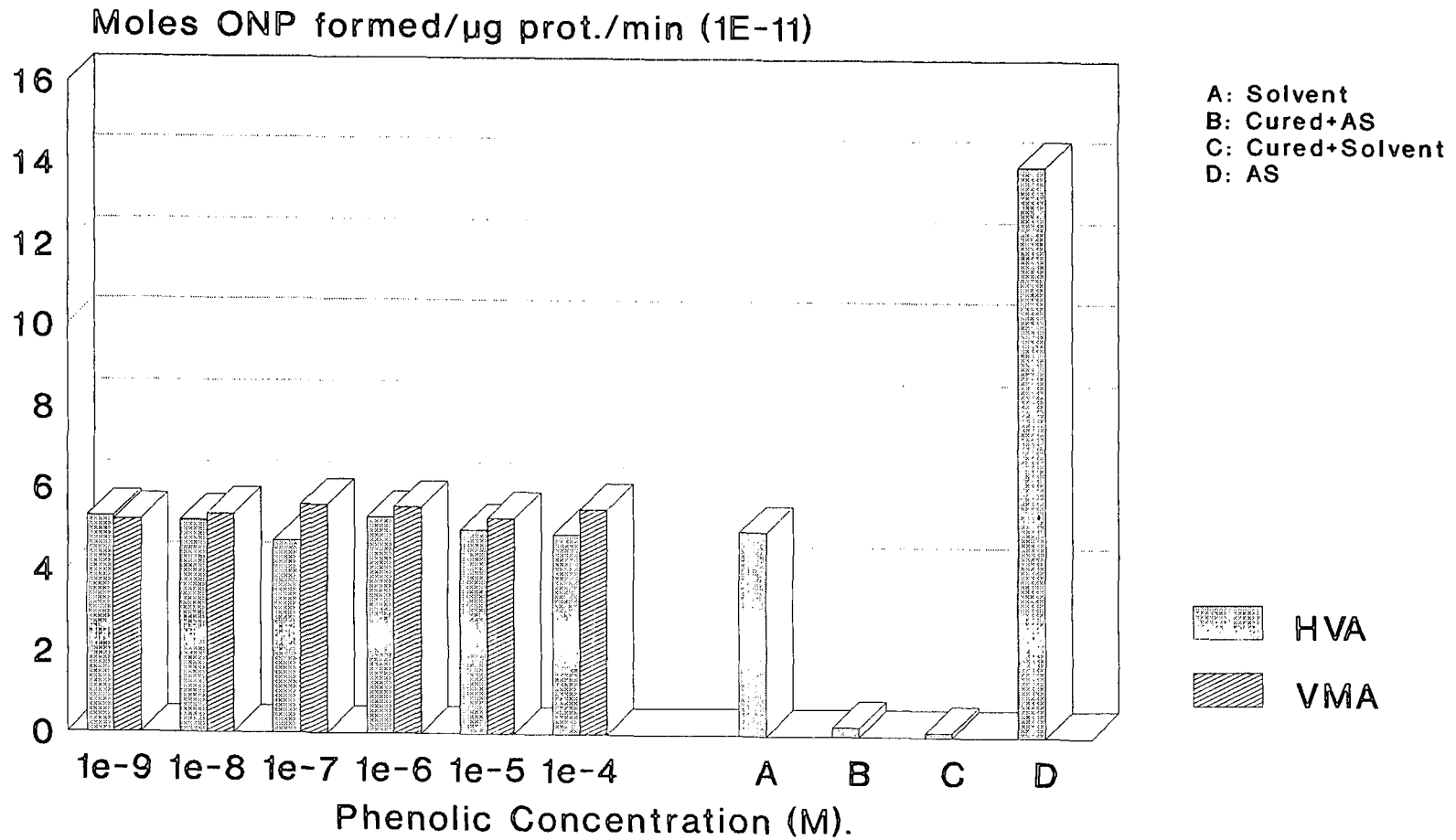


Figure 3.4.3.A

Stationary Culture Density
C58C1Rif (pVK257, pUCD1187).

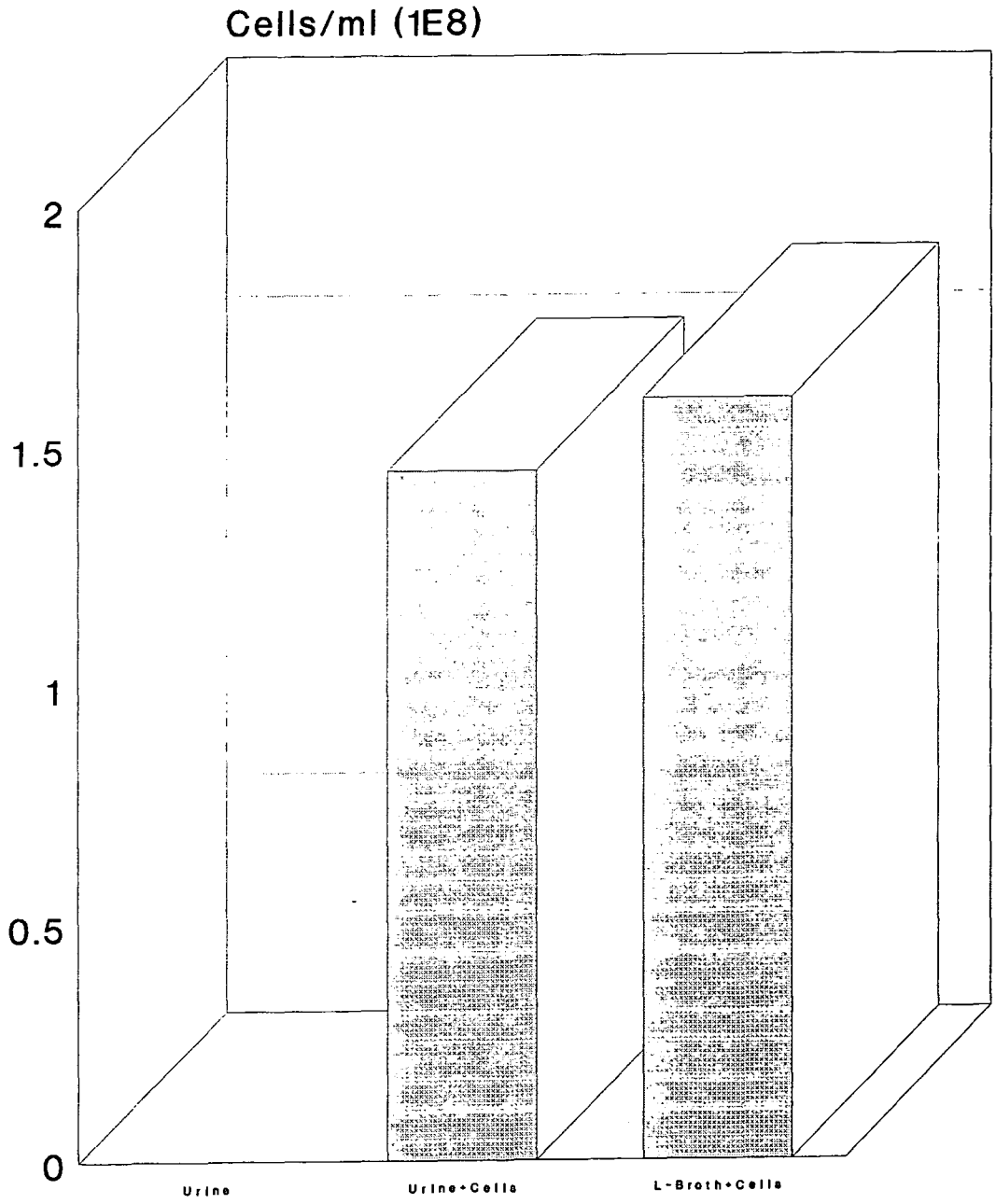


Figure 3.4.4.A

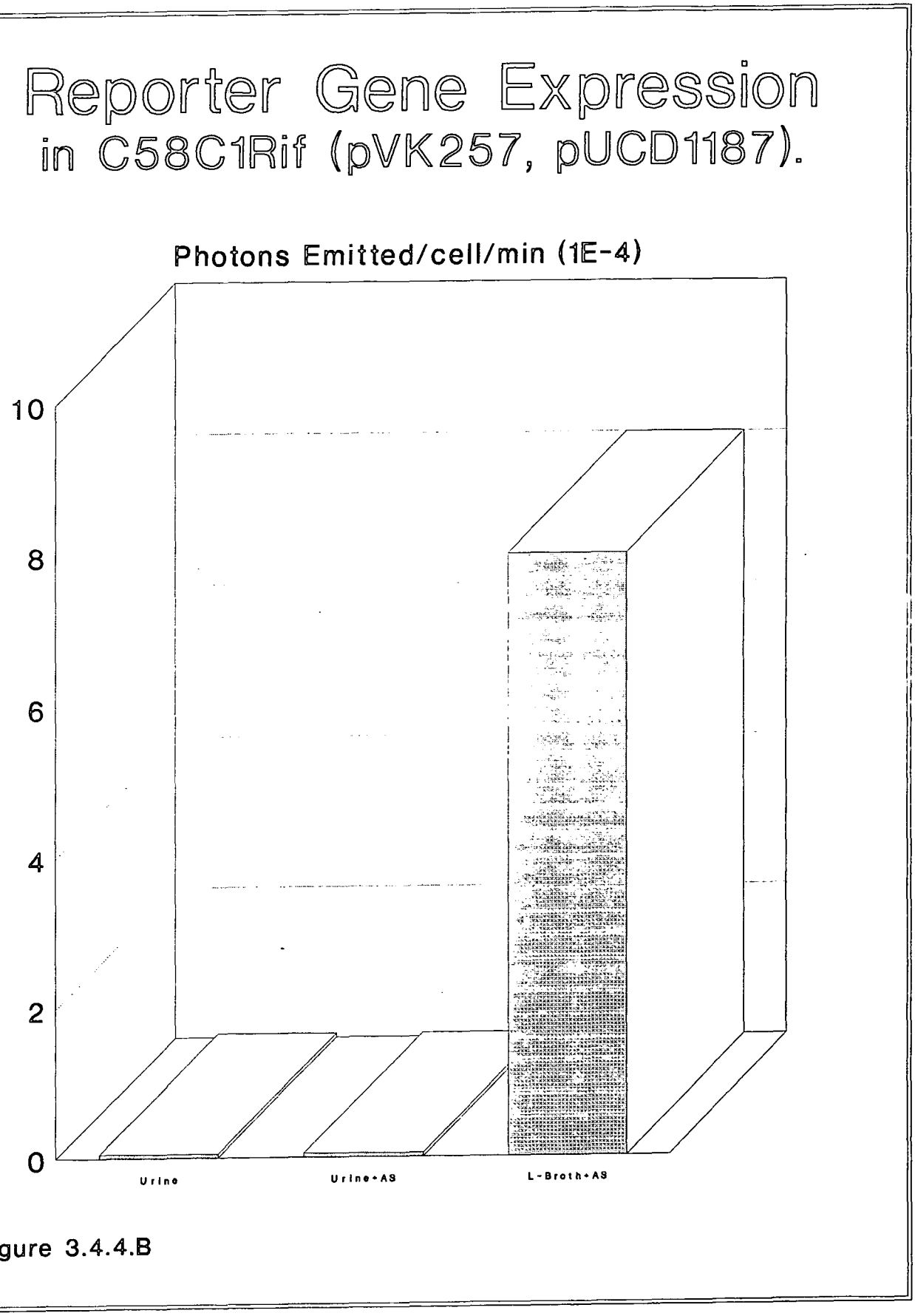

Reporter Gene Expression
in C58C1Rif (pVK257, pUCD1187).
Photons Emitted/cell/min (1E-4)
10
8
6
4
2
0
Urine
Urine+AS
L-Broth+AS

Figure 3.4.4.B

4. Agrobacterium's Chemotactic Response to Phenolic Compounds.

4.1 Introduction.

As previously mentioned, *Agrobacterium* responds to phenolic compounds in 2 ways, by gene induction and by chemotaxis - the nature of the response depending on the perceived phenolic concentration. Chemotaxis is the movement of organisms toward or away from a chemical (Adler, 1966). Having failed to detect agrobacterial *vir* induction as a result of the action of catecholamine metabolites, it was decided to see if the bacterium responded chemotactically to HVA and VMA. Positive attraction of *Agrobacterium* to AS has been demonstrated by Ashby *et al* (1987, 1988) and a number of compounds with similar structures have been shown to act as chemoattractants (Ashby *et al*, 1988).

4.2 Chemotaxis in Bacteria.

The majority of chemotaxis related genes are involved in flagella production and assembly (MacNab, 1987a). The rotation of helical flagella provides the impetus for most bacterial motion (MacNab, 1987a). The flagella itself is a complex structure, composed of many different proteins. Essentially however, there are motor and switching regions, a transmembrane basal body which incorporates structures allowing rotation and a 50-100μm filament on the outside of the cell joined to the basal body by a hook (Shaw, 1991, Armitage, 1992). In the best studied example, *E.coli*, counter clockwise proton driven flagella rotation causes the flagella filaments to wrap into a bundle, driving the bacterium (MacNab, 1987b, Armitage, 1992). However, if the perceived conditions change, cytoplasmic components interact with the flagella switch (FliG,M,N) (Stock and Lukat, 1991), altering the direction of flagella rotation (Armitage, 1992). Clockwise rotation causes the

filament bundle to fly apart and the bacterium is induced to tumble, randomly reorientating itself (Armitage, 1992, MacNab and Ornston, 1977). Counter-clockwise rotation is resumed and the bacterium sets off in a new direction. Environmental conditions change the frequency of tumbling, making it less likely if the direction of movement is favourable, more frequent if the cell travels along an unfavourable gradient (Armitage, 1992). The result of this is a tactic movement towards favourable stimuli or away from repellents (Armitage, 1992, Stock *et al*, 1989). In *Agrobacterium* the flagella are only capable of clockwise rotation, driving the cells at speeds of up to 60μm/s in runs as long as 500μm (Shaw, 1991). In this case simple stops of flagella rotation allow re-orientation (Armitage, 1992). As already mentioned *Agrobacterium* is envisaged to be chemotactically attracted towards plant wound sites prior to *vir* gene induction (Ashby *et al*, 1987, 1988). The observations of Hawes and Smith (1989) and Hawes *et al* (1988) that mutants deficient in chemotaxis are less virulent under certain conditions supports this hypothesis.

Agrobacterium has been shown to be attracted to 3 classes of plant wound exudates. Loake *et al* (1988) reported a highly sensitive attraction of the bacterium towards a range of sugars. Sucrose, an abundant plant saccharide evokes the strongest response, with an optimum concentration of 10^{-6}M. Other sugars exhibit equally low attraction thresholds (10^{-9}M) but provoke a smaller aggregation (Loake *et al*, 1988). In addition the amino acids arginine and valine act as chemoattractants (Loake *et al*, 1988). The majority of genes involved in chemotaxis are chromosomally encoded (Shaw *et al*, 1991) and *Agrobacterium's* response to these 2 classes of molecule is independent of Ti plasmid presence (Loake *et al*, 1988). This may explain the prevalence of both virulent and avirulent strains in the rhizosphere (Kerr *et al*, 1969). Attraction towards the third class of molecules, phenolics, however requires the presence of the Ti-plasmid (Ashby *et al*, 1987) and more specifically VirA and G (Shaw *et al*, 1988). This smaller response is thought to operate only in close proximity to the wound site (Shaw *et al*, 1989, Shaw, 1991) and

is therefore probably more important in guiding virulent cells to a suitable infection site once they are close to the plant roots. The strong response to sugars and amino acids benefits both virulent and avirulent cells by directing them to nutrient sources and additionally aids tumourigenesis by bringing virulent cells nearer to the wound site. The results of Parke *et al* (1987) indicate that the importance of phenolic chemotaxis appears to show some strain specificity whilst Hawes *et al* (1988) and Hawes and Smith (1989) emphasise the importance of chemotaxis in general during virulence.

4.3 Chemotaxis Information Signal Transduction and Flagella Rotation Switching.

The events associated with the perception and transduction of information, its integration and mode of action in *Agrobacterium* are not as well studied as the model systems *E.coli* and *Bacillus subtillis*. There is however a degree of similarity between *Agrobacterium* and especially *E.coli* (Shaw, 1991).

Bacteria are too small to perceive spatial gradients, thus they sample their environment temporally. Implicit and essential for this behaviour is an ability to 'remember' and adapt to conditions (Schnitzer *et al*, 1990). Furthermore since bacteria are simultaneously subjected to a wide range of varying stimuli that may be contradictory, there must be a facility for signal integration allowing a consensus response (Borkovich and Simon, 1990, Armitage, 1992).

4.3.1 Methyl-Accepting Chemotaxis Proteins.

In 1969 Adler demonstrated that chemoeffectors exerted their effect without being transported (Adler, 1969). This indirectly implied the existence of surface receptors (Armitage, 1992). In *E.coli* and closely related *Salmonella typhimurium* 4 such receptors (see table 4.3.1.A) have been identified (MacNab, 1987b) although there is also scope for receptor independent signalling (Armitage, 1992).

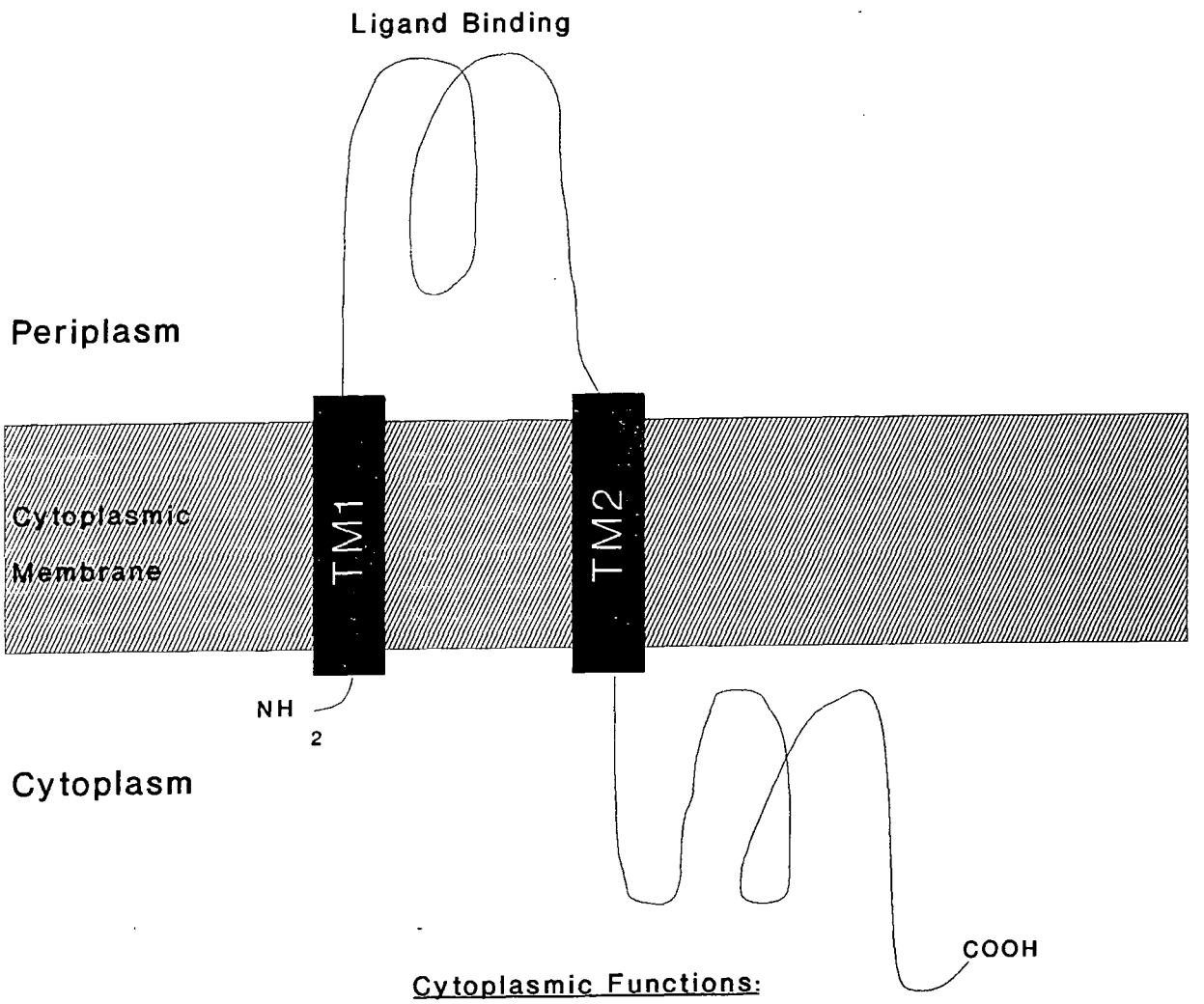
Receptor	Mediates Response to:
Tsr	Serine, temperature, pH
Tar	Aspartate, ribose
Trg	Maltose, galactose
Tap	Dipeptides

Table 4.3.1.A.

These surface receptors are known as methyl accepting chemotaxis proteins or MCP's. The response to sugars is indirect and occurs through the periplasmic sugar binding proteins of the sugar transport system (Armitage, 1992). For example Tar can interact directly with aspartate or can respond to direct binding of an occupied ribose periplasmic binding protein. The MCP's act as both receptors and signal transducers.

From sequence analysis many predictions have been made about the structure and possible mechanism of action of the MCP's (Hazelbauer *et al*, 1990) (see figure 4.3.1.B). There is strong sequence homology between the proteins and interspecific antibody cross reactivity, suggesting a common evolutionary heritage (Bollinger *et al*, 1984, Krikos *et al*, 1983). As would be expected, this homology is strongest in the cytoplasmic signalling region whilst there is greater variation in the ligand binding domains (Armitage, 1992). Each transducer has 2 membrane spanning regions, a periplasmic loop involved in response to the signal and a cytoplasmic loop which incorporates the signalling and methylation domains (Armitage, 1992). This is very reminiscent of the *Agrobacterium* VirA structure (Leroux *et al*, 1987) as already described and moreover there is a degree of sequence conservation between *virA* and *cheA* of *E.coli*, a protein in the signal transduction cascade (Stock *et al*, 1989). Furthermore there is strong homology between the cytoplasmic signalling regions of MCP's and 2 component regulator system receptors. Functional regions associated with methylation and adaption and flagella signalling/ switching are to be found in both groups (Armitage *et al*, 1992). The transducers exists as dimers in the membrane (Milligan and Koshland, 1988). VirA is

MCP Monomer Structure and Function.



Cytoplasmic Functions:

Autophosphorylation

Signalling

Methylation

Figure 4.3.1.B

therefore especially interesting since it acts both as an MCP and a 2 component regulator receptor.

4.3.2 Dynamic Protein Phosphorylation.

Four *E.coli* cytoplasmic proteins are involved in the transmission of sensory information from the MCP's to the flagella motors, namely: CheA, CheW, CheY and CheZ (Wolfe *et al*, 1987). Sensory adaptation (Goy *et al*, 1977) is mediated by two cytoplasmic enzymes, CheR and CheB which covalently modify the MCP's, thus modulating signalling (Borkovich and Simon, 1990, Parkinson, 1988) (see figure 4.3.1.B). Although this diagram is useful as a model there is evidence for the existence of MCP-CheA-CheW complexes (Liu and Parkinson, 1989, McNally and Matsumura, 1991) and it must also be remembered that MCP's form dimers *in vivo* (Milligan and Koshland, 1988).

Phosphate fluxes through a pathway involving, CheA, CheB and CheY (Borkovich and Simon, 1990, Parkinson, 1988, Shaw, 1991). CheA, an autophosphorylating protein kinase receives the *gamma* phosphate from ATP (Shaw, 1992, Borkovich and Simon, 1990, Bourret *et al*, 1991) and covalently binds it to histidine residue 48 (Hess *et al*, 1988c). Phospho-CheA passes phosphate to CheB and CheY aspartate 57 (Sanders *et al*, 1989), converting them to their active forms (Borkovich and Simon, 1990, Stock *et al*, 1989). The rate of CheB and CheY phosphorylation is dependent on ligand binding at the MCP's (Borkovich *et al*, 1989). CheY phosphate acts at the flagella switch to induce clockwise rotation and is thus known as the 'tumble factor' (Stock *et al, 1989*). CheB phosphate acts to remove methyl groups from the MCP's (Borkovich and Simon, 1990). CheZ constitutively dephosphorylates CheY (Hess *et al*, 1988a,b). CheR constitutively methylates the MCP's at specific glutamate residues (Springer and Koshland, 1977). Regulation of CheA kinase activity thus indirectly alters the net amount of 'tumble factor' present in the cytoplasm and the net methylation state of the MCP's. CheW is

necessary for the transfer of information concerning ligand binding from the MCP's to CheA (Borkovich *et al*, 1989).

The binding of attractant to the periplasmic receptor region of an MCP causes a conformational change which is propagated to the membrane spanning and signalling domains of the same polypeptide (Milligan and Koshland, 1991). In the presence of CheW, the rate of CheA autophosphorylation decelerates, causing decreases in CheB and CheY activation (Borkovich *et ak*, 1989, Stock *et al*, 1989). Constitutive CheZ activity lowers the net concentration of CheY phosphate, decreasing the probability of a tumble (Shaw, 1991). Concurrently constitutive CheR activity competes more effectively with lowered CheB methylesterase activity leading to greater MCP methylation allowing adaptation to prevailing conditions and restoring the conformational change (Shaw, 1991, Armitage, 1992). Each MCP has 4 (5 in some cases) methylatable glutamate residues, 2 of which are usually filled (Park *et al*, 1990). Acquisition of these residues alters MCP conformation and favours smooth swimming (Armitage, 1992).

A more recent model proposed by Armitage (1992) allows for the existence of an MCP-CheA-CheW complex from which CheA-CheW is released following similar conformational changes resultant from less ligand binding. Released CheA-CheW is more capable of autophosphorylation and subsequentlty more CheY phosphate is produced, promoting tumbling. CheZ subsequently dephosporylates the 'tumble factor'.

In *Bacillus subtillis* the situation has been demonstrated to be slightly more complex (Bischoff and Ordal, 1992) with the main chemotaxis proteins from *E.coli* being present but playing slightly different roles. In *B.subtillis* additional intermediates are also present and MCP methylation does bring about adaptation. There is also a possibility of signal transduction by methylated intermediates although the exact differences between the 2 models are not yet clear.

The role of dynamic phosphorylation in mediating *vir* induction has been demonstrated (Huang *et al*, 1990, Jin *et al* 1990a,b,c). In these papers, the authors demonstrate that the C terminus of VirA can be autophosphorylated at histidine residue 474 and can transfer this phosphate to aspartate 52 in the N terminus of VirG, rendering it a sequence specific DNA binding protein and transcriptional activator through its C terminus (Huang *et al*, 1990, Jin *et al* 1990a,b,c, Pazour and Das, 1990a, Powell *et al*, 1989). It was thus thought interesting to investigate the role of VirA/G phosphorylation in mediating chemotaxis since it is not clear how the VirA/G system is capable of mediating both chemotaxis and *vir* induction at different concentrations of the same ligand. This is especially interesting in the light of Lukat *et al's* 1991 paper which describes the conservation of an acidic pocket containing a phosphate receiving aspartate residue amongst response regulators (Asp57 in CheY (Sanders *et al*, 1989), Asp52in VirG (Jin *et al* 1990a)). This structural similarity amongst regulators makes 'cross talk' or interaction with sensor proteins other than their normal partner possible (Aoyama *et al*, 1991, Wanner, 1992). It is also of note that CheB has a DNA binding domain although no transcriptional activation has yet been attributed to this protein (Stock *et al*, 1989). In addition little is known about the role of dynamic phosphorylation in bacterial signal transduction pathways other than in the model systems described above.

4.4 Agrobacterium's Chemotactic Response to HVA and VMA.

Initially, attempts to measure *Agrobacterium* chemotaxis towards phenolic compounds relied upon the use of attractant supplemented swarm plates (see section 2.13.1). The results were however very disappointing, and this was suspected to be because the bacteria were incapable of metabolising the compounds under study (a requisite for this tyoe of assay). Metabolism of the agar used to solidify the plates, resulting in decreased medium viscosity was taken as evidence of this (Lippincott J.A. *et al*).

Ashby *et al* (1987, 1988) and Shaw *et al*, 1988 had shown that the attractant metabolism-independent Blind Well assay was applicable to the study of *Agrobacterium* chemotaxis towards phenolic compounds. Strain C58C^1 (pVK 257) was made motile and used to test the assay, chemotaxis experiments towards acetosyringone being performed as in section 2.13.2. This strain was chosen as suitable since it harboured essential *virA* and *G* functions and because it has previously been demonstrated to be attracted towards acetosyringone (Shaw *et al*, 1988). The results of this work are shown in figure 4.4.A and illustrate a peak of positive attraction to acetosyringone at 10^{-7}M. Other AS concentrations are markedly less attractive to the bacteria, and these results correspond well to those of Ashby *et al* (1987, 1988) and Shaw *et al* (1988) who report similar response.

In a similar manner positive reproducible attraction to HVA and VMA was also demonstrated (see figures 4.4.B and 4.4.C). The most attractive concentrations of HVA and VMA attraction were 10^{-6}M and 10^{-7}M respectively. As with chemotaxis to AS other attractant concentrations were caused markedly smaller changes in bacterial behaviour. Significance should not be attatched to the actual numbers of bacteria attracted since no account of the number of cells initially introduced into the assay chambers has been taken. It is however of note that VMA causes strongest attraction at the same concentration as AS (within the sensitivity of these assays) and that a 10 fold higher concentration of HVA is required to provoke a similar response This could be taken to indicate a weaker interaction with the bacterial chemosensory system.

To test the mechanism of attraction these experiments were repeated using motile strains C58C^1 (211MxCb), C58C^1 (226MxCb) and C58C^1 (19MxCb), respectively *virA*, *virA* and *virG* Tn3HoHo transposon disrupted mutants. This data is presented in figures 4.4.E and 4.4.F (AS data shown for comparison, figure 4.4.D). Comparison of the chemotactic response of C58C^1 (211MxCb) and C58C^1 (226MxCb), carrying non functional VirA molecules to C58C^1 (pVK257) with a wild

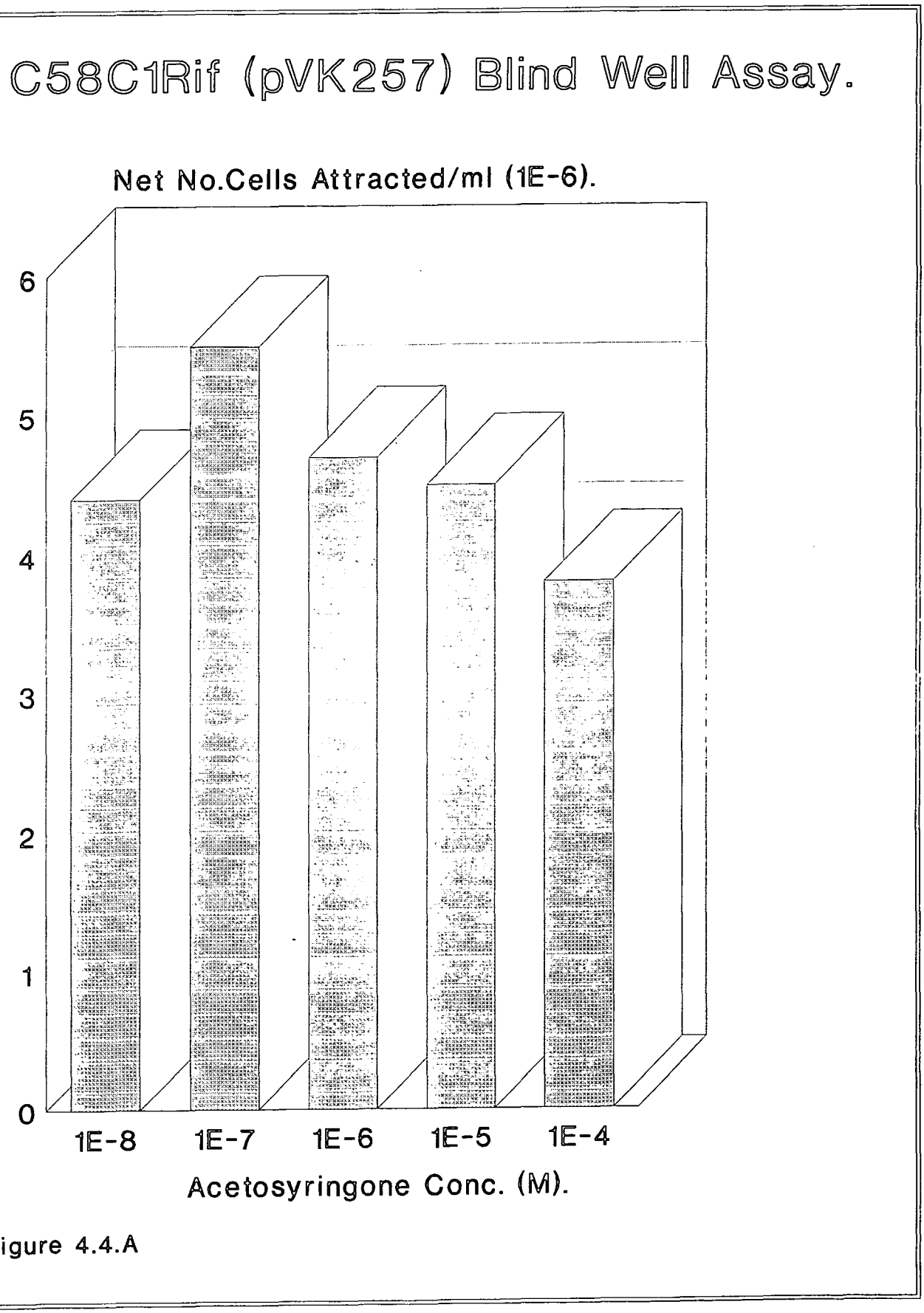

C58C1Rif (pVK257) Blind Well Assay.
Net No.Cells Attracted/ml (1E-6).
6
5
4
3
2
1
0
1E-8
1E-7
1E-6
1E-5
1E-4
Acetosyringone Conc. (M).

Figure 4.4.A

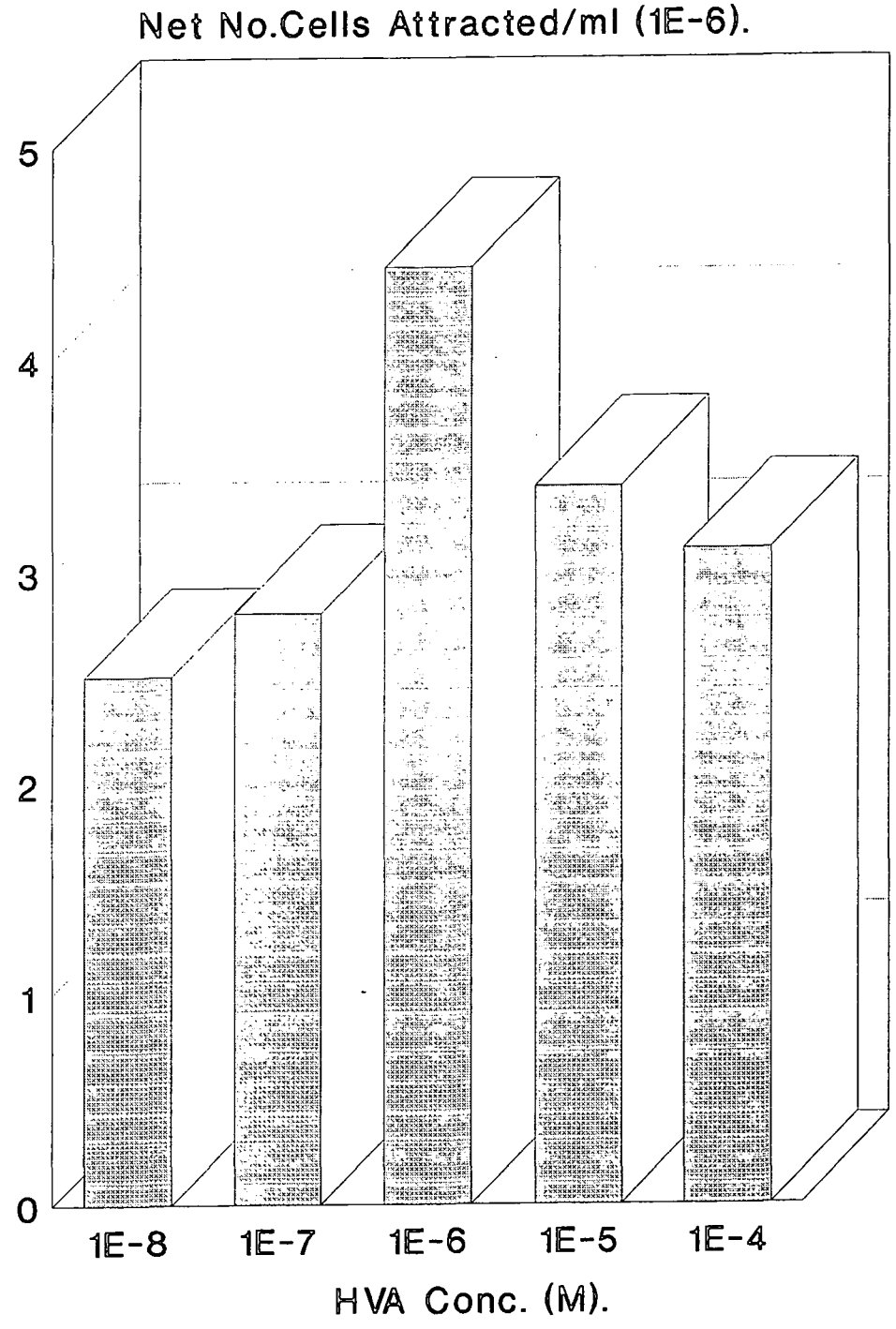

C58C1Rif (pVK257) Blind Well Assay.
Net No.Cells Attracted/ml (1E-6).
5
4
3
2
1
0
1E-8
1E-7
1E-6
1E-5
1E-4
HVA Conc. (M).

Figure 4.4.B

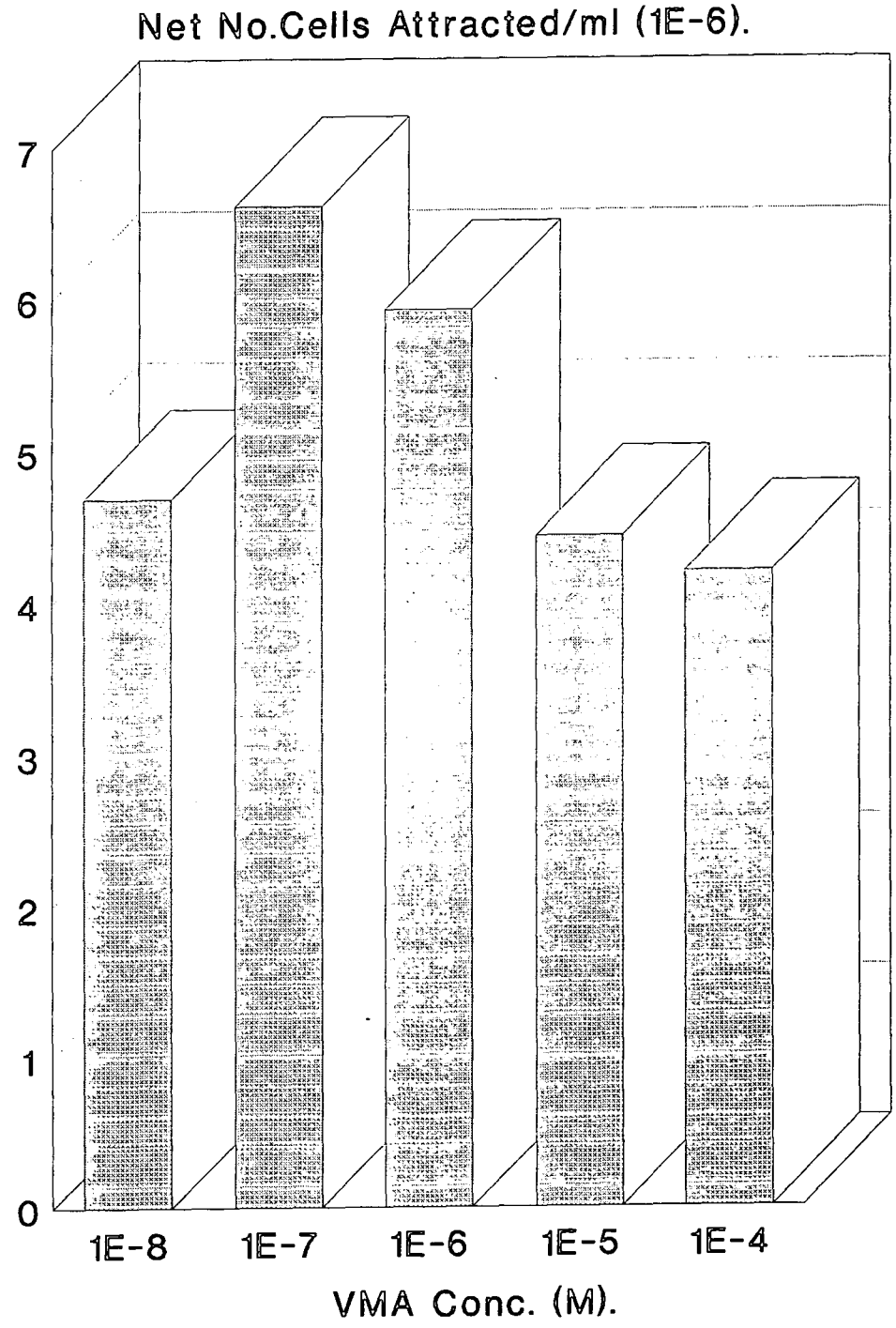
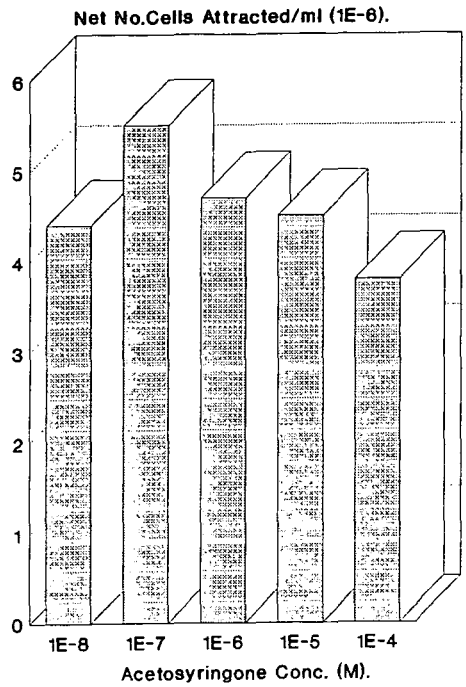
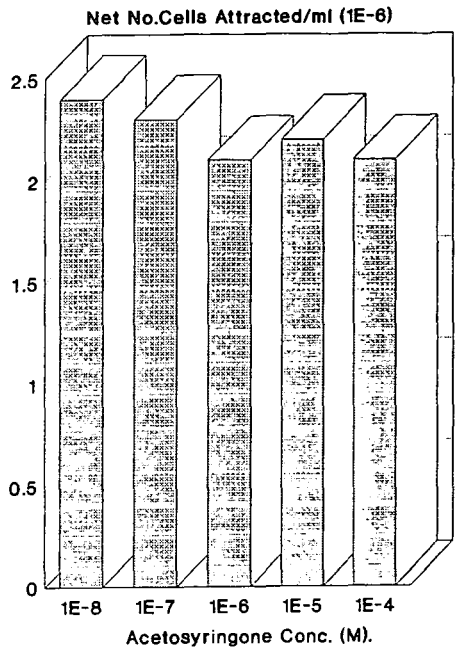

C58C1Rif (pVK257) Blind Well Assay.
Net No.Cells Attracted/ml (1E-6).
7
6
5
4
3
2
1
0
1E-8
1E-7
1E-6
1E-5
1E-4
VMA Conc. (M).

Figure 4.4.C

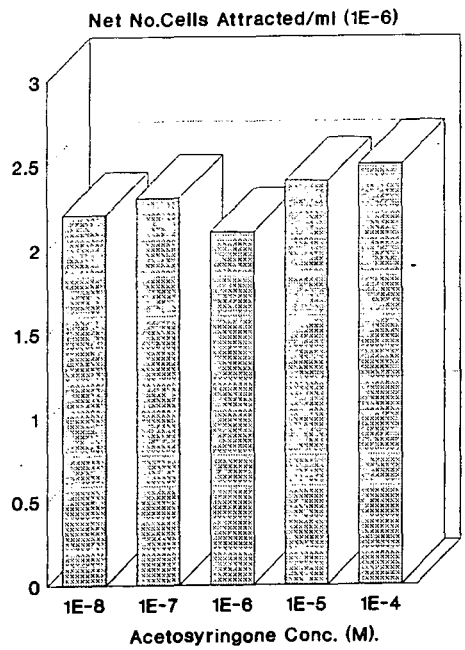
C58C1Rif (pVK257) Blind Well Assay.



C58C1 (19MxCb) Blind Well Assay. (VirG Mutant).



C58C1 (211MxCb) Blind Well Assay. (VirA Mutant).



C58C1 (226MxCb) Blind Well Assay. (VirA Mutant).

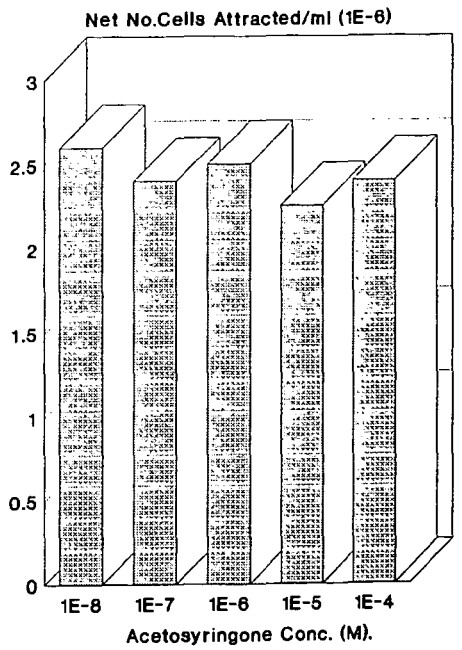
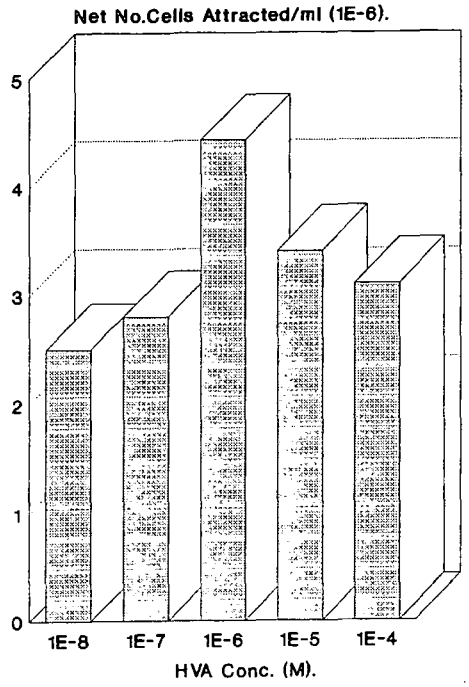
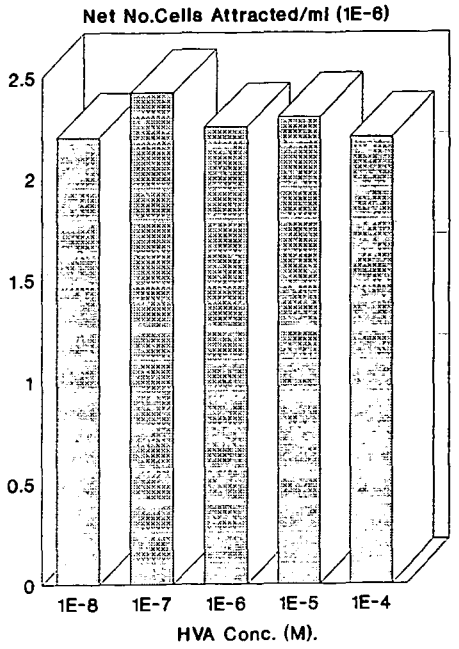


Figure 4.4.D

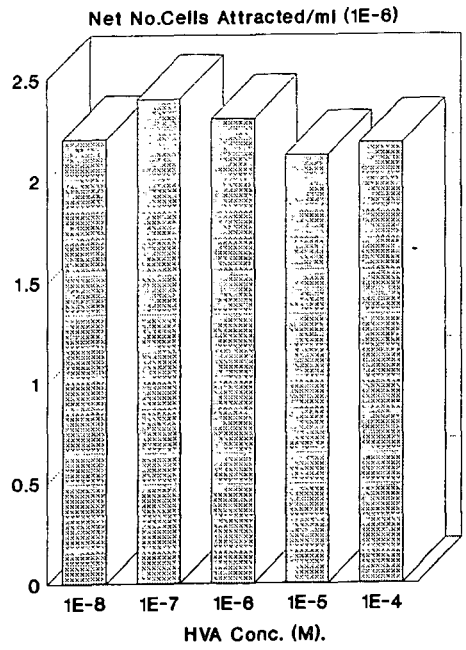
C58C1Rif (pVK257) Blind Well Assay.



C58C1 (19MxCb) Blind Well Assay. (VirG Mutant).



C58C1 (211MxCb) Blind Well Assay. (VirA Mutant).



C58C1 (226MxCb) Blind Well Assay. (VirA Mutant).

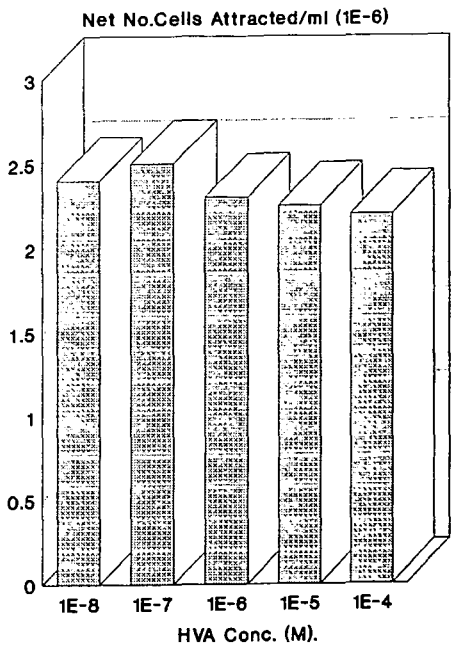
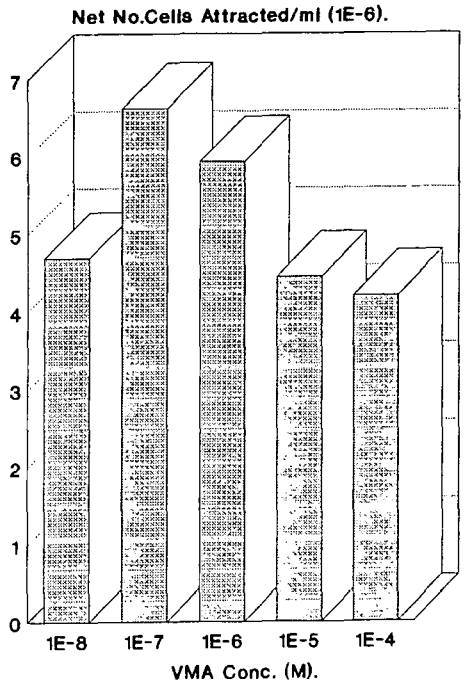
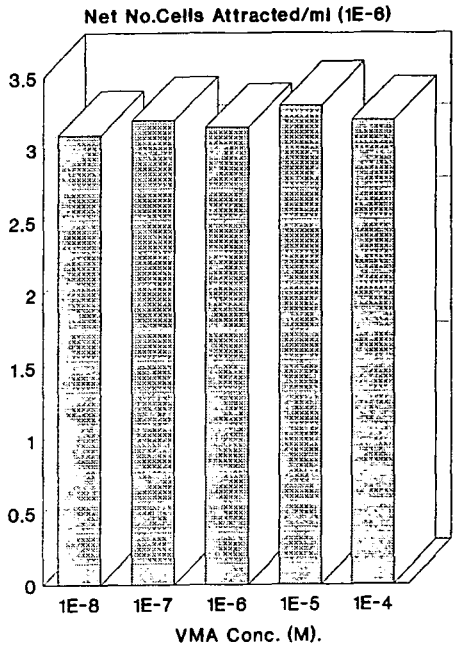


Figure 4.4.E

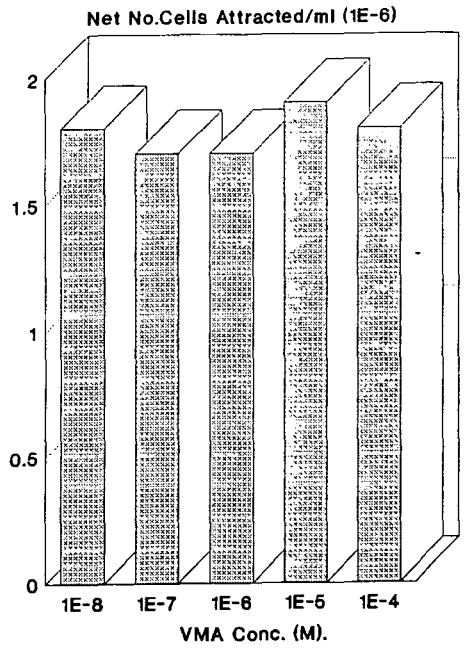
C58C1Rif (pVK257) Blind Well Assay.



C58C1 (19MxCb) Blind Well Assay. (VirG Mutant).



C58C1 (211MxCb) Blind Well Assay. (VirA Mutant).



C58C1 (226MxCb) Blind Well Assay. (VirA Mutant).

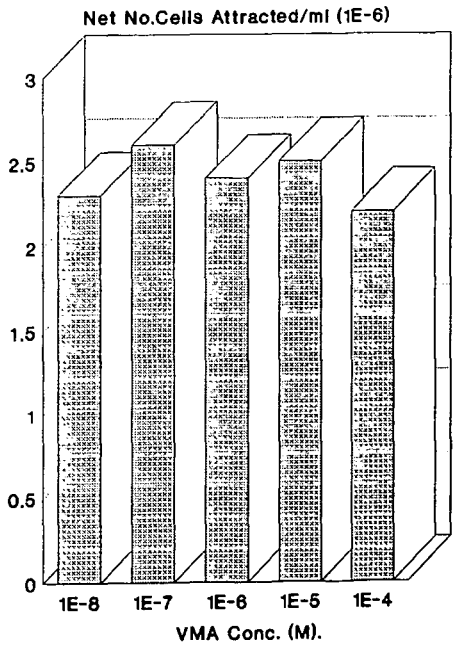


Figure 4.4.F

type clone indicate loss of chemotactic competancy. Similarly transposon insertion into *virG* causes a reduction in the chemotactic ability of C58C^1 (19MxCb) compared to C58C^1 (pVK257). It should be noted that these graphs are plotted to different scales and as before, the density of cells attracted is not significant in these experiments. The shapes of the graphs are however important, and the reader is encouraged to note the lack of a chemotactic peak in the data produced in experiments using transposon disrupted mutants when compared to the C58C^{1}Rif (pVK257) graphs.

These results imply that VirA and G function are essential in the mediation of *Agrobacterium's* chemotactic response to catecholamine metabolites. This indicates that the nature of the response is strongly reminiscent of that to acetosyringone. Furthermore this data was interesting since it demonstrates that the *virA/G* system is capable of mediating chemotaxis but not *vir* gene induction in response to HVA and VMA. The latter response presumably requires greater stimulation of the bacterial response mechanisms than the former since *vir* induction has an AS concentration peak some 1000 fold higher than that of chemotaxis toward the same ligand (see earlier). It was decided to study the role of dynamic protein phosphorylation in mediating the chemotactic response, as alteration of phosphorylation patterns has been implicated in both *vir* induction and bacterial chemotaxis (see above). In addition it is not clear how the *virA/G* system is capable of mediating both chemotaxis and *vir* induction in response to different concentrations of AS and it was felt these experiments might illuminate the subject.

4.4.1 Chemotaxis Towards Infants' Urine.

Using filter sterilized children's urine adjusted to neutral pH, response of motile *Agrobacterium* to 3 healthy childrens' and 1 neuroblastoma patient's urine was compared (see figure 4.4.1.A). All samples acted as agrobacterial attractants.

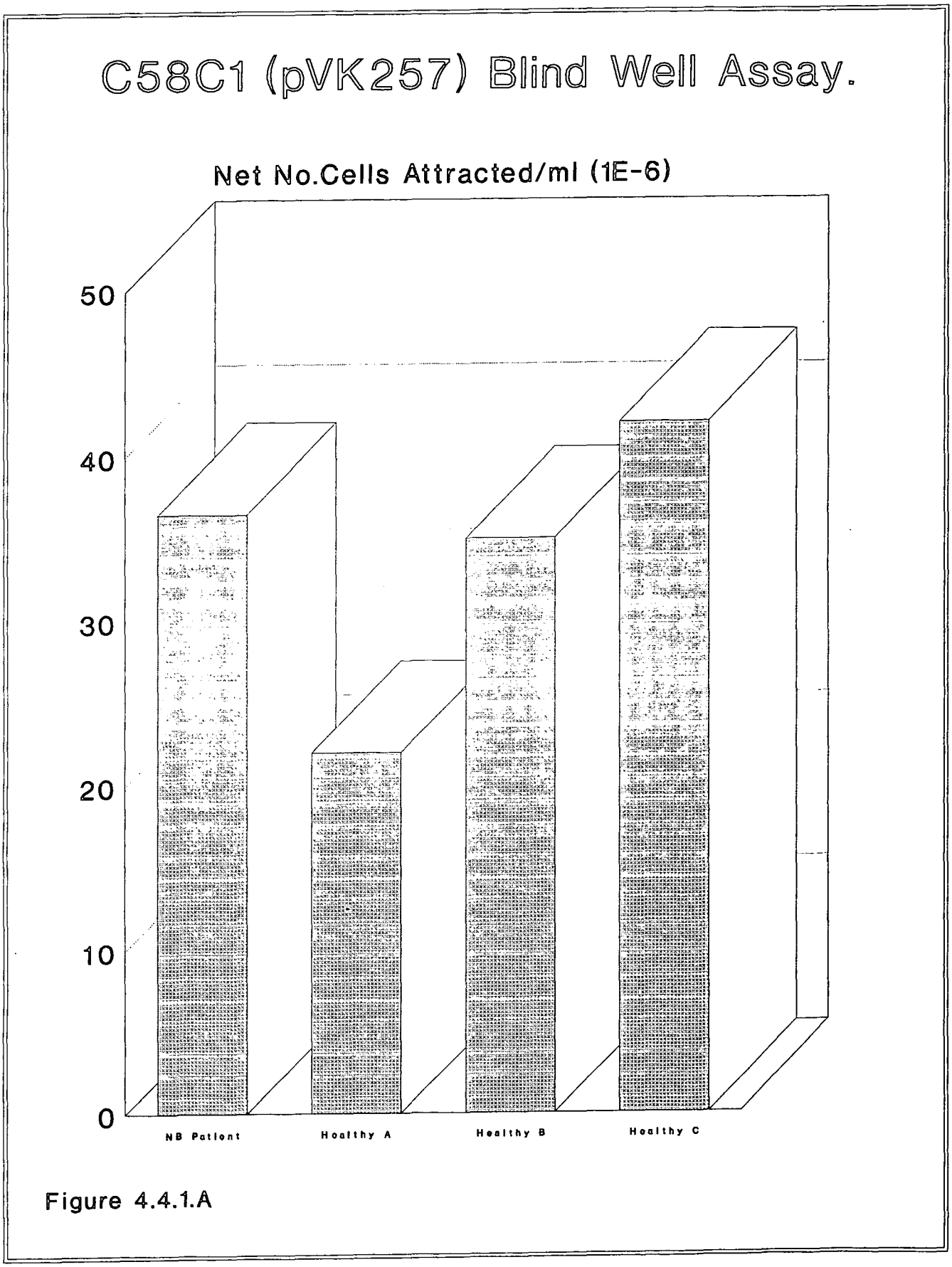

C58C1 (pVK257) Blind Well Assay.
Net No.Cells Attracted/ml (1E-6)
50
40
30
20
10
0
NB Patient
Healthy A
Healthy B
Healthy C

Figure 4.4.1.A

Nothing distinguished the response to the urine of the patient, suggesting that the bacterium was responding to some stimulii other than HVA or VMA concentration. This is largely as expected since the predominant chemotactic response of *Agrobacterium* is to sugars and amino acids rather than phenolic compounds (Loake *et al*, 1988). Samples were generously donated by J.Seviour of Newcastle General Hospital.

4.5 The Role of Dynamic Phosphorylation in *virA/G* Mediated Phenolic Chemotaxis.

4.5.1 Micro-electroporation (Palmer and Shaw, 1992).

To allow construction of the large number of strains required for these experiments a new transformation protocol was devised. Tri-parental (section 2.7.1.1) and 2 step matings (section 2.7.1.2) had proved very inefficient and time consuming to perform. Freeze thaw (section 2.7.2.1) allowed greater ease of introduction of DNA into *Agrobacterium* but the most promising method had been shown to be electroporation, especially the protocol published by Nagel *et al* (1990). This method however, was still relatively slow although it provided almost certain success. A modified version of this protocol was devised that maintained the success rate of *Nagel et al's* work whilst cutting the time required for transformation down to around 10 minutes.

Plasmids were purified from 100ml overnight cultures of *E.coli* C2110 using Qiagen plasmid midi columns. Plasmids carrying copies of *virA* and *virG* were electroporated into *Agrobacterium* backgrounds carrying cosmid clones. 5ml cultures of the recipient strain were grown overnight with antibiotic selection. 2 successive 1.5ml aliquots of cells were pelleted in the same Eppendorf tube by centrifugation for 10 seconds in an MSE Microcentaur bench top centrifuge. The sample was washed 3 times in ice cold 1mM HEPES buffer, pH7.0 and once in 1ml chilled sterile 10% glycerol. The cells were resuspended in 40μl 10% glycerol (4°C) and 3μl

plasmid miniprep DNA mixed in. The mixture was transferred to an ice cold Biorad 0.2cm electroporation cuvette and subjected to a 2.5kV pulse dissipated at 200Ω, 25μF using a Biorad Gene Pulser. Successful transformations were associated with a time constant in the order of 4.7 milliseconds. Immediately after transformation, 1ml nutrient broth was added to the cuvette and the cells incubated with shaking at 27°C for 2 hours. Following pelleting and resuspension in 100μl nutrient broth, the cells were plated out and grown with appropriate selection for transformants. Prior to use plasmid content and culture purity was confirmed as before.

4.5.2 Modifications to the Blind Well Assay (Palmer and Shaw, 1992).

The nature and complexity of the Blind Well assay made it impossible to perform all the desired experiments concurrently. It was thus felt that an attempt should be made improve the comparibilty of data gathered on different days. A new estimate of chemotaxis, the Chemotactic Index (CI) (Palmer and Shaw, 1992) was derived which corrects for differing numbers of cells being added to the chamber and for attraction/repulsion by factors other than the compound of interest. To these ends the assay procedure was modified somewhat from section 2.13.2 and would now allow comparison of the strength of responses to different treatements.

For later experiments greater numbers of cells were used. 50ml Min A + glucose cultures were grown overnight with shaking at 27°C. The cells were harvested by centrifugation for 10 minutes at 7000g in an MSE 18 High Speed Centrifuge and washed in 40, 30, 20 and 15ml chemotaxis medium before final resuspension in 10ml of the same. The density of the preparation introduced into the bacterial chamber was ascertained using a Coulter Electronics Multisizer II.

4.5.2.1 The Chemotactic Index (Palmer and Shaw, 1992).

The chemotactic index is a measure of the proportion of cells in the bacterial population attracted towards (or repelled by) a particular concentration of attractant. The CI corrects for differing initial cell density, and arises from the observation that for a given strain, the proportion of cells attracted to a particular attractant concentration is a constant, over the range of cell densities used experimentally (see figure 4.5.2.1.A).

To calculate the CI, Multisizer II readings were converted to number of cells/ml. Control figures were subtracted from each point, to give a figure for number of cells attracted solely by the phenolic compound. To correct for differences in original culture density, the data were then divided by the cell density of the suspension initially introduced into the chambers. Averaged figures were then converted to percentages of cells attracted by the compound (the CI). The CI is thus a reproducible value which permits comparison from one experiment to the next.

$$\text{C.I.} = \frac{[\text{Cells in upper chamber}]-[\text{Cells in control assay upper chamber}]}{[\text{Cells initially introduced into lower chambers}]} * 100\%$$

4.5.3 Chemotaxis Assays Using Phosphorylation Deficient VirA and G Mutants.

pSG687 and pRS0824 encode mutant VirG and VirA proteins, respectively, in which the phosphorylated residue has been altered by site specific mutation to a similar but non-phosphorylatable amino acid (see section 2.3). Neither protein can mediate *vir*-induction (Jin, *et al*, 1990a; Jin, *et al*, 1990b). pTB108 and pSG689 express wild-type VirA and VirG respectively.

To investigate the pathway effecting acetosyringone chemotaxis, the ability of these mutant proteins to confer this phenotype upon *A.tumefaciens* was investigated. Due to plasmid incompatibility, it was necessary to analyse the wild-type and mutated *virA* and *virG* (on IncW plasmids) in *A.tumefaciens* backgrounds carrying

The Linear Relationship Between the Number of Cells Introduced to Blindwell Assay Chambers and the Number Attracted to AS.

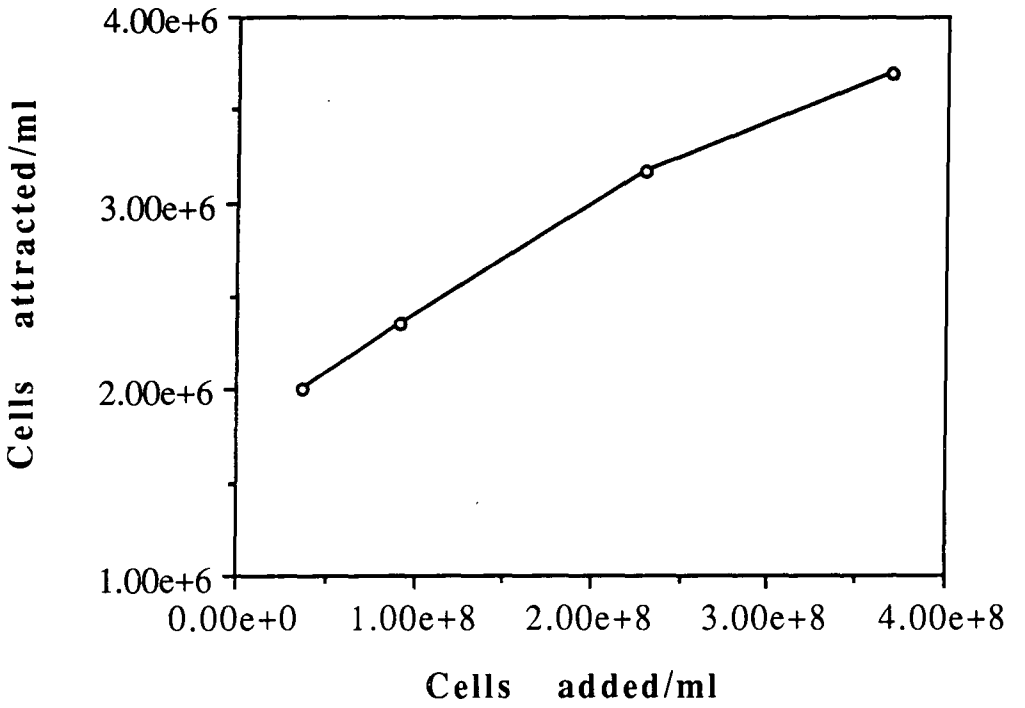


Figure 4.5.2.1.A

vir-gene combinations on the IncP cosmids pVK219 (*virA, B*) pVK225 (*virG, C, D, E*) and pVK257 (*virA, B, G, C*). Plasmids were introduced into these backgrounds by micro-electroporation. Motile populations of bacteria were then selected by swarm plate, and assayed for chemotaxis by modified Blind Well Assay against several acetosyringone concentrations.

4.5.3.1 The Requirement for VirG Phosphorylation in Mediating Chemotaxis.

Figure 4.5.3.1.A depicts the results of acetosyringone chemotaxis experiments, utilising *A.tumefaciens* harbouring either pVK219 (*virAB*) or pVK257 (*virABGC*) alone, and strains harbouring both pVK219 and pSG687 (*virG*D52N) or pVK219 and pSG689 (*virG*). In this and the following figures, error bars indicate one standard deviation from the mean. As before pVK257 could confer chemotaxis to acetosyringone, with a peak of attraction at 10^{-7}M. The fact that pVK219-harbouring cells lack this response, shows that *virA* alone is insufficient to mediate chemotaxis to acetosyringone. However, the introduction into pVK219-harbouring cells of pSG687, demonstrates that the non-phosphorylatable mutant of VirG, is not able to complement this deficit, when compared to the wild-type VirG encoded by pSG689. This indicates that phosphorylation of VirG is necessary for acetosyringone chemotaxis.

4.5.3.2 The Requirement for VirA Phosphorylation in Mediating Chemotaxis.

Figure 4.5.3.2.A illustrates chemotaxis assays in which the test strains all harboured pVK225 (*virGCDE*) either alone, or in combination with pRS0824 (*vir*AH474Q), or pTB108 (*virA*). pVK257 data from figure 4.5.3.1.A is shown by way of comparison. In accordance with previous results (Shaw *et al*, 1988) pVK225 is incapable of conferring chemotaxis towards acetosyringone, demonstrating that *virG* alone is insufficient for this response. Addition of the non-phosphorylatable VirA (encoded by pRS0824) provides very marginal chemotactic competency compared to

Figure 4.5.3.1.A

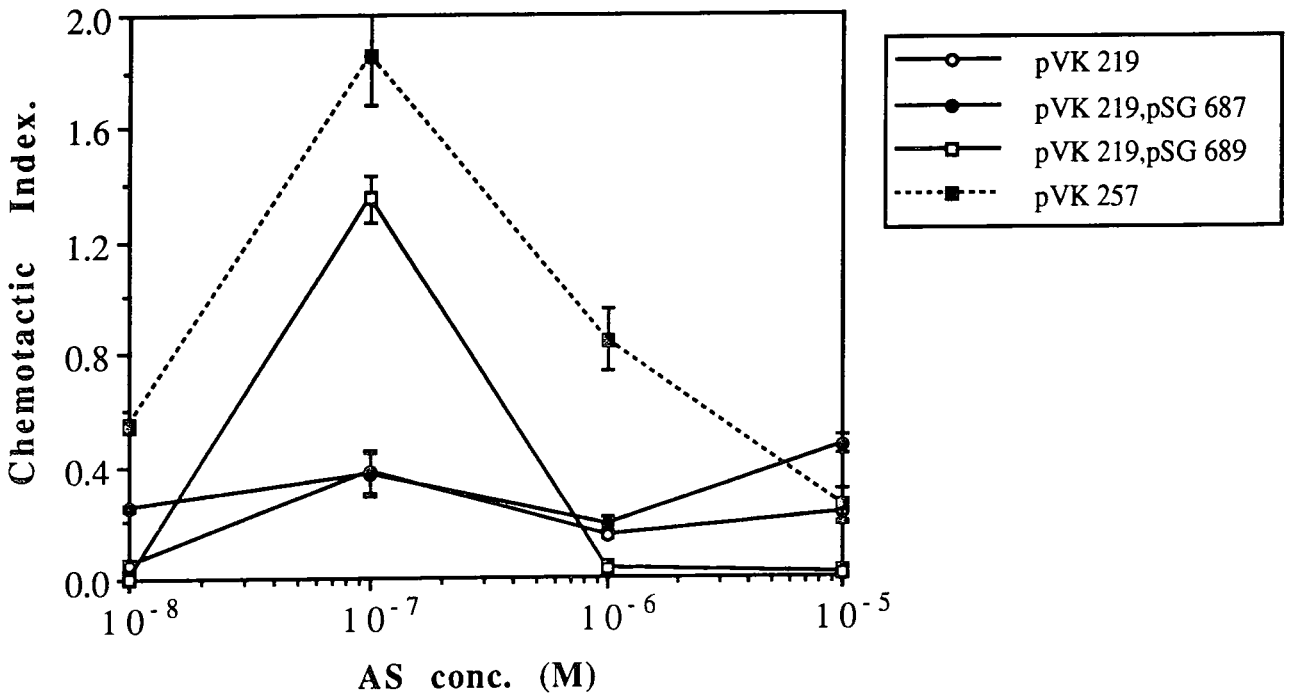
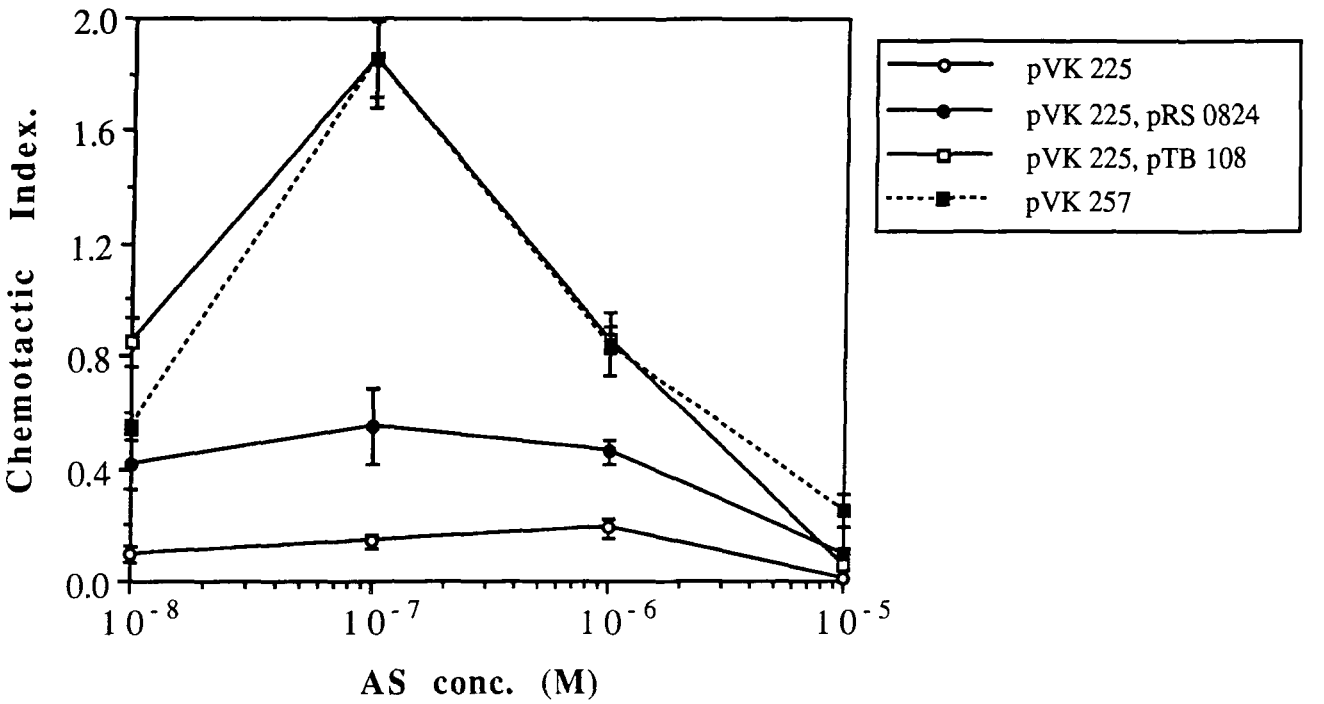


Figure 4.5.3.2.A



the wild-type situation conferred by pVK257 or pVK225 and pTB108 in combination. The non-phosphorylatable derivative of *virA* is thus incapable of complementing for the wild-type protein, suggesting that phosphorylation of VirA is required for acetosyringone chemotaxis.

In addition, the method of Dunnett (1955) was used to show that the peaks of attraction observed at 10^{-7}M AS were statistically significant (within 95% confidence limits) increases in the CI whilst the same could not be said of other treatments. Using motile C58C^{1}Rif with and without pIB100 attraction to HVA and VMA was also shown to be statistically significant (see figures 4.5.3.2.B and 4.5.3.2.C) and further confirming that the *virA* and *G* functions encoded by pVK257 were sufficient to mediate chemotaxis as described by Shaw *et al* (1988). It is also of note that HVA provokes a stronger chemotactic response (max. C.I. 1.2) than VMA (max. C.I. 0.45) but that the most atractive concentration of the former is 10 times higher than that of the latter.

4.5.3.3 The Interaction Between Wild Type and Mutant 2 Component Regulatory System Molecules.

From figure 4.5.3.3.A it is apparent that the addition of mutant copies of VirA and VirG (encoded by pRS0824 and pSG687 respectively) to a background harbouring their wild-type counterparts (encoded by pVK257) decreases chemotactic competency. Using half-logarithmic increments, it appears that the addition of mutant copies of VirA and VirG in a wild-type background cause a shift in the chemotaxis optimum towards higher attractant concentrations (see figure 4.5.3.3.B). This figure determines the optimum AS concentration for *Agrobactrium* chemotaxis more accurately than previosly published estimates (Ashby *et al*, 1988, Shaw *et al*, 1988) at $3.16*10^{-8}$M. As the *vir* genes on these plasmids are expressed from their normal promoters, it is to be expected that wild-type and mutant proteins would be transcribed at equivalent levels. This suggests

Figure 4.5.3.2.B

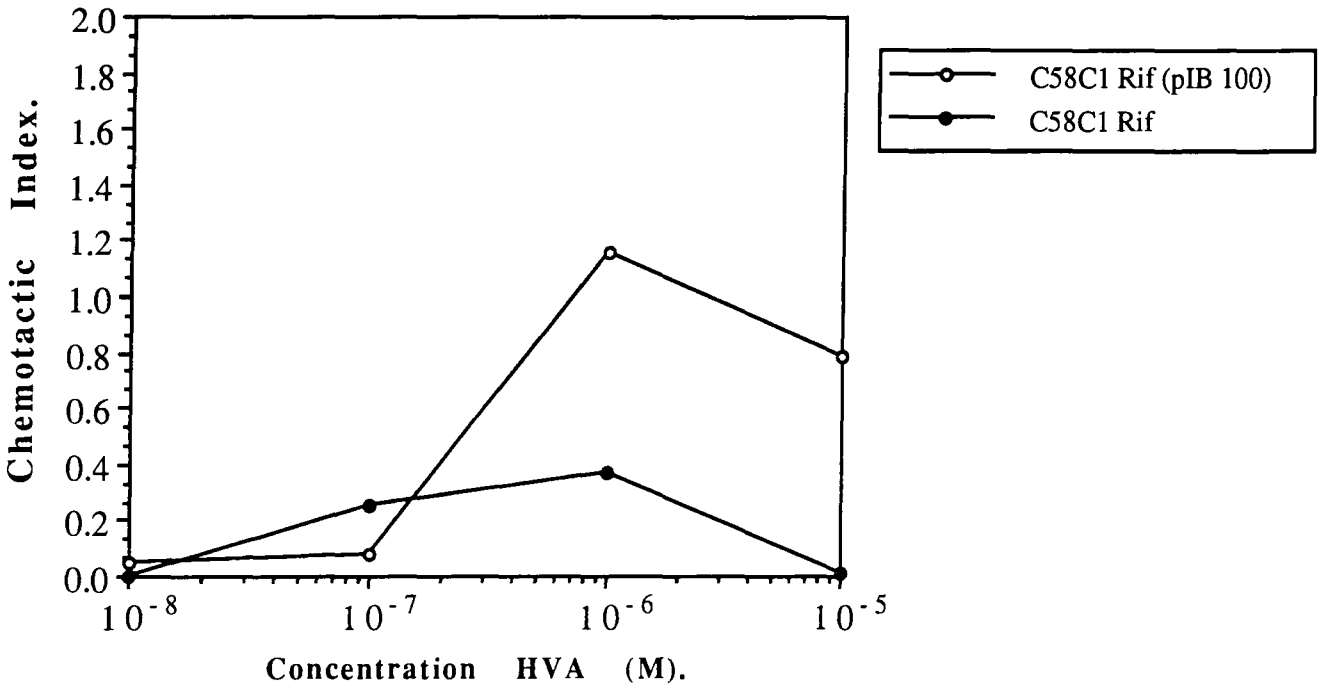


Figure 4.5.3.2.C

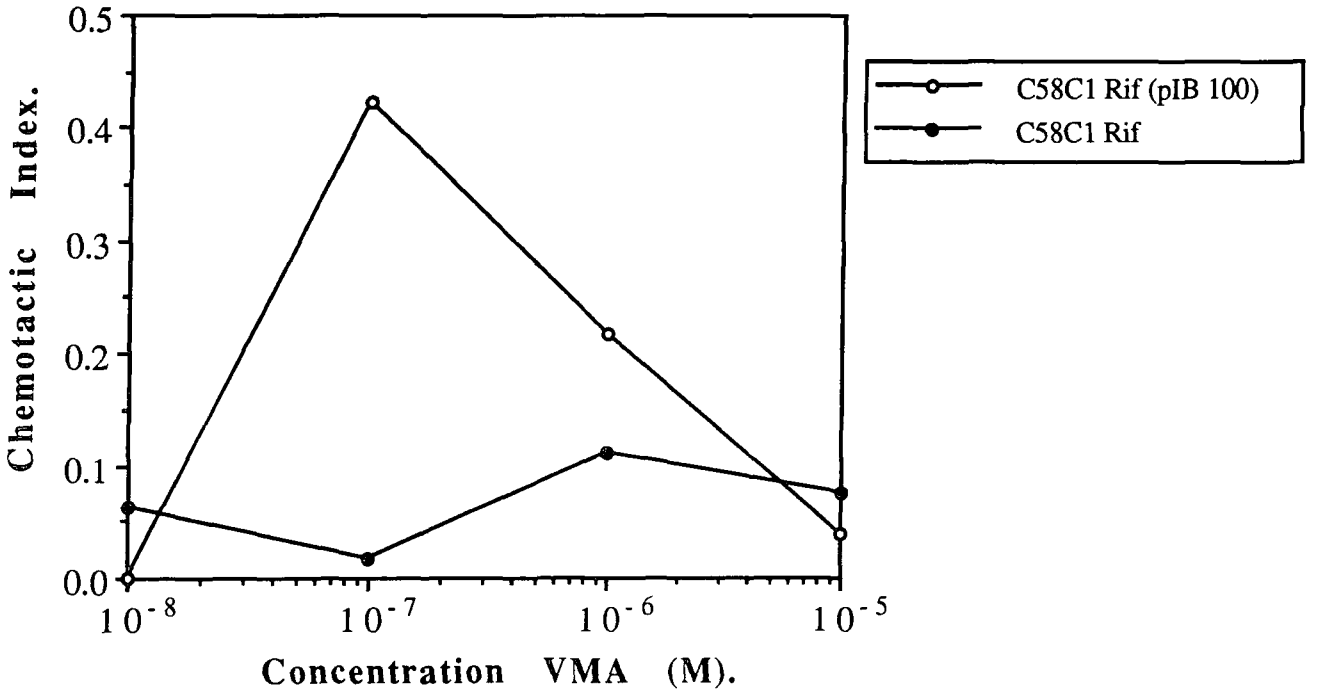


Figure 4.5.3.3.A

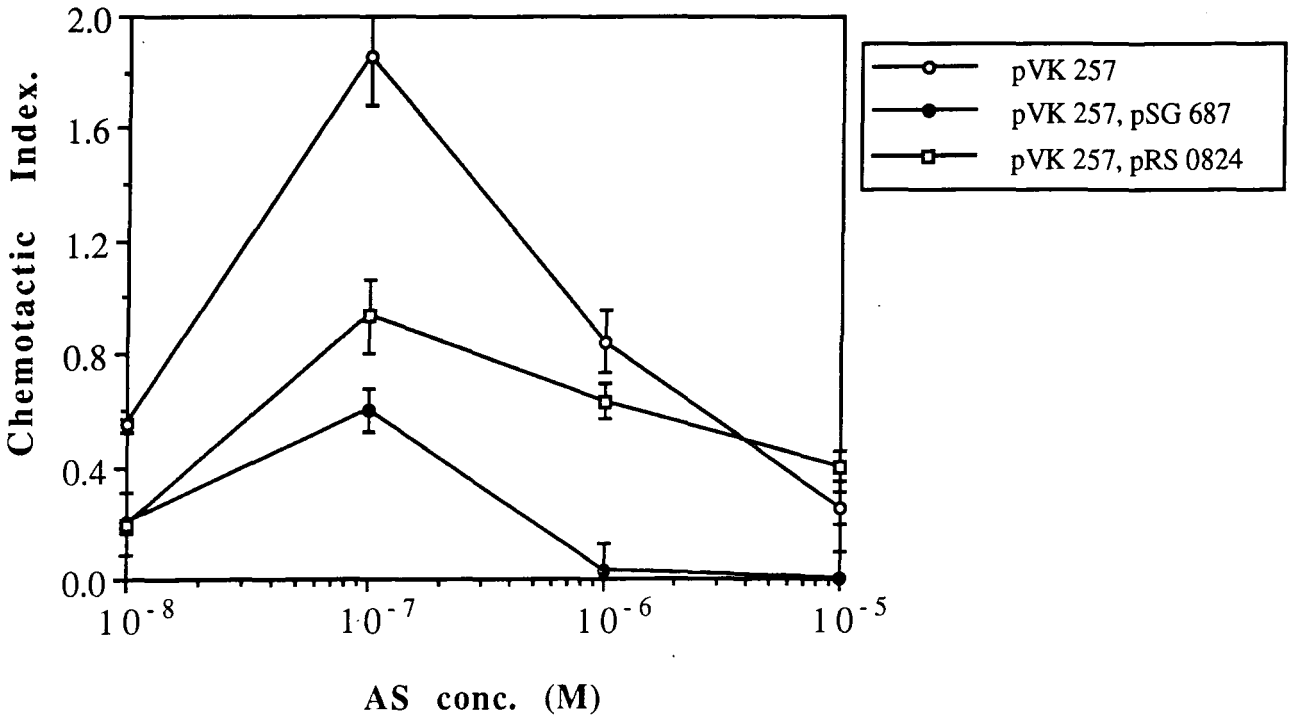
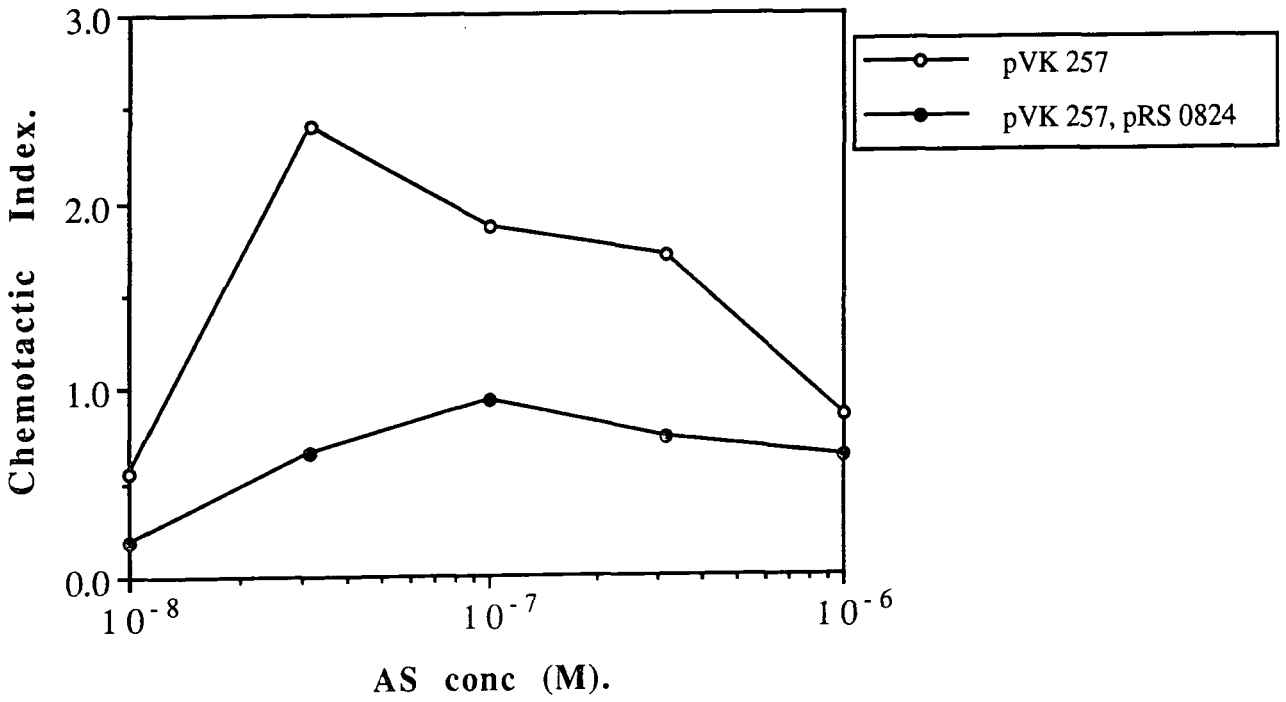
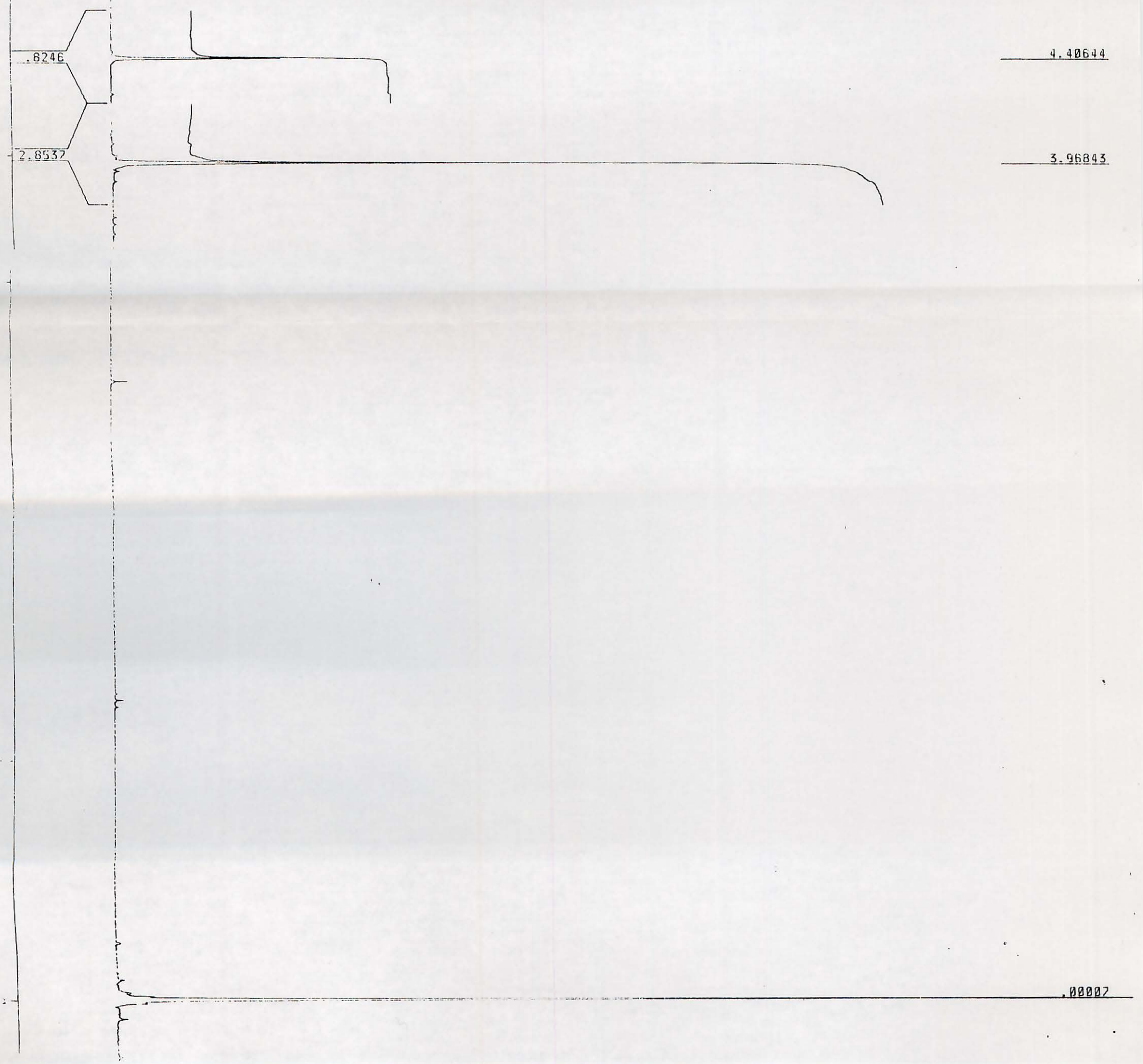


Figure 4.5.3.3.B




.8246
2.8532
4.40644
3.96843
.00007

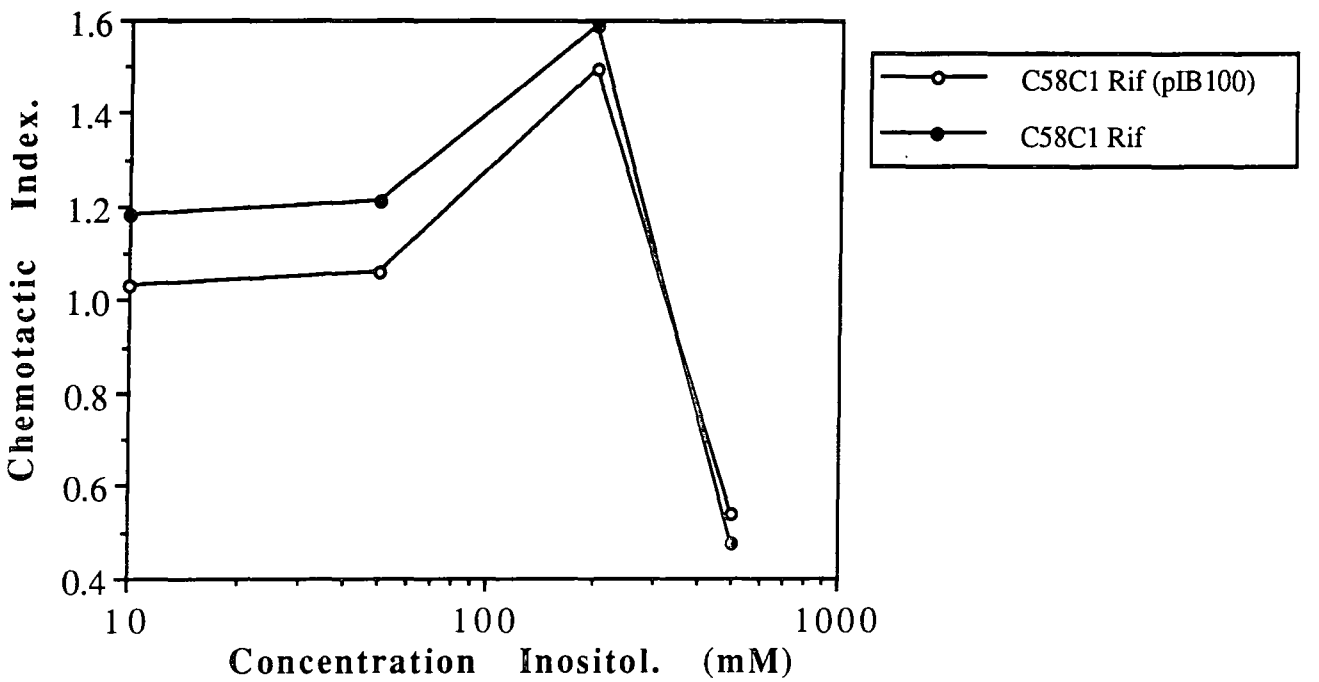
that the mutant copies of VirA and VirG interfere with normal signalling in a wild-type background.

4.6 The Effects of *vir* Induction Inhibitors and Saccharides on Phenolic Chemotaxis.

Bromo-acetosyringone is a potent and specific inhibitor of *vir* induction at micromolar concentrations (Hess *et al*, 1991). The molecule is thought to bind covalently and irreversibly to the AS receptor site (Hess *et al*, 1991, D.O'Hagan, personal communication). 2.4g of AS-Br was synthesized as in section (2.18). This is equivalent to a 34% yield. TLC showed the reaction had gone to completion, the halogenated phenolic being more polar and thus more mobile when run on a chromatogram than the starting material. The melting point of the compound produced was 125°C, somewhat higher than the 118-120°C reported by Miksche implying the sample produced in this study was either purer than that reported by the previous author or that the wrong product had been produced. A small quantity of the sample was taken up in chloroform and subjected to NMR spectroscopy as in section 2.18. This spectrum (figure 4.6.A) shows that the reaction had gone to completion as there is no evidence of -H bonding at 2ppm. The presence of CH_3H, H-Br, C-H bonds in a 6:2:2 ratio confirms that the product is indeed bromo-acetosyringone. The sample was run relative to a TMS standard (0 ppm).

Inositol interacts synergistically with AS and AS-OH to induce *vir* gene expression (Song *et al*, 1991a). These authors proposed that the sugar relieved some form of inhibition to gene induction, with maximal activity at a concentration of 50mM. It was thought worthwhile to study the effects of AS-Br and inositol both singly and in combination on *Agrobacterium* chemotaxis and to compare this data with the published studies on *vir* gene induction. The nature of the chemotactic response with and without pIB100 (*virA, G*), to a range of inositol concentrations was first investigated (figure 4.6.B) and indicate a C.I. of around 1.1 at 50mM

Figure 4.6.B


1.6
1.4
1.2
1.0
0.8
0.6
0.4
Chemotactic Index.
10
100
1000
Concentration Inositol. (mM)
C58C1 Rif (pIB100)
C58C1 Rif

concentration for both strains. There appears not to be a significant difference in the response of cells that expressing and not expressing VirA and G since a peak of attraction of similar size is noted at 200mM in both cases. This implies that *Agrobacterium* does not respond chemotactically to inositol via the *virA/G* system and the sugar is probably sensed by the chromosomal system reported by Loake *et al* (1988). Studies on the effect of inositol on bacterial growth followed (section 2.16.2) and indicated that *Agrobacterium* was unable to metabolize insositol. This was taken to show that any aggregation of bacteria noted in assays was a result of taxis rather than increased nutrients allowing greater cell division or random motility.

The action of ASBr was investigated, see figure (4.6.C+ key following). These results show that the nature of the bacterium's response to this molecule is strongly dependent upon the concentration. At 10^{-5}M concentration, (a concentration reported as strongly inhibiting *vir* gene induction (Hess *et al*, 1991) ASBr acts a *Agrobacterium* repellant (C.I. -0.6, bar A), whilst at lower concentration (10^{-7}M) it exhibits attractant behaviour (C.I. 0.85, bar B). When 10^{-7}M AS (strong attractant) and 10^{-5}M ASBr (repellent) are simultaneously exposed to the bacteria, replusion is observed (bar C). This is in contrast to the control situation when 10^{-7}M+ 10^{-5}M ($1.01*10^{-5}$M) AS was tested for its reaction on the bacteria (bar D). In this case mild attraction (marginally less than for 10^{-5}M AS, bar E) was noted. When studying the AS and inositol together, no synergysm of response is indicated by the combination of 10^{-7}M AS and 50mM Inositol (C.I. 1.9 (bar H)), compared to separate treatments (10^{-7}M AS, 1.8 (bar F), 50mM inositol, 1.1 (bar G)). This is in contrast to the improved induction responce when optimally inducing AS is coincubated with 50mM inositiol. Furthermore 50mM inositol does not counter the repulsive nature of 10^{-5}M ASBr (bar I, 50mM inositol + 10^{-5}M ASBr), reinforcing the idea that inositol does not interact with the VirA/G system's chemotaxis mediating functions. A possible mechanism for this effect is proposed in the discussion.

Motile C58C1 (pVK257) Blind Well Assays.

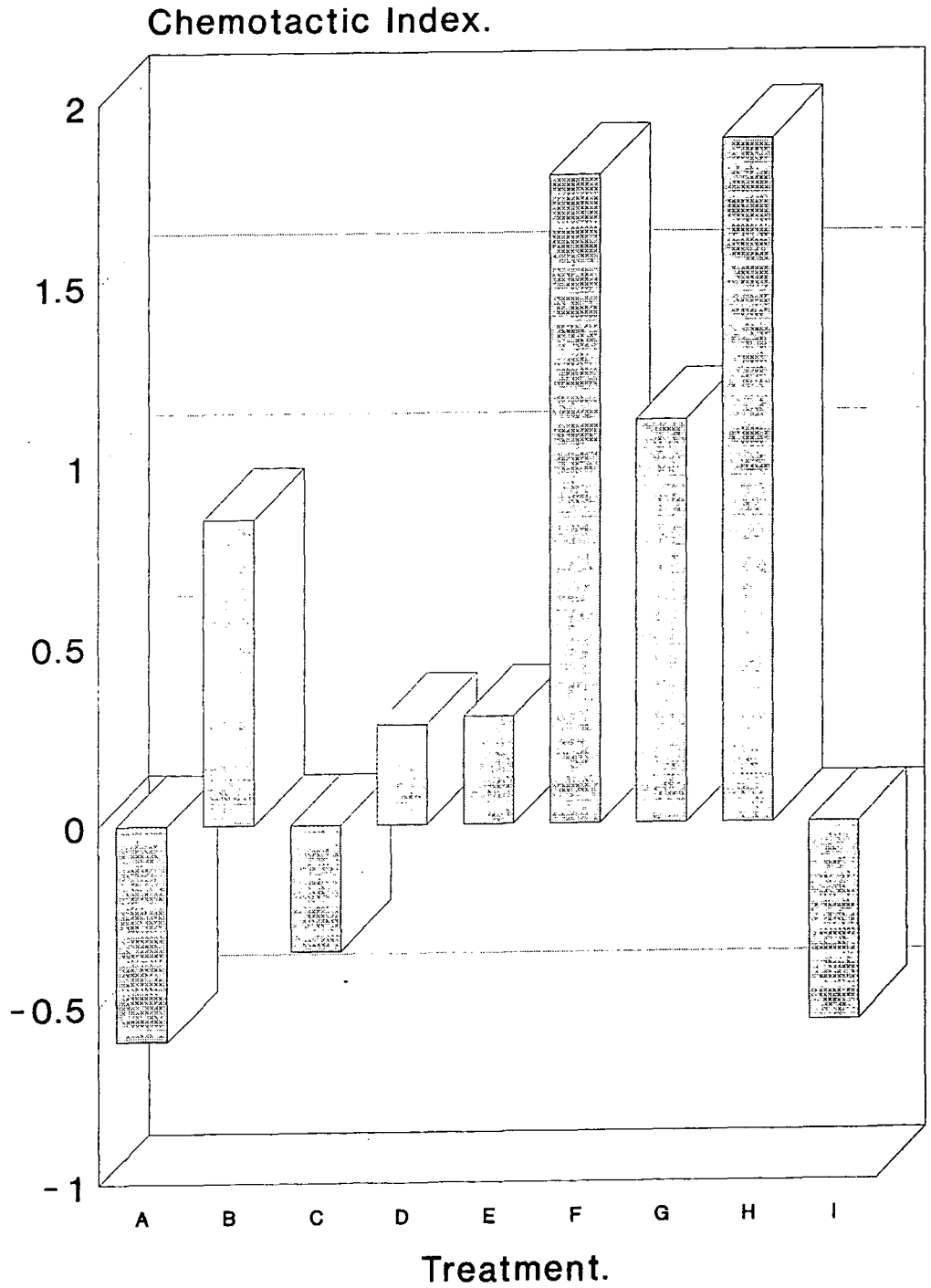


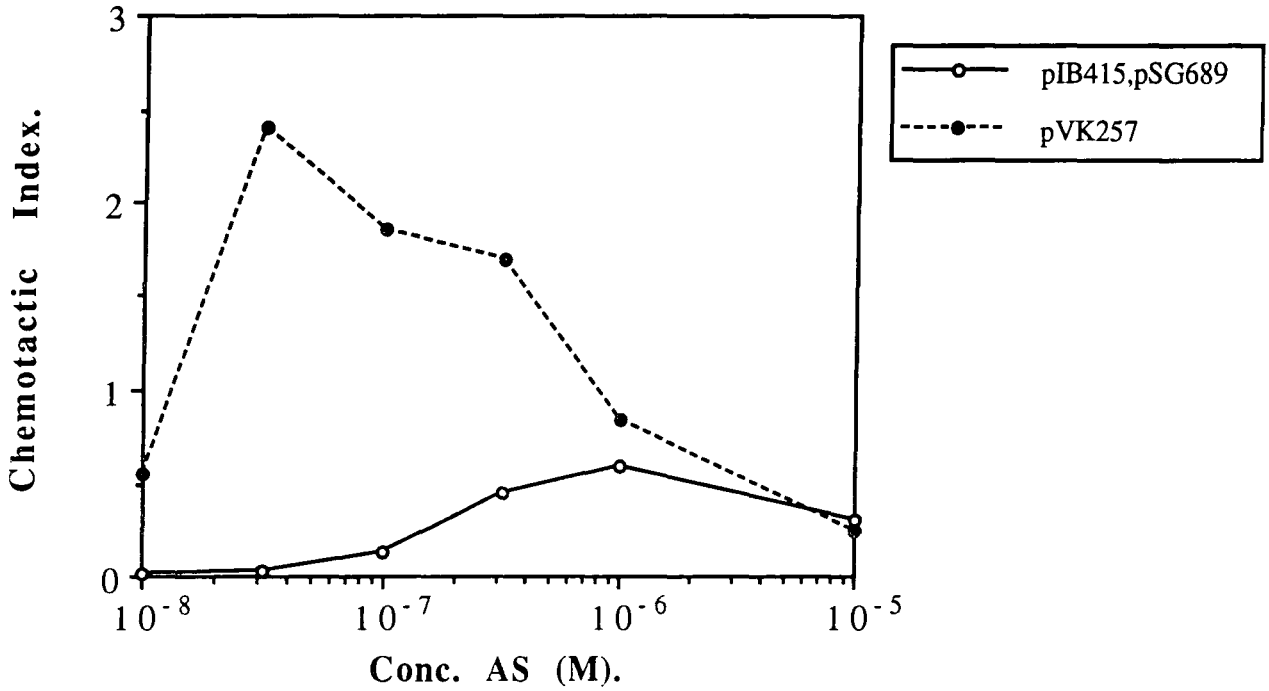
Figure 4.6.C

Key to Figure 4.6.C.	
Bar A	10^{-5}M AsBr
Bar B	10^{-7}M ASBr
Bar C	10^{-7}M AS + 10^{-5}M ASBr
Bar D	10^{-7}M AS + 10^{-5}M AS
Bar E	10^{-5}M AS
Bar F	10^{-7}M AS
Bar G	50mM inositol
Bar H	50mM inositol + 10^{-7}M AS
Bar I	50mM inositol + 10^{-5}M ASBr

4.7 The Role of the Periplasmic Region of VirA in Mediating Chemotaxis.

The ligand binding site of MCP's and most 2 component regulator system sensors has been localised to the periplasmic region (Armitage, 1992). Whilst this region is essential for VirA pH, sugar and temperature responsiveness, deletion of this region does not abolish the ability to detect phenolic compounds (Winans, 1991) and respond by *vir* gene induction (Cangelosi *et al*, 1990). As it has been shown that *virA* is necessary for AS chemotaxis (Shaw *et al*, 1988) and for sensitivity to HVA and VMA (this work) it was thought interesting to investigate the role of the periplasmic region of VirA in mediating chemotaxis. Plasmid pIB415 (*virA*▲63/240) expresses a mutant VirA protein, in which residues 63-240 (essentially the whole periplasmic domain) have been deleted. In figure 4.7.A (10/8/92), the effect of the periplasmic deletion of *virA* upon chemotaxis is shown in comparison with the data for pVK257. pIB415 is able to only partially restore chemotactic ability in concert with wild-type *virG* carried by pSG689. The peak is substantially smaller and shifted to a higher concentration, relative to the wild-type situation. This suggests that while the periplasmic domain of VirA is non-essential for *vir*-induction by acetosyringone, it is important in acetosyringone chemotaxis.

Figure 4.7.A


Chemotactic Index.
3
2
1
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Conc. AS (M).
pIB415,pSG689
pVK257

5. Improving the Sensitivity of the Bacterium's Response.

5.1 Introduction.

The results of previous experiments had shown that the *Agrobacterium virA/G* two component regulatory system was capable of detecting and responding to the phenolic compounds HVA and VMA. Unlike the 'natural' ligand, AS, exposure to catecholamine metabolites only provoked positive chemoattraction, and not *vir* gene induction (even at higher phenolic concentrations). Experiments were thus undertaken to attempt to improve the sensitivity of response to HVA and VMA, in the hope that a catecholamine metabolite inducible strain could be produced.

5.2 Two Component Regulatory Systems.

As already mentioned, VirA and G are part of a family of proteins, known as 2 component regulators (Winans *et al*, 1986). Most members of this group encode positive regulatory elements (Winans *et al*, 1986), although CheA and CheY which regulate chemotaxis and not gene expression are also strongly homologous to this group (Bourret *et al*, 1989). DNA sequence analysis has shown an extremely high level of conservation amongst this family (Ronson *et al*, 1987). This homology extends to the secondary and tertiary structures of gene products (Ronson *et al*, 1987). These authors suggest that during bacterial evolution the 2 component system has been adapted many times for sensing and responding to different environmental changes (Ronson *et al*, 1987). One component acts as a sensor and signal transducer, interacting with the second, responsive component. Response time can vary from milliseconds in the case of chemotaxis to hours required for sporulation in *Bacillus subtillis* (Bourret *et al*, 1991). Members of the family include the *E.coli* proteins EnvZ/OmpR responding to osmolarity, NtrB/C responding to nitrogen limitation,

PhoR/B, phosphate limitation, CpxA/SfrA, toxic compounds and in *Rhizobium leguminosarum*, DctB/D which regulates expression of a transport protein (Ronson *et al*, 1987, Bourret *et al*, 1991). Stock *et al* (1990) expect about 50 such systems to operate in *E.coli* alone.

Approximately 200-250 amino acids in the C terminal region of the sensor molecules show strong conservation amongst all members of the family. The greatest sequence variation is in the regions that have the least conserved roles *ie* the ligand binding domains (Armitage, 1992). Hydropathy data suggest that the sensors generally have 2 hydrophobic transmembrane regions bordering a positively charged N terminal periplasmic domain (Ronson *et al*, 1987, Winans, 1991). In addition there is a highly conserved C terminal cytoplasmic domain, making the structure manifestly reminiscent of that of MCP's (Ronson *et al*, 1987, Bourret *et al*, 1991). Furthermore the requirement of ChvE for *Agrobacterium* VirA response to sugars is mirrored by the interaction of sugar binding proteins with *E.coli* MCP's. The sensor component is an autophosphorylating protein kinase that receives *gamma* phosphate from ATP and transfers it to a histidine residue (Bourret *et al*, 1991) (see above). Ligand binding is thought to occur at the N terminal periplasmic domain and result in a conformational change which is propagated to the C terminus allowing the signal to be transmitted further *via* regulator phosphorylation. Interestingly, deletion of the periplasmic region of VirA does not abolish *Agrobacterium's vir* induction response to acetosyringone (Melchers *et al*, 1989) and a number of workers have suggested the second transmembrane region as being involved in phenolic binding (Melchers *et al*, 1989). Winans (1991) casts doubt on this assumption however, considering this stretch of amino acids to be too short and of the wrong shape to constitute a ligand binding receptor site. Alternative binding sites could be formed by a pocket between the 2 halves of VirA dimers or on the cytoplasmic side of the molecule, accessibility being aided by as yet unidentified permeases. Another

attractive possibility would be an acetosyringone binding protein that interacts with the transmembrane or cytoplasmic portion of VirA to mediate its effect.

The regulator class also shows strong homology between its elements (Ronson *et al*, 1987). All proteins in this group have a conserved region of around 120 residues in their N terminus, including an aspartate residue in an acidic pocket which together are responsible for receiving phosphate from the sensor (Bourret *et al*, 1991, Lukat *et al*, 1991, Albright *et al*, 1989) (see above). In addition there is a conserved C terminus that falls into one of two groups which show strong homology between themselves and also DNA binding proteins and transcriptional activators. This region is thought to interact with RNA polymerase or its σ factors (Ronson *et al*, 1987). It is suggested that response regulator phosphorylation stabilises a conformational change and thus indirectly mediates the increase in transcriptional activity (Bourret *et al*, 1991).

5.3 Mutagenic Agents.

The range of mutations commonly encountered include: deletions, frameshifts by both deletion and addition and base substitutions (Miller, 1972).

Base substitutions include transitions (the substitution of a purine for a purine or a pyrimidine for a pyrimidine) and transversions, the substitution of a pyrimidine for a purine or *vice-versa* (Miller, 1972).

Most mutagens are toxic. A calibration of mutagenic activity is thus required to allow a suitable compromise between survival whilst maximising chances of mutagenesis in the gene of interest.

5.3.1 Spontaneous Mutation.

Bacterial populations are subject to spontaneous random mutation. The frequency of specific events is generally less than 10^{-5} in *E.coli* (Miller, 1972). This

necessitates the study of large numbers of colonies. Spontaneous transitions, transversions, insertions, deletions and frameshifts are reported by Miller (1972).

5.3.2 U.V. Light.

Ultra violet radiation induces both subsitutions and deletions of bacterial genetic material (Yanofsky *et al*, 1966, Schwartz and Beckwith, 1969). Berger *et al* (1966) report the weak induction of frameshifts. Other effects may include insertions and internal chromosome rearrangements (Miller, 1972). Mutations are thought to arise due to errors in the repair of photochemical damage caused by UV exposure and the procedure is best carried out in the dark to prevent efficient DNA repair (Miller, 1972).

5.3.3 Hydroxylamine.

In vitro HA is specific for the transition of G:C to A:T (Freese *et al*, 1961a,b). However, *in vivo* both G:C to A:T and A:T to G:C transitions are found, stemming from side reactions with different cellular compounds (Tessman *et al*, 1967). In some cases the mutagenic effect is only weak (Miller, 1972).

5.3.4 EMS.

EMS is an alkylating agent that modifies DNA bases by the addition of alkyl groups to ring nitrogens (Lawly, 1966). It acts powerfully *in vivo* to induce transitions and transversions (Yanofsky *et al*, 1966, Miller, 1972).

5.3.5 Nitro-S-Guanidine.

This potent mutagen induces high frequency mutations whilst causing little killing. The agent primarily causes base substitutions, although deletions are also

found. Mutagenic activity is thought to stem from the production of diazomethane, which acts mainly at the site of replication, leading to clustering of mutations and high frequencies of multiple mutations (Miller, 1972).

5.3.6 Nitrous Acid.

Nitrous acid deaminates nucleic acids and results in transitions *in vitro*, but *in vivo*, transversions and deletions are also caused (Schwartz and Beckwith, 1966, Yanofsky *et al*, 1966, Freese, 1959, Bautz-Freese and Freese, 1961). High killing is required for good mutagenesis (Miller, 1972).

5.4 Attempts to Increase Responsiveness to HVA and VMA.

The rationale behind these experiments was to expose *Agrobacterium* cells carrying clones of *virA* and *G* (with reporter gene constructs under their control) to conditions which it was hoped would prove inducing. Cells that were induced under these conditions would be selected for further analysis. The conditions selected were the "cutoff" values for diagnosis of neuroblastoma employed by workers in Newcastle-upon-Tyne (Cropper, 1989). It was thus hoped that mutant bacteria that were induced by low HVA and VMA concentrations could be selected.

Catecholamine metabolite concentrations of $2.4*10^{-5}$M HVA and $1.4*10^{-5}$M VMA were derived from the cut offs of 39μg/mg creatinine and 25μg/mg creatinine respectively, assuming 1μM creatinine (typically 0.5-3.0 μM, J.Seviour, personal communication, comparible with findings of Tuchman *et al* (1989), molecular weight creatinine: 113 (Varley, 1967)) and HVA and VMA molecular weights of 182.2 and 198.2. Appropriate concentrations of antibiotic for selection were used and plates X-Gal supplemented, so *lacZ* expression could be readily assayed.

The U.V. calibration curve (figure 5.4.A) gave a 95% killing time of 2'30" for the strain used (A348 (pSM30) (*virB::lacZ*)). This length of exposure was used for

Agrobacterium U.V. Mutagenesis Dose Response Curve.

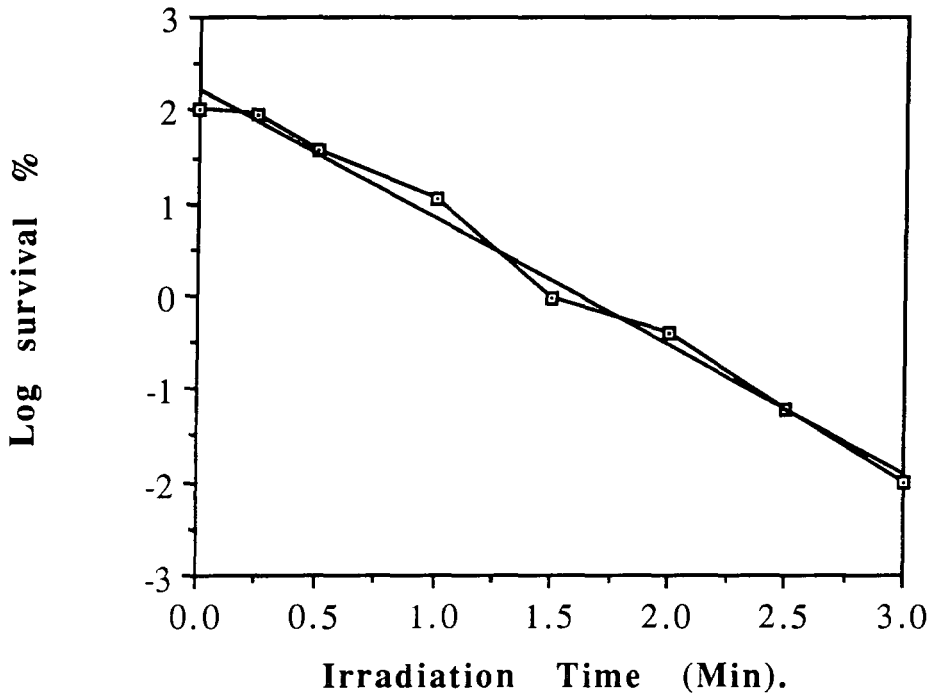


Figure 5.4.A

future experiments but whilst giving suitable numbers of survivors only generated constitutive (promoter) mutants although the experiment was repeated several times. To test the inducibility of blue colonies by HVA and VMA, seleted cells were plated out on similar media that only contained solvent and not catecholamine metabolites, in the expectation that truly inducible cells would appear white, whilst constitutive *lacZ* expressing cells would remain blue.

Since the U.V. mutagenic approach provided poor results, it was proposed to try and employ the natural variation in the bacterial population to select a suitable mutant cell line. In several experiments, hundreds of thousands of cells were plated out under mutant selection conditions on 22cm sided phage plates. In these experiments, no mutagenic agent was employed. No suitable colonies were detected.

It had become clear that the desired mutation would occur at extremely low frequency. A method for screening very large numbers of bacteria was thus needed to isolate the desired clone. It was thought that using a *vir* inducible antibiotic resistance reporter gene construct could be a feasible method of fulfilling this requirement. Exposure of very large numbers of mutagenised cells to low catecholamine metabolite concentrations in the presence of antibiotic would then only allow growth of induced and constitutive cells. Constitutive mutants could then be screened out of the mutant enriched population. To this end a *vir* inducible CAT fusion was constructed.

The *SalI virB* promoter fragment of V21 (see section 2.3) was purified by fragment isolation (section 2.9.5) from an agarose gel (section 2.9.4) that had been used to separate plasmid restriction products (section 2.9.3). Following DNA concentration estimation (section 2.9.6) of both vector and insert, the insert was ligated (section 2.9.8) to alkaline phosphatase (section 2.9.7) treated pIJ3100 *SalI* digest (see table in section 2.3) in the appropriate ratio. *SalI* was used as pIJ3100 has a suitable restriction site upstream of its CAT coding region and because the *virB* promotor can be most conveniently exised from V21 on a *SalI* fragment. The

resultant mixture was used to transform *E.coli* DH5α (section 2.8.1). Since no selection was available for inserts, recombinants were selected by colony hydridisation using the Boehringer Mannheim non radioactive detection kit and DNA bound to solid support membranes (sections 2.9.9). Insert orientation was determined in a number of independent isolates by restriction analysis. This line of research was abandoned before implementation due to the availability of a more elegant approach.

The pIB50/pIB100 system was employed. pIB50 encodes a dual reporter gene construct with *lacZ* and *CAT* fusions under the control of different *vir* promoters (see section 2.3). Induced pIB50 harbouring cells will thus grow on chloramphenicol and cleave XGal to a blue product. This ability was used to screen for undesired promoter mutations since a constitutive mutation in either promoter would not affect the other and so the desired phenotype (Cm^R, blue cells on X-GAL) would not be expressed. The probability of 2 independent promoter mutations was thought to be extremely low. Plasmid pIB100 encodes wild type *virA* and *G* clones. This plasmid was mutagenised *in vitro* with HA (section 2.14.2), purified and electroporated (section 2.7.2.2) into GMI9023(pIB100) before exposure to screen conditions. This strategy was favoured since the only material to be exposed to mutagenesis was pIB100, theoretically decreasing the chances of unwanted mutations, increasing the chance of desired changes and aiding characterisation of selected mutants.

In 2 separate experiments, 11 possible independent mutants were isolated. These were confirmed to be *Agrobacterium* by the *Agrobacterium* plate test (section 2.15) and then grown on mutant selection media that lacked HVA and VMA but contained an equal concentration of solvent to previous experiments. An absence of resultant blue colonies was initially encouraging and it was decided to test the mutants inducibility using the pUCD1187 (*virB::lux*). In these and following experiments mutant selection plates were supplemented with 50mM inositol

following the report of Song *et al* (1991a) that this treatment improved induction response to phenolics.

It was not possible to electroporate pUCD1187 into the mutant *Agrobacteria* directly as it would not be possible to select for novel Km^R pUCD1187 transformants due to the kanamycin resistance marker of pIB50. pIB50 (IncP) was however dispensable for these experiments and thus it was decided that plasmid incompatibility should be used to cure the mutants of this plasmid. To this end RPI-85 (Amp^R, Tc^R,Km^S, IncP) was introduced into W3110 using a plate mate conjugation technique (section 2.7.1.3), transconjugants being selected by ability to grow on thiamine supplimented M9 minimal media and Amp+Tc. Using a second plate mate conjugation RPI-85 was transferred to the mutant *Agrobacterium* strains and selected for by Amp(Cb)+Tc resistance. Resultant transconjugants were cured for pIB50 due to IncP incompatibility and thus no longer Km^R. This allowed the introduction of pUCD1187 by micro-electroporation (section 4.5.1). *Vir* induction of mutant *lux* transformants by HVA and VMA was tested as before and dissapointingly showed no improvement.

The results of previous experiments (see Discussion), Cangelosi *et al* (1990) and Winans (1991) have suggested the existence of an acetosyringone binding protein which interacts directly with the ligand and VirA. In light of the failure to generate the required mutants by the above procedures, it was decided to alter the strategy. Virulent Ti plasmid harbouring *Agrobacterium* were considered to be the organism of choice for further mutagenesis experiments. A putative AS binding protein would in all probability be chromosomally encoded since the entire Ti plasmid has been analysed (Rogowsky *et al*, 1990). Furthermore this location is consistent with the work of Ashby *et al* (1988) who reported a chromosomally encoded phenolic sensing ability. For future mutagenesis experiments strain $C58C^1$ Ery Cm(pDUB1003▲31, pIB50) was constructed by micro-electroporation, allowing

mutagenesis of wild type cells with marked Ti plasmid and selection with discrimination against promoter mutants as above.

It was decided that the nature of the mutagen should be altered and several additional mutants were isolated following exposure of C58C^{1} Ery Cm(pDUB1003*delta*31, pIB50) to U.V.light, HA *in vivo*, EMS, NG and nitrous acid (see section 2.14). However, none had the desired phenotype.

6. Discussion.

6.1 Introduction and Project Aims.

This project set out to test the hypothysis that the common soil prokaryote *Agrobacterium tumefaciens* would sense and respond to the catecholamine metabolites HVA and VMA *via* the VirA/G two component regulatory system. This hypothesis is based on the marked structural similarity between HVA, VMA and AS, a phenolic compound to which the bacterium naturally responds (see figures 1.3.2.A and 1.3.2.B). Two main classes of potential response were tested, *vir* gene induction and chemotaxis, and it was hoped that these would form the basis of a bacterial test for neuroblastoma. This test would be based upon the observation that a high proportion of neuroblastoma patients excrete elevated urinary concentrations of HVA and VMA. A cheap, reliable and reproducible bacterial screening test was thought to be of great benefit in improving the prognosis of neuroblastoma patients, whose age at time of diagnosis is strongly correlated with their clinical outlook.

During the period of research, the initial optimism aroused by pilot neuroblastoma screening projects has settled to a reserved caution. It is felt that such programmes may not be as beneficial as first thought, and the results of more carefully conducted studies are eagerly awaited. There is growing evidence that suggests that mass neuroblastoma screening should not be recomended for countries not already involved in such research.

Likewise much has been discovered about the biology of *Agrobacterium tumefaciens* in the last few years. Research illuminating the control of virulence has been especially interesting, and the role of dynamic protein phosphorylation and sugar binding proteins that interact with VirA, the two component sensor have been described. The role of VirG as a sequence specific DNA binding protein and transcriptional activator has also been elucidated. Most recently it has been

suggested that a phenolic binding protein may exist and this has clear implications for a bacterial neuroblastoma screening test.

6.2 Consequences of Neuroblastoma Screening.

Since the implementation of the Japanese national neuroblastoma screening programme in 1985 it has detected over 250 cases (Sawada *et al*, 1991). In the fourteen years between 1974 and 1988 Dr Sawada and collegues have recorded 55 neuroblastoma cases in the Kyoto Prefecture. 22 of these cases were detected by 6 month VMA testing and HPLC screening (Sawada *et al*, 1991). Data from this study has shown that VMA testing increased the number of patients diagnosed under 1 year of age and decreased the number of patients older than 2 years at the time of diagnosis (Sawada *et al*, 1991). Concurrent with these changes was an increase in the proportion of cases diagnosed during favourable stages (I,II and IVS) and a reduction in stage III and IV presentations (Sawada *et al*, 1991). The implementation of the screening is considered by its proponents to have halved the annual number of neuroblastoma deaths in Kyoto (Sawada *et al*, 1991).

Implementation of HPLC screening in the same prefecture caused an apparent 1.5 fold increase in the annual diagnosis of neuroblastoma and raised the number of patients detected before 1 year of age to 5.5 times that of the same population before screening (Sawada *et al*, 1991). A similar trend towards earlier diagnosis due to screening is noted by Nishi *et al* (1990c) in Sapporo City. The number of cases diagnosed after 2 years of age had fallen to half the pre-screening figure (Sawada *et al*, 1991). Sawada and collegues (1991) expect the annual rate of neuroblastoma deaths in an HPLC screened population to be half that of the population before screening. Summarising their experiences, these authors describe mass neuroblatoma screening as leading to an increase of neuroblastoma incidence in children under 1 year of age, a shift to diagnosis at earlier ages and stages, and a decrease in the number of deaths. These authors however concede that HPLC

screening may detect the small proportion of cases that would otherwise spontaneously regress and feel that this explains the higher observed incidence of neuroblastoma in screened populations compared to untested ones. Ishimoto *et al* (1990) and Matsumura *et al* (1991) have observed spontaneous remission in patients detected by mass screening. Parker *et al* (1992) and Nishi *et al* (1990c) also feel screening will detect benign tumours and Hanawa *et al* (1990) and Goodman (1991) point out that the detection of cases with good prognosis may improve the apparent effectiveness of screening in improving prognosis. An additional explanation for the increased neuroblastoma incidence rates observed after the introduction of screening would be a greater awareness of the disease in the medical profession and a consequent increase in neuroblastoma diagnosis (Sawada *et al*, 1991, Tuchman, 1991a). Nishi *et al* (1990c) however claim that screening has not increased the recorded incidence of neuroblastoma in Sapporo City.

Japanese workers claim their most important acheivement has been the reduction of annual neuroblastoma deaths following the introduction of screening (Sawada *et al*, 1991). However, the significance of this figure is debated by several authors (Goodman, 1991, Parker *et al*, 1992) who feel that this is a misleading comparison. Sawada *et al* (1991) contrast contempory annual death rate in the Kyoto prefecture with the same population many years ago, thus ignoring improvements in therapy during this period (Sawaguchi *et al*, 1990). The Japanese study would thus be of far greater significance if data could be compared to a similar unscreened population during the same period (Goodman, 1991, Parker *et al*, 1992) and there is little convincing evidence of a fall in neuroblastoma death rate as a result of screening (Hanawa *et al*, 1990). This initial report (Hanawa *et al*, 1990) of reduction in Japanese neuroblastoma mortality attributed to screening by the authors, produces little evidence for optimism since an equivalent fall is noted in the UK unscreened population (Cole *et al*, 1992).

Further complications in the interpretation of the Japanese studies arise due to the lack of proper controls. Length time bias derives from the observed increase in survival due to the higher proportion of good prognosis cases in screened populations. This can be as a result of the sensitivity of the test or its timing, if poor prognosis cases present clinically before screening (Goodman, 1991, Parker *et al*, 1992, Prorok, 1992). Lead time bias originates however due to the earlier detection of screened cases giving rise to a longer surival period compared to clinical cases, even though death may occur at the same age (Goodman, 1991, Parker *et al*, 1992, Prorok, 1992). To assess the feasibilty of GC-MS screening for a properly controlled UK study, workers in Newcastle-upon-Tyne undertook a more carefully designed trial (Parker *et al*, 1992).

This GC-MS study has detected 2 cases of neuroblastoma with good prognosis both of whom have been sucessfully treated. However, 3 false negative cases with poor prognosis were missed despite the use of HVA and VMA cut off values biased for sex and creatinine concentration. One of these patients succumbed to disease whilst the other 2 responded positively to treatment. In addition 8 false positive cases were noted from the 20829 babies screened. Employment of more recent practises would have reduced the number of false positives to 3 (Parker *et al*, 1992). This and other studies (Hanawa *et al*, 1990, Tuchmann *et al*, 1990, Sawada *et al*, 1990) have shown that neuroblastoma screening is technically feasible but it remains to be seen whether screening *per se* improves the long term survival and mortality of neuroblastoma patients (Murphy *et al*, 1991). Indeed it is suggested that the overall neuroblastoma mortality rate may not be significantly different in the Japanese screened and UK unscreened populations (Murphy *et al*, 1991, Cole *et al*, 1992).

Furthermore it has been demonstrated that neuroblastoma cases detected by mass screening frequently have good prognosis markers (Tuchman *et al*, 1990b, Kaneko *et al*, 1990, Lavalotte, 1991, Bessho *et al*, 1991, Parker *et al*, 1992) and

indeed screening detected cases are reported to have a 97% survival rate (Bessho *et al*, 1991). Conversely poor prognosis markers are often associated with clinically diagnosed tumours that thave been missed by screening (Woods *et al*, 1992, Tuchman *et al*, 1990b). This further questions the effectiveness of screening by suggesting that the cases detected as a result of screening are probably amenable to therapy even if detected clinically, and that those missed are less so (Woods *et al*, 1992). Implicit in this observation is evidence to refute the claim that patients diagnosed by screening fare better than clinically discovered patients in the same poulation. This means that a potential screening test may not fulfill the requirement of Prorok (1992) that individuals identified as true positives should benefit from a more favourable prognosis. It has been suggested (Kankeo *et al*, 1990, Parker, 1991, Ishimoto *et al*, 1990) that screening at 6 months of age is thus too young and that a 12 or 18 month screen would be more appropriate. Later screening would aim to detect a higher proportion of poor prognosis cases and reduce false negatives whilst not adversly affecting the outlook for the group of patients currently diagnosed by 6 month screening. Reduction in the false positive rate would occur by allowing small tumours time to develop sufficient volume to produce detectable HVA and VMA levels (Ishimoto *et al*, 1990) and becuase poor prognosis tumours missed by screening are more strongly associated with older children (Kaneko *et al*, 1990). Norman *et al* (1987) question whether subsequently diagnosed tumours missed at 6 months are actually present at the time of screening, although as already mentioned the general assumption is that neuroblastomas are congenital. A further explanation would be that aggrressive tumours are congenital, but have the ability to remain quiescent for several years (Weinblatt, 1988) perhaps awaiting a second mutation in genes controlling cell division.

The progression of poor prognosis neuroblastomas to aggrressive disease has yet to be demonstrated and contary to this, it appears that *N-myc* amplification and DNA ploidy levels are temporally constant (Brodeur *et al*, 1987, Taylor *et al*, 1988).

This inability to detect poor prognosis cases and stable heterogeneity of the tumours accounts for the unchanged mortality rate in screened poulations (Carlsen, 1992) when compared to controls. About 50% of the Japanese tumours missed by screening have been diagnosed at later than 1 year of age and were poor prognosis tumours that killed the patients (Kaneko *et al*, 1990, Ishimoto *et al*, 1990, Murphy *et al*, 1991). It has yet to be demonstrated that the target group of tumours that later screening would aim to detect would benefit from earlier detection (Murphy *et al*, 1991), once again violating the guidelines of Prorok (1992). Indeed it has been demonstrated that once *N-myc* amplification and DNA ploidy have been subjected to multivariate analysis, age no longer has an independent effect on prognosis (Bourhis *et al*, 1990). A further alternative would be to screen only high risk infants, such as those whose parents have had assisted conceptions or who have been exposed to strong electromagnetic fields (Toren *et al*, 1992).

In conclusion the early optimism aroused by the Japanese screening programmes may have been premature. Whilst it has been demonstrated that neuroblastoma mass screening is technically and logistically practical and that the cost is acceptable (Scriver *et al*, 1987, Takeda, 1989, Parker *et al*, 1992, Nishi *et al*, 1991, Sawada *et al*, 1991), it is not clear that screening for neuroblastoma in 6 month old infants can significantly decrease mortality resulting from the disease. In spite of this, further study may prove fruitful (esp the results of the cooperative study undertaken in Canada and North America with population based controls) although it will be essential to consider the design of further trials more carefully to include essential controls and refine the timing of testing. Ideally screening methodology should be improved to detect patients with a poor prognosis. Additionally, important questions concerning the progression of disease associated with good prognosis markers to aggressive neuroblastoma need to be answered, before further national scereening could be wholeheartedly recomended. If disease progression is

discounted as currently seems possible, improvements in therapy for advanced patients will be of paramount importance in improving overall prognosis.

6.3 Evaluation of the *Agrobacterium Virulence* Gene System as a Potential Diagnostic Test for Neuroblastoma.

6.3.1 vir Gene Induction.

The National Catalogue of Industrial and Marine Bacteria (1990) lists no organisms capable of metabolising HVA or VMA. This coupled with the presence of many other metabolites in urine and its solvent extracts precludes the use of bacterial growth utilizing catecholamine metabolites as the basis for a screening test. Likewise the observed supression of *Agrobacterium* growth by HVA and VMA (figure 3.4.1.D) would not be sufficiently reliable to form the foundation of such work due to the presence of interfering compounds.

A study based upon the use of *vir* promotor controlled reporter genes would be the most suitable way of developing a bacterial neuroblastoma screening test. *Agrobacterium* was shown to grow well in filter sterilised urine (figure 3.4.4.A) and not to be induced in normal samples (figure 3.4.4.B). A potential screening scenario would have the test bacterium grown in either fresh liquid sample or in aqueous resuspension of dried urine collected on filter paper. If necessary to improve the test specificity, bacteria could be exposed to aqueous solutions of solvent extracted sample, thus removing some inhibitory impurities (figure 3.4.4.B). It was hoped that the bacterium would be induced by catecholamine metabolites in positive samples and that this induction could easily be detected using a colourimetric or light production assay dependent on *lux, GUS* or *lacZ* reporter activity.

However, studies with these reporter genes failed to show any induction response by the bacterium on exposure to HVA and VMA (see figures 3.4.1.A, 3.4.2.C and 3.4.3.A) when compared to controls. Furthermore HVA and VMA did not interfere with the biological activity of acetosyringone (figures 3.4.1.B and 3.4.2.D). It had been thought that catecholamine metabolites might supress induction

in cells exposed to inducing concentrations of AS due to competition between HVA/VMA and AS for phenolic receptor sites. Alternatively these experiments could have detected possible synergism of response between HVA and VMA and AS in cells sensitised to phenolic presence by the 'natural' ligand.

These results were disappointing despite the optimisation of *vir* induction conditions (figure 3.4.1.C) and the use of quantitative assays of reporter expression in all cases. However, the extremely sensitive *lacZ* assay was taken to indicate that the wild type VirA/G system was incapable of mediating a *vir* induction response to catecholamine metabolites (figure 3.4.3.A). These results could be a result of the inability of HVA and VMA to interact with the phenolic sensing functions of *Agrobacterium* to elicit a responce.

Such an inability could arise because the sensor does not recognise the catecholamine metabolites. Alternatively the shape of the catecholamine metabolites or their larger size when compared to AS could prevent access to the active moiety of the receptor. On the basis of these results alone it would also be possible that the bacterial outer membrane presents a physical barrier to HVA and VMA, through which they are not able to pass. AS would however be actively or passively able to transverse the outer membrane and gain access to the phenolic receptor site - traditional associated with the inner membrane. This last suggestion is however the least attrractive since a chemotactic response to catecholamine metabolites was later demonstrated.

6.3.2 Chemotaxis.

These experiments confirm the previously reported observations of *Agrobacterium* chemotaxis towards phenolic compounds (Ashby *et al*, 1987, 1988, Shaw *et al*, 1988), see figure 4.4.A. In addition the optimum acetosyringone concentration for taxis was determined more precisely than in previously published reports (3.16* 10^{-8}M as compared to 10^{-7}M). HVA and VMA were reproducibly

shown to be positive chemoattractants in a number of experiments (figures 4.4.B, 4.4.C, 4.5.3.2.B, 4.5.3.2.C). In addition transposon disruption and the use of cured strains (figures 4.4.E, 4.4.F, 4.5.3.2.B, 4.5.3.2.C) showed that this response was mediated by the *virA/G* system suggesting a strong similarity with the response to AS. A further experiment designed to test this assumption would have been to synthesise brominated HVA and VMA which it was hoped would covalently bind to their sites of action and block them. If these sites were the same as for acetosyringone, chemotaxis and *vir* induction would be expected to be suppressed in response to the 'natural' ligand. However, consultation with Dr D. O'Hagan (University of Durham, Chemistry Department) lead to the abandonment of this idea since it was felt that the differing chemistry of these molecules would mean that halogenated forms would not bind covalently to their receptor sites.

The non phosphorylatable versions of VirA and VirG proteins are not competent to mediate chemotaxis towards acetosyringone. This deficiency when compared to their wild-type counterparts implies a requirement for phosphorylation if chemotaxis is to occur efficiently (see figs 4.5.3.1.A and 4.5.3.2.A). The implicit suggestion that phospho-VirA and phospho-VirG are required for chemotaxis is consistent with the previously reported phosphorylation of VirG by phospho-VirA during the induction of *vir*-gene expression (Jin *et al*, 1990a,b). It would appear that VirA, the sensor, is capable of sensing a broad band of acetosyringone concentrations, and effecting two responses, depending upon the sensed concentration. Phospho-VirG is the active, stable, transcriptional activator, capable of sequence specific, high affinity DNA binding (Jin *et al*, 1990a,c, Pazour & Das, 1990a,b, Powell *et al*, 1989, Powell & Kado, 1990). How it can fulfil this role at high concentrations of acetosyringone and mediate chemotaxis at lower concentrations is not clear.

A possible explanation for the ability of VirA and VirG to mediate two effects, in response to differing concentrations of the ligand would be that phospho-

VirG has a high affinity for a component of the cytoplasmic chemotaxis signalling pathway (possibly a CheA homologue) and a somewhat lower affinity for *vir*-box DNA. Thus at low concentrations of the ligand ($<<1$ molecule/receptor) the small amounts of phospho-VirG produced would preferentially interact with the chemotaxis pathway. At higher concentrations (>10 molecules/receptor) sufficient phospho-VirG is produced to participate in significant *vir*-box binding, and hence *vir*-induction.

The periplasmic region of VirA has been shown to be essential for *vir*-induction by sugars through the ChvE pathway (Cangelosi, *et al*, 1990). It has also been shown not to be involved in chemotaxis towards sugars, where a mechanism involving ChvE and a putative Trg analogue are thought to effect the response. The role of this region of the molecule in the cell's response to acetosyringone is unclear. The putative acetosyringone binding site of VirA is thought to be associated with the transmembrane regions of the molecule (Cangelosi, *et al*, 1990, Melchers, *et al*, 1989). Deletion of the periplasmic region is reported to have a variety of effects on *vir*-induction, with one report of no change (Melchers, *et al*, 1989) and another describing increasing sensitivity of response (Cangelosi, *et al*, 1990). The reported increased sensitivity to acetosyringone is thought to be because the molecule adopts a non-wild type conformation in the membrane. The periplasmic deletion appears to severely curtail chemotaxis towards acetosyringone (figure 4.7.A), which may be simply an effect of the altered conformation. However, the data does indicate that the periplasmic domain, while inessential for *vir*-induction, is required for chemotaxis. Most recently Chang and Winans (1992) have suggested that the periplasmic region whilst being dispensable for induction is essential for virulence. This coupled with the absolute requirement for chemotaxis in virulence in certain soils (Hawes and Smith, 1989) lends weight to the requirement for this region of the molecule in mediation of chemotaxis. This suggests that VirA may possess more than one site with differing affinities for acetosyringone. It is also possible, and perhaps

more likely that acetosyringone does not interact directly with VirA, but rather with one or more binding proteins in the periplasm, with different ligand affinities. Differential concentration effects would then be achievable by these proteins interacting with different domains of VirA (see next section).

The addition of mutant copies of VirA and VirG to the wild-type chemotaxis system encoded by pVK257 decrease its effectiveness (figure 4.5.3.3.A). The ability to respond to the stimulus is reduced, with a shift in the chemotaxis peak towards higher ligand concentrations (figure 4.5.3.3.B). In the case of *virA* mutants it is hypothesized that of the relatively small number of acetosyringone molecules interacting with cell surfaces, a proportion interact with mutant molecules to no effect and a proportion elicit a chemotaxis inducing signal through the wild-type. This decreases the number of cells attracted. The explanation of the results following the addition of mutant *virG* is thought to be that the cells ability to respond to acetosyringone is depleted because its proportion of competent signal intermediates is lessened. Mutant non-phoshorylatable VirG molecules compete with wild-type intermediates for association with VirA, meaning that although acetosyringone presence is detected by VirA the chemotactic signal is transmitted at lower levels.

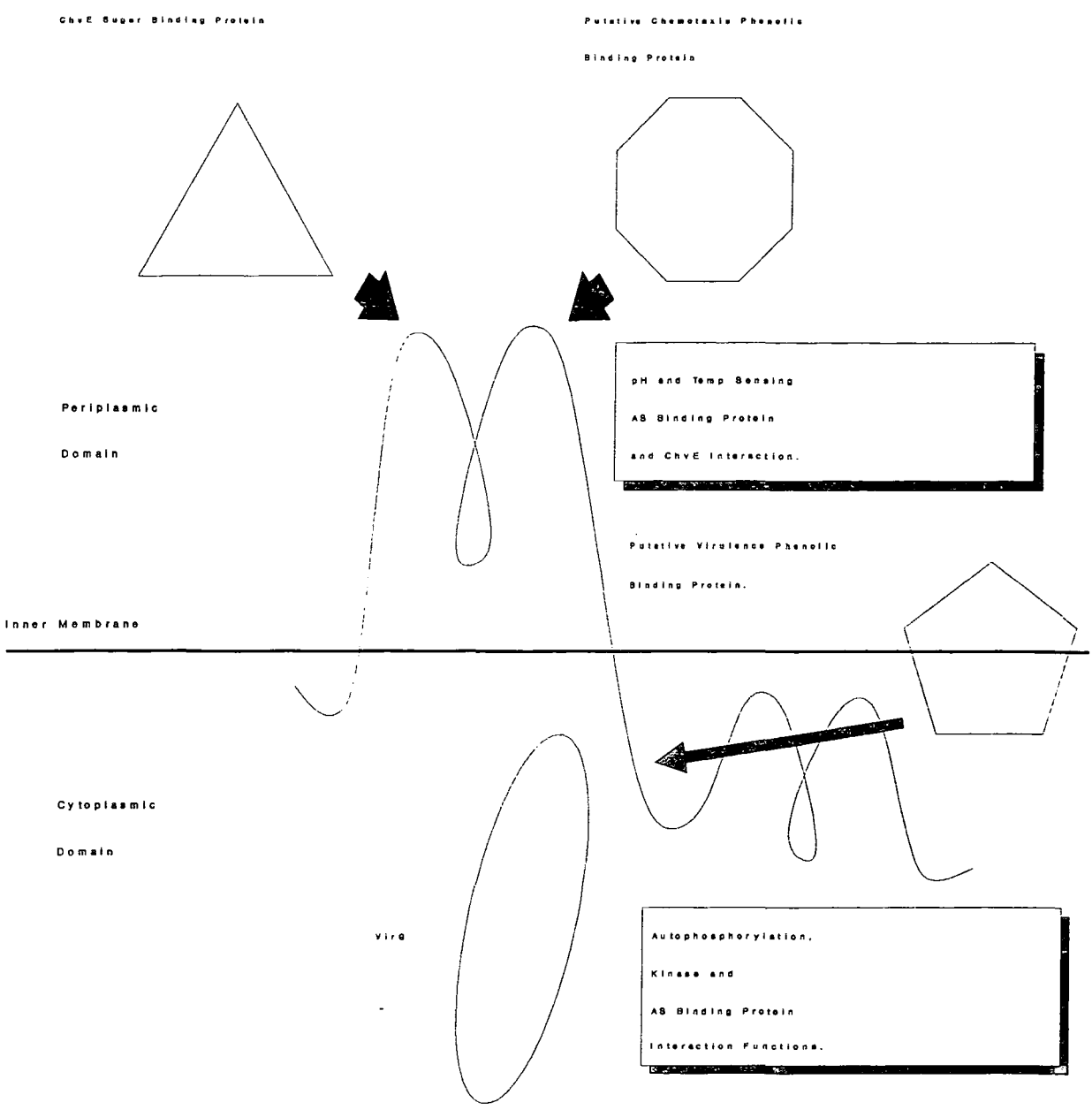
6.3.3 A New Model for *Agrobacterium* Phenolic Sensing.

The result of experiments with the specific *vir* induction inhibitor, ASBr suggest a new model for the interaction of phenolics with the *virA/G* system. This model proposes that AS interacts indirectly with VirA to bring about its effects. This new model takes account of the suggestion that there may be more than one phenolic receptor site and seeks to provide a further hypothetical explanation for the ability of VirA and G to mediate two responses to different concentrations of the same ligand.

Bromo-acetosyringone is interesting because it acts as a chemoattractant at low (10^{-7}M) concentration whilst, at higher levels (10^{-5}M) it is a repellent or chemo-suppresant (see figure 4.6.C). The new model is in keeping with previous reports (see earlier) and attributes pH and temperature sensing functions to the periplasmic domain of VirA. As before sugars bring about their response through ChvE and indirect association with the periplasmic loop. The new model however differs from previous suggestions (Melchers *et al*, 1989, Cangelosi *et al*, 1990) by attributing the phenolic binding ability to separate and as yet unidentified binding proteins rather than to VirA itself. This is similar to the requirement of analagous proteins involved in MCP and VirA sugar responsiveness. It is proposed that *Agrobacterium* has 2 such proteins, a high affinity, possibly relatively highly expressed periplasmic binding protein that is responsible for mediating chemotaxis and a lower affinity, inner membrane protein that controls induction response (see figure 6.3.3.A).

The periplasmic protein would either as a result of its higher affinity for the ligand, its higher copy number or its mobility in the periplasmic space be able to detect lower concentrations of phenolics than the inner membrane protein. This would account for the lower ligand concentration required for optimum chemotactic response compared with that of induction. This molecule would when complexed with ligand interact with the periplasmic domain of VirA to mediate a chemotactic response, explaining the requirement of this portion of the 2 component regulatory sensor in phenolic chemotaxis. The possibility of this being a higher affinity receptor than its membrane bound counterpart could explain the ability of *Agrobacterium* to respond to catecholamine metabolites chemotactically but not by *vir* induction.

In contrast the association of the *vir* responsive binding protein with the inner bacterial membrane and a possible lower level of expression would lower its ability to bind available ligand. This lower affinity could also be a function of the receptor site itself. The lower affinity for ligand would explain the 1000 fold higher AS concentration required for *vir* induction compared to chemotaxis. This protein would



New Model for VirA Mediated Phenolic

Sensing and Response.

Figure 6.3.3.A

be expected to interact with the second transmembrane region of VirA in traditional dogma, but after the observations of Chang and Winans (1992), an association with the cytoplasmic portion of the molecule is to be expected. There would be no requirement for interaction with the periplasmic domain of VirA in the new model. If such a model is true then its goes someway to explaining the results of Parke *et al* (1987) who report that a compounds capacity to cause a chemotactic response is not always proportional to its *vir* inducing abilities. This could be explained if the 2 binding proteins had slightly different specificities, perhaps as a result of responding to different functional groups or portions of the ligand. This in turn offers another explanation for the observed chemotactic response of *Agrobacterium* to HVA and VMA whilst induction can not be detected.

ASBr is proposed under the new model to interact at low (10^{-7}M) concentration in much the same way as the non-halogenated ligand, thereby inducing chemotaxis. However, at higher concentration (10^{-5}M) it also interacts with the inner membrane binding protein. This causes specific inhibition of *vir* gene induction (Hess *et al*, 1991) and would also alter the ability of VirA to mediate chemotaxis, thus causing the observed suppression of response (figure 4.6.C). Supression of chemotaxis by 'high' phenolic concentrations has been observed before (Shaw *et al*, 1988). ASBr, is thought to bind covalently and irreversibly to its receptor (D.O'Hagan, personal communication). There is scope in this new model for hypothetical phenolic binding proteins to interact with signal transducers other than VirA, in a similar way to that in which ChvE interacts with both VirA and a Trg analogue.

The results of the chemotaxis experiments to urine (figure 4.4.1.A) indicate that other compounds in samples make this an unsuitable basis for a screening test. The time consuming nature and practical difficulty of such assays mean they also do not lend themselves to a mass screening programme.

6.3.4 Mutagenesis.

Non of the mutagenic treatments employed gave rise to the desired mutations. The mutants isolated in early experiments were most likely to be promoter mutants that lead to constitutive expression of the reporter genes under their control. In the experiments using, pIB50, the dual reporter construct, the mutants generated could have possibly been of a similar type, but this is thought to be most unlikely as the frequency of a single cell obtaining 2 independent constitutive promotors mutations would be expected to be very low. An alternative explanation is that mutations arose in the genes encoding the RNA polymerase sigma factors involved in transcription of the reporter genes. This could possibly lead to constitutive expression without promotor mutation.

The inability of pIB100 (*virA, G*) mutagenesis to generate the desired phenotype is not contradictory to the new model for phenolic sensing. As this plasmid cannot encode any putative phenolic binding protein, its mutagenesis and consequently *virA/G* mutagenesis would not be expected to improve the ability of the bacterium to sense HVA and VMA (if the receptor site is elsewhere). Furthermore there is no reason to suppose that binding proteins should be subject to a high rate of turnover if ligand binding is irreversible. This would suggest that in a bacterial culture not undergoing rapid division the rate of binding protein gene expression would be relatively low. Depending upon the gene's proximity to other highly expressed regions of DNA this could suggest that the DNA encoding putative binding proteins would be in a highly condensed state and thus less accessible to mutagenic agents. This coupled with the highly specific nature of mutation required to improve sensitivity to HVA and VMA could go part way to explaining the lack of these strategies success.

6.4 Conclusions and Future Prospects.

In summary *Agrobacterium* has been shown to be unable to respond to the catecholamine metabolites HVA and VMA by *vir* gene induction. However, the bacterium can respond chemotactically to these compounds, and the mechanism of this response has been elucidated to some extent. This response is not thought to be amenable to the development of a neuroblastoma screening test, the need for which is becoming increasingly uncertain. Attempts to improve the ability of *Agrobacterium* to sense HVA and VMA failed, and explanations for this have been suggested. In light of experiments which have shown flaws in the accepted model of *Agrobacterium's* phenolic sensing functions, a radical new hypothetical model has been suggested.

If further reseach time and funds were available it would be interesting to try and gain evidence to reinforce or disprove these suggestions. One key experiment could be to attempt isolation and purification of acetosyringone binding proteins. A possible method for doing this would be to disrupt and fractionate *Agrobacterium* cells and to incubate these fractions with tritiated or ^{14}C labelled bromo-acetosyringone. Using protein purification techniques it should be possible to purify the AS binding functions, identified by means of the covalently bound radiolabelled ligand. If sufficient sample could be obtained, it should be possible to sequence the proteins and isolate the genes encoding them using deduced degenerate sequence. Cloned binding proteins could then be subjected to mutagenesis in a similar way to that which pIB100 was, in the hope of isolating HVA and VMA sensitive mutants. If insufficient protein to sequence was available, and alternative strategy would be to screen and *Agrobacterium* expression library with mouse antibody raised to the binding protein and to isolate the gene from this.

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