# $C_4$-DICARBOXYLIC ACID TRANSPORT GENES IN *R. MELILOTI*

# ANALYSIS OF $C_4$-DICARBOXYLIC ACID TRANSPORT GENES IN *RHIZOBIUM MELILOTI*

By

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ABSTRACT:

*Rhizobium meliloti* mutants defective in $C_4$-dicarboxylic acid transport ($Dct^-$) were previously isolated by Tn*5* mutagenesis, and divided into two groups based on complementation of $Dct^-$ with cosmid clones. In this work further characterization was carried out on the two loci. Group I mutants were found to be defective in dicarboxylate transport ($Dct^-$), nitrate utilization, and symbiotic nitrogen fixation. Subcloning and complementation work confined the Group I mutations to a 3.5 kbp *Bam*HI-*Eco*RI fragment containing the *ntrA* gene. Group V mutants were defective in dicarboxylate transport and demonstrated varying levels of nitrogen fixation. Complementation and site-directed Tn*5* mutagenesis revealed three transcriptional units, corresponding to *dctA*, *dctB*, and *dctD*, localized within a 6 kbp *Hind*III fragment. The use of *dctA*::Tn*phoA* fusions determined the expression of *dctA* to be *ntrA*, *dctB*, and *dctD* dependent. $Dct^+$ revertants of *dctB* and *dctD* mutants were selected which carried second-site mutations responsible for restoring the $Dct^+$ phenotype.

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| | |
|---|---|
| bp | base pairs |
| *dct* | in reference to the gene |
| Dct | in reference to the phenotype |
| DCT | in reference to the protein |
| Gm | gentamycin |
| hr | hour |
| kb | kilobase |
| kbp | kilobase pairs |
| Km | kanamycin |
| Nm | neomycin |
| OD | optical density |
| Ot | oxytetracycline |
| RT | room temperature |
| Sm | streptomycin |
| Sp | spectinomycin |
| Tc | tetracycline |
| *Tn5* | transposon *Tn5* |

**Dedicated to my family**

# CHAPTER 1

# INTRODUCTION

## Importance of the Legume-Rhizobium symbiosis

In the agricultural industry there exists a continual problem of efficiently supplying a nitrogen source to plants. Although nitrogen gas, $N_2$, constitutes 78% of the atmosphere, it is inert and must be converted by nitrogen fixation to mononitrogen forms such as ammonia or nitrate to become biologically active. Biological processes are responsible for fixation of 175 million metric tons (67.7% out of total produced) of nitrogen per year (Langenheim and Thimann 1982); out of all the biological processes, the legume-bacterial symbiosis produces up to 700 kilograms of nitrogen per hectare per year (Evans and Barber 1977). Alfalfa (Medicago sativa) has been promoted as an almost ideal crop for maintaining and improving soil nitrogen content and is adaptable to various soil and temperature conditions providing more protein per hectare than any other crop (Goplen et al. 1987). But it is the symbiotic association with bacteria of the genus Rhizobium that is responsible for the fixation of nitrogen.

## Biology of the Rhizobiaceae

The bacterial genera Rhizobium, Bradyrhizobium, Agrobacterium, and Phyllobacterium, sharing several morphological properties, comprise the Rhizobiaceae family. Many of the species in the family can co-exist in a symbiotic relationship by forming cortical hypertrophies on roots (referred to as nodules) or on leaves of certain plants (Jordan 1984). The cells are gram-negative, aerobic, utilize a wide range of carbohydrates, and, in the free-living form, are rod-shaped and motile. Rhizobium and Bradyrhizobium are differentiated on the basis of growth rate and carbohydrate utilization. Specifically, Bradyrhizobium, classifed as slow-growing, can usually produce an alkaline reaction in mineral salts-mannitol medium whereas Rhizobium, known as fast-growing, produces acid in this medium.

Both Rhizobium and Bradyrhizobium genera form nodules on the roots of plants of the Leguminosae family. In this symbiotic state the bacteroids have the ability to fix atmospheric nitrogen. The nodulation process has been characterized as host-specific i.e. Rhizobium meliloti has the capacity to successfully nodulate plant species of Medicago, Melilotus, and Trigonella, while Bradyrhizobium japonicum (previously known as Rhizobium japonicum) can nodulate Glycine max and Macroptilium atropurpureum. The genus Rhizobium predominates on plants found in the temperate zone and bacteria grow optimally between 25 and 30 $^{o}$C in pH 6

-7 soil conditions. Free-living forms obtain nitrogen from ammonia salts, nitrate, or nitrites. Members of the genus include Rhizobium leguminosarum biovar trifolii (also known as Rhizobium trifolii), Rhizobium leguminosarum, and Rhizobium meliloti.

## Nodule Biology

The plant infection process by Rhizobium is being actively characterized by several research groups. Several reviews present information gathered regarding the interaction (Bauer 1981, Long 1984, Djordjevic et al. 1987). In most legumes bacterial infection occurs through developing root-hair cells. Both nodulation (nod) and host-specific nodulation (hsn) genes are involved in nodule induction: the nod genes determine root-hair curling, infection thread synthesis, and nodule initiation whereas the hsn genes determine the host range of the bacterium. Expression of the nodABC genes requires bacterial nodD, constitutively expressed, as well as the secretion of flavenoid compounds by the plant (Rossen et al. 1985, Redmond et al. 1986). Rhizobia then release compounds that stimulate root-hair curling and/or induce cortical cell division. Bacteria are thought to be trapped in the curled root-hairs and enter the root via a tube-like structure of plant origin termed the infection thread. Cortical cells are stimulated to divide and form the nodule tissue into which the infection thread

penetrates. Upon release of the bacteria from the infection thread the bacteria remain surrounded by a plant-derived membrane and "differentiate" into $N_2$-fixing pleomorphic structures termed bacteroids. The plant-derived membrane surrounding the bacteroids is called the peribacteroid membrane.

Effective nitrogen fixation requires many Rhizobium genes. The nif genes are needed for the synthesis and activity of the nitrogen-fixing enzyme nitrogenase. These genes show homology to the nif genes of Klebsiella pneumoniae (Buikema et al. 1985 and 1987). The fix genes are also involved in nitrogen fixation. In R. meliloti these genes directly involved in symbiosis are located on two megaplasmids, pRmeSU47a and pRmeSU47b (Banfalvi et al. 1981 and 1985, Batut et al. 1985, Finan et al. 1986, Hynes et al. 1986).

## $C_4$-dicarboxylic acids are required for nitrogen fixation

Several areas of research has demonstrated that $C_4$-dicarboxylic acids succinate, fumarate, and malate are the main carbon sources supplied by the plant to the bacteroid for subsequent conversion to energy required for nitrogen fixation. These consist of: (1) addition of $C_4$-dicarboxylic acids to bacteroid fractions resulting in increased nitrogen fixation, (2) isolation of $C_4$-dicarboxylic acid transport

mutants that resulted in ineffective nodules (no $N_2$-fixation).

Bergersen and Turner (1967) examined soybean root nodules and found the addition of succinate, fumarate, or pyruvate to the bacteroid fraction in a sucrose-free medium could stimulate up to a 10-15 fold increase in nitrogen fixation. Tuzimura and Meguro (1960) reported high rates of $C_4$-dicarboxylate oxidation by soybean bacteroids. Active and inducible accumulation of $C_4$-dicarboxylates was demonstrated for R. leguminosarum (Glenn et al. 1980, Finan et al. 1981, McKay et al. 1988), R. meliloti (Engelke et al. 1987), and Bradyrhizobium japonicum (McAllister and Lepo 1983, San Francisco and Jacobson 1985). It has been demonstrated that, once in the bacteroid, $C_4$-dicarboxylates are catabolized via the TCA cycle, and acetyl CoA is thought to be generated via malic enzyme and pyruvate dehydrogenase (McKay et al. 1988).

The requirement for active transport of $C_4$-dicarboxylates in effective symbiosis was demonstrated by the isolation of $C_4$-dicarboxylate transport mutants of several Rhizobium species. Mutants were isolated by transposon Tn5 mutagenesis or by mutagenesis with such chemicals as NTG (N-methyl-N-nitro-N-nitrosoguanidine); mutants failed to grow on defined media supplemented with succinate, malate, or fumarate as carbon source and to actively transport [$^{14}$C]-labelled $C_4$-dicarboxylates. Subsequent inoculation of the mutant strains onto respective host plants resulted in plant

nodules failing to fix nitrogen. This has been reported for R. leguminosarum (Finan et al. 1981 and 1983, Arwas et al. 1986, McKay et al. 1988), R. trifolii (Ronson et al. 1981), and R. meliloti (Bolton et al. 1986, Engelke et al. 1987, Watson et al. 1988). This data confirmed the requirement of a functional $C_4$-dicarboxylate transport (Dct) system for nitrogen fixation in Rhizobium.

Other carbohydrates have been eliminated as potential energy sources for nitrogen fixation. Both Hudman and Glenn (1980), and De Vries et al. (1982) have demonstrated that R. leguminosarum bacteroids exhibited no uptake of glucose. Ronson and Primrose (1979) obtained R. trifolii mutants defective in enzymes of the Entner-Doudoroff and pentose phosphate pathways that remained effective symbiotically. Duncan (1981) demonstrated that Tn5 mutants of R. meliloti unable to grow on ribose, xylose, or fructose successsfully nodulated alfalfa plants. Later examination of R. leguminosarum Tn5 mutant strains (Glenn et al. 1984, Arwas et al. 1985, McKay et al. 1985) defective in gluconeogenesis enzymes (phosphoenolpyruvate carboxykinase), and metabolism enzymes (glucokinase and fructokinase) remained effective symbiotically. This demonstrated that the ability to synthesize sugars via gluconeogenesis, or to utilize particular $C_6$ and $C_{12}$ sugars was not essential for bacteroid development nor for the establishment of effective nitrogen fixation.

## $C_4$-dicarboxylate transport in other bacteria

$C_4$-dicarboxylate transport systems have been reported for several microorganisms (Kay 1978): Escherichia coli (Lo et al. 1972, Lo and Sanwal 1975a and 1975b), Pseudomonas putida (Dubler et al. 1974), and Bacillus subtilis (Fournier et al. 1972, Ghei and Kay 1972 and 1973). Evidence for E. coli suggests an active carrier-mediated transport of $C_4$-dicarboxylates involving three genetic loci: dctA, dctB, and cbt (Lo and Sanwal 1975a). Linkage was established between cbt and dctB; dctA maps elsewhere on the E. coli chromosome. $C_4$-dicarboxylate transport is induced by succinate and repressed by glucose (Lo et al. 1972). Two succinate-binding proteins, SBP2 and SBP1, have been purified together and found to correspond to products of dctA and dctB loci, respectively (Lo and Bewick 1978). The cbt gene product corresponds to a periplasmic binding protein (PBP) that binds dicarboxylates (Lo and Sanwal 1975b). In the absence of any one of these proteins, no effective transport occurs. Properties of carboxylate transport in Pseudomonas putida were similar to E. coli. Competitive inhibition studies and $K_m$ determinations indicated the order of affinity for uptake was succinate>malate>fumarate. No further characterization has been accomplished on the dicarboxylate transport systems in these bacteria.

## Molecular studies of Rhizobium $C_4$-dicarboxylic acid transport genes

To date, most of the research published concerning the molecular struture of $C_4$-dicarboxylate transport genes has been carried out in R. leguminosarum. R. leguminosarum $C_4$-dicarboxylic acid transport (dct) genes were localized to a 5 kbp region of DNA (Ronson et al. 1984, Ronson and Astwood 1985). The complementation of an R. trifolii dct mutant by recombinant plasmids from an R. leguminosarum clone bank enabled the isolation of the dct region (Ronson et al. 1984). Three Dct classes were identified in further complementation studies with Rhizobium and E. coli dct mutants; the loci were designated dctA, dctB, and dctC. Results suggested that dctA, encoding the dct structural gene, was transcribed divergently from dctB and dctC, both encoding regulatory elements. Later, recombination of Tn5 insertions from the complementing fragment into the genome led Ronson and Astwood (1985) to report another gene, dctD, between dctB and dctC. Site-directed mutagenesis showed that insertions in dctA, dctB, and dctD gave a $Dct^-$ phenotype whereas insertions in dctC resulted in a $Dct^+$ phenotype.

Inoculation of R. leguminosarum Tn5 mutants onto pea plants resulted in the reduction or elimination of nitrogen fixation: mutants of dctA formed ineffective nodules (no $N_2$ fixation), dctB and dctD mutants were partially effective, while dctC mutants were effective (Ronson and Astwood 1985). The identification of the dctC region as part of the dct

locus is thought to be an artifact. Preliminary work with the regulation of the dct region was carried out with dct transcriptional fusions to lacZ (Ronson and Astwood 1985). Results showed that the dctA-lacZ fusion was induced 10-fold by succinate whereas dctB-lacZ and dctD-lacZ fusions were constitutively expressed at a low level. Introduction of dctA-lacZ into dct mutant backgrounds indicated dctA-lacZ induction was dependent on functional DCTB and DCTD. In a dctA mutant background, the dctA-lacZ fusion was constitutively expressed.

Sequence analysis (Ronson et al. 1987a) confirmed the transcriptional arrangement of the dct genes. At the protein level DCTA was hydrophobic suggesting a highly embedded position in the membrane. Further analysis of dctA revealed an NTRA-like promoter sequence suggesting a requirement for NTRA (sigma factor 60) in the expression of dctA.

The ntrA gene (also known as rpoN) was first identified as glnF involved in nitrogen regulation (Magasanik 1982, Gottesman 1984, Magasanik and Neidhardt 1987, Reitzer and Magasanik 1987). NTRA was recently shown to be a sigma factor (Hirschman et al. 1985, Hunt and Magasanik 1985) which enabled RNA polymerase to recognize the consensus promoter sequence -26 CTGGYAYR-N4-TTGCA -10 (Gussin et al. 1986). The consensus sequence for NTRA binding has been identified in the promoter regions of R. leguminosarum dctA (Ronson and Astwood 1985), genes involved in Azotobacter vinelandii and

E. coli nitrogen regulation (Hirschman et al. 1985, Hunt and Magasanik 1985), Klebsiella nitrogen fixation (Ausubel 1984, Buck 1986), and in some E. coli genes responsible for anaerobic metabolism (Birkmann et al. 1987).

Selection was made for R. meliloti ntrA mutants on the basis that they would be unable to activate transcription of a plasmid-localized R. leguminosarum dctA-lacZ fusion (Ronson et al. 1987b). As the plasmid contained copies of dctB and dctD it was reasoned that mutants isolated would be mutated in the R. meliloti ntrA gene. R. meliloti strains mutated in ntrA and cured of the R. leguminosarum dct-containing plasmid were examined for further phenotypic properties. The ntrA strain failed to grow on defined media containing succinate as carbon source or potassium nitrate as sole nitrogen source, and failed to form $Fix^+$ nodules. This confirmed a role for NTRA in regulating several genetic loci.

Further sequence analysis of dctB and dctD (Ronson et al. 1987a) has identified DCTB and DCTD as members of a conserved family of two-component regulatory systems: one component (DCTB) is responsible for perceiving an environmental stimulus, and transmitting this signal to the second component (DCTD) for activation of the response usually at the transcriptional level. Other two-component gene systems have been found to respond to osmolarity (envZ/ompR; Matsuyama et al. 1986, Ikenaka et al. 1988), chemotaxis (cheY/cheB; Hess et al. 1988, Ninfa et al. 1988,

Parkinson 1988), nitrogen limitation (ntrB/ntrC; Drummond et al. 1986, Nixon et al. 1986), phosphate limitation in Escherichia coli (phoR/phoB; Wanner 1987, Wanner et al. 1988), and plant exudate as it affects virulence of Agrobacterium tumefaciens (virA/virG; Winans et al. 1986).

Both components of these systems have shown areas of amino acid conservation: similarities in the carboxy-terminal regions of sensor proteins such as DCTB and NTRB, and in the amino-terminal regions of the activator proteins such as DCTD and NTRC (Ronson et al. 1987a and 1987c). Sensor proteins have a conserved stretch of 200 amino acids in the carboxy-terminal region. Analysis of the R. leguminosarum DCTB amino acid sequence revealed residues 25 to 42, and 321 to 338 to contain uncharged, hydrophobic regions suggesting transmembrane localization (Ronson et al. 1987a). Regulatory proteins share some homology throughout the amino acid sequence; a region of 120 amino acids exhibit high homologies at the N-terminal of the proteins. Ronson and colleagues (1987a) have indicated that R. leguminosarum DCTD is conserved with NTRC and NIFA proteins in a central domain of the proteins and shares a helix-turn-helix motif that resembles DNA-binding domains; these regions are thought to play a role in the functioning of these proteins.

The regions of homology have suggested common mechanisms of activation of the response. Work on the NTRB-NTRC ($NR_{II}/NR_{I}$) system of E. coli by Ninfa and Magasanik

(1986) revealed NTRB to be a kinase/phosphatase. In conditions of decreased ammonia in the media (less than 1 mM), high intra-cellular 2-ketoglutarate/glutamine ratios stimulate uridylyl transferase (UTase) to uridylate protein $P_{II}$ to $P_{II}(UMP)_4$ which, unlike $P_{II}$, cannot block the phosphorylation and activation of NTRC ($NR_I$) by NTRB ($NR_{II}$) (Magasanik and Neidhardt 1987, Reitzer and Magasanik 1987). Activated NTRC then promotes transcription at other NTR promoters recognized by RNA polymerase containing NTRA (sigma factor 60). It has been suggested that similar modifications of the regulatory protein by the sensory protein result in the activation of osmolarity, phosphate, and virulence systems (Nixon et al. 1986). The conservation of amino acid sequence and the identification of similar mechanisms of activating responses to an environmental (or internal) stimulus has led Nixon et al. (1986) to propose that a sensory component of one system may activate (or repress) the regulatory component of another system resulting in a cross-communication of messages termed "crosstalk". A recent example of "crosstalk" (Ninfa et al. 1988) was demonstrated between chemotaxis signal transduction proteins and proteins regulating transcription of the Ntr regulon.

Based on the NTRB-NTRC system a model has been proposed by Ronson et al. (1987a) outlining dctA activation by the DCTB-DCTD system resulting in $C_4$-dicarboxylate transport in Rhizobium leguminosarum (Figure 1). Both DCTB

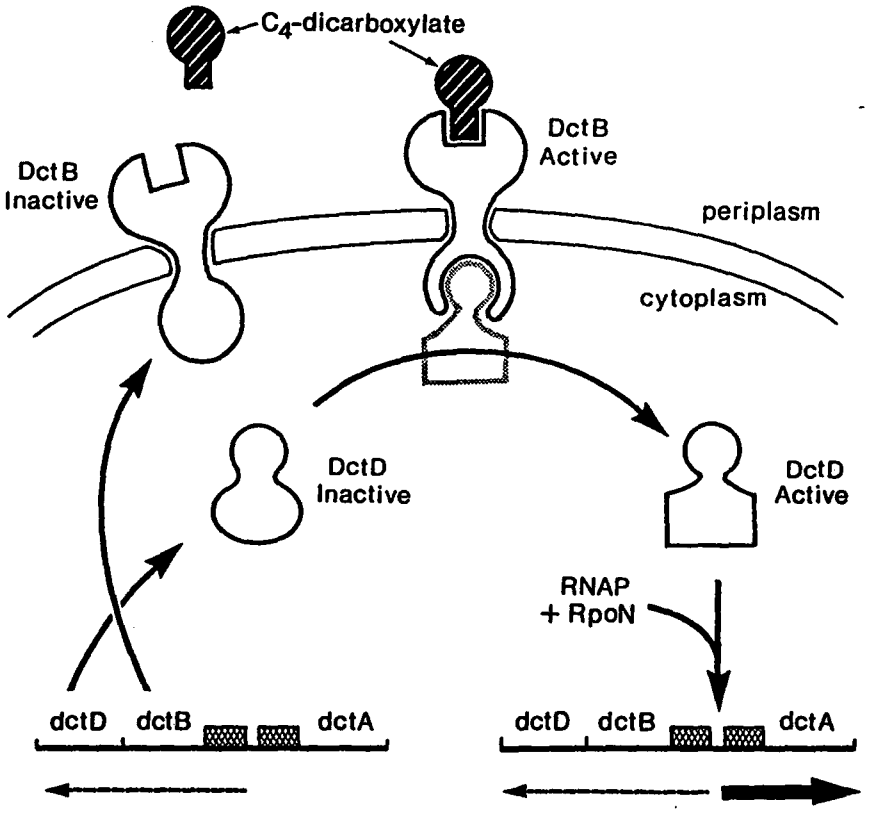



Figure 1: Model for dctA activation. From Ronson et al. (1987a).

and DCTD are constitutively expressed at a low level. The periplasmic region of DCTB binds $C_4$-dicarboxylates; this binding changes the conformation of the cytoplasmic tail and thereby activates DCTB. DCTB can then interact with the conserved N-terminal region of DCTD resulting in activation. The exact nature of this interaction is not known though an allosteric or covalent modification may be possible. It may be that DCTB exhibits kinase/phosphorylase activities as was identified with NTRB though this, as yet, has not been

demonstrated. Active DCTD interacts with the NTRA sigma factor to promote transcription at dctA. ATP may be involved in this regulation as an ATP-binding site was localized to the conserved central region of the DCTD protein.

Work is underway to characterize Dct in R. meliloti. Engelke et al. (1987) have reported two Dct loci in R. meliloti SU47. Watson et al. (1988) have found that a clone complementing Dct mutants in R. meliloti JJ1c10 could hybridize to R. meliloti SU47 megaplasmid pRmeSU47b DNA. In R. meliloti JJ1c10 the Dct locus was localized to 5.9 and 2.9 kbp EcoRI fragments.

In this work we characterized two groups of R. meliloti Dct mutants isolated by Finan et al. (1988). The two mutant classes were designated groups I and V, and differed on the basis of plasmid complementation and map location. Our initial studies focused on the chromosomally located group I mutations. Shortly after this work was initiated Ronson et al. published a detailed molecular characterization of the ntrA gene of R. meliloti (1987b). We then demonstrated that our group I mutants carried ntrA mutations. We subsequently analysed the group V mutations which map close to thi on the SU47 megaplasmid pRmeSU47b. Our data show that this region is organized and regulated in a similar manner to the dct locus described by Ronson et al. (1984, 1987a), and Ronson and Astwood (1985).

# CHAPTER 2

# MATERIALS

## Bacterial strains, phage, and plasmids

Bacterial strains utilized are listed in Table 1 along with applicable genotypes and references. Bacteriophage ΦM12 (Finan et al. 1984) was utilized for generalized transduction of *Rhizobium meliloti*. Plasmids utilized for cloning and for complementation work, and their descriptions are found in Table 2.

## Transposons

Transposon mutagenesis of *Rhizobium* DNA and of complementing DNA cloned in pRK7813 was carried out utilizing neomycin/kanamycin/streptomycin (Nm/Km/Sm) resistance-containing Tn*5* (Berg and Berg 1983) and its derivatives: Tn*5*-233, gentomycin/spectinomycin (Gm/Sp) resistant (De Vos et al. 1986); Tn*5*-132, oxytetracycline (Ot) resistant (Berg et al. 1980); Tn*V*, Nm/Km resistant, and containing the origin of replication of *E. coli* plasmid pSC101 (Furuichi and Inouye 1985); Tn*5*-*lac*, Nm/Km resistant (Kroos and Kaiser 1984); Tn*phoA*, Nm/Km resistant and containing the alkaline phosphatase gene (Manoil and Beckwith 1985).

TABLE 1: BACTERIAL STRAINS

| Strain | Genotype | Reference |
|---|---|---|
| *Escherichia coli* | | |
| MT607 | MM294A *recA-56* | Finan et al. 1986 |
| MT609 | *polA*$^-$, Thi$^-$, Sp$^r$, | T. M. Finan |
| MT614 | MT607 Ω::Tn*5* | T. M. Finan |
| MT616 | MT607 pRK2013 *npt*::Tn*9* | Finan et al. 1986 |
| MT621 | MM294A MalF::Tn*phoA* | Finan & Sorce |
| CC202 | CC118/F42 lacI3 zzf-2::Tn*phoA* | Manoil & Beckwith 1985 |
| DH5α | F$^-$, *endA1*, *hsdR17*($r_k^-$,$m_k^-$), *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*,Δ(*argF-lacz*ya)U169, Φ80d*lacz*,ΔM15 | BRL Inc. |
| *Rhizobium meliloti* [1] | | |
| Rm1021 | SU47 *str-21* | Meade et al. 1982 |
| Rm5011 | *ntrA71*::Tn*5* | Finan et al. 1988 |
| Rm5074 | *ntrA72*::Tn*5* | Finan et al. 1988 |
| Rm5300 | *thi502*::Tn*5*-11 | Finan et al. 1986 |
| Rm5312 | *dctD11*::Tn*5*-*lac* | Finan et al. 1988 |
| Rm5314 | RCR2011, *dctD16*::Tn*5* | Finan et al. 1988 |
| Rm5419 | *ntrA74*::Tn*5* | Finan et al. 1988 |
| Rm5421 | *dctB17*::Tn*5*-233 | Finan et al. 1988 |
| Rm5422 | *ntrA75*::Tn*5* | Finan et al. 1988 |
| Rm8002 | Rm1021, *phoA*$^-$ | Long et al. 1988 |
| RmF121 | *dctD16*::Tn*5* | Finan et al. 1988 |
| RmF122 | *ntrA73*::Tn*V* | Finan et al. 1988 |
| RmF152 | Ω5069::Tn*5*-132 | A. Bottacin |
| RmF153 | *dctB12*::Tn*5*-132 | A. Bottacin |
| RmF154 | Ω5071::Tn*5*-132 | A. Bottacin |
| RmF155 | Ω5072::Tn*5*-132 | A. Bottacin |
| RmF173 | Independent Dct$^+$ second-site revertant from Rm5421 | This work |
| RmF174 | Independent Dct$^+$ second-site revertant from Rm5421 | This work |
| RmF175 | Independent Dct$^+$ second-site revertant from Rm5421 | This work |
| RmF176 | Independent Dct$^+$ second-site revertant from Rm5421 | This work |
| RmF177 | Independent Dct$^+$ second-site revertant from Rm5421 | This work |
| RmF188 | Dct$^+$ second-site revertant from Rm5422 | This work |

| | | |
|---|---|---|
| RmF193 | Independent Dct$^+$ second-site revertant from Rm5419 | This work |
| RmF194 | Independent Dct$^+$ second-site revertant from Rm5419 | This work |
| RmF208 | Dct$^+$ second-site revertant from Rm5314 | I. Oresnik |
| RmF215 | Ω5085::Tn*5*-233, 93% linked to second-site mutation in RmF208 | I. Oresnik |
| RmF222 | Rm8002 (Rm1021, *phoA*$^-$) | Long et al. 1988 |
| RmF250 | Tn*5*-233 replacement of Rm5419 (*ntrA74*::Tn*5*-233) | Finan et al. 1988 |
| RmF283 | Independent Dct$^+$ second-site revertant from RmF121 | I. Oresnik |
| RmF284 | Independent Dct$^+$ second-site revertant from RmF121 | I. Oresnik |
| RmF287 | Homogenote of pSF1::Tn*5* #4, Ω5183::Tn*5* | This work |
| RmF288 | Homogenote of pSF1::Tn*5* #5, *ntrA76*::Tn*5* | This work |
| RmF289 | Homogenote of pSF1::Tn*5* #6, Ω5184::Tn*5* | This work |
| RmF291 | Homogenote of pSF1::Tn*5* #20 Ω5185::Tn*5* | This work |
| RmF314 | RmF215 x Rm1021, (Ω5085::Tn*5*-233) | I. Oresnik |
| RmF332 | *dctB18*::Tn*5* | Finan et al. 1988 |
| RmF365 | Independent Dct$^+$ second-site revertant from RmF288 | This work |
| RmF367 | Independent Dct$^+$ second-site revertant from RmF288 | This work |
| RmF369 | Independent Dct$^+$ second-site revertant from RmF288 | This work |
| RmF403 | Ω5129::Tn*5*-233, 53% linked to *ntrA71*::Tn*5* | I. Oresnik |
| RmF460 | Rm5300 x RmF154, (*thi502*::Tn*5*-11, Ω5071::Tn*5*-132) | This work |
| RmF481 | Rm8384 (Rm8002, *fix-384*::Tn*phoA*), *dctA13*::Tn*phoA* | Long et al. 1988 |
| RmF502 | RmF403 x RmF193, (Ω5129::Tn*5*-233, *ntrA74*$^+$) | This work |
| RmF504 | RmF403 x RmF188, (Ω5129::Tn*5*-233, *ntrA75*$^+$) | This work |
| RmF506 | RmF403 x RmF365, (Ω5129::Tn*5*-233, *ntrA76*$^+$) | This work |
| RmF612 | RmF332 x RmF222, (*dctB18*::Tn*5*, *phoA*$^-$) | This work |
| RmF613 | Rm5421 x RmF222, (*dctB17*::Tn*5*-233, *phoA*$^-$) | This work |
| RmF614 | RmF121 x RmF222, (*dctD16*::Tn*5*, *phoA*$^-$) | This work |
| RmF615 | Rm5312 x RmF222, (*dctD11*::Tn*5*-*lac*, *phoA*$^-$) | This work |

| | | |
|---|---|---|
| RmF617 | RmF250 x RmF222, (ntrA74::Tn5-233, phoA$^-$) | This work |
| RmF619 | RmF173 x RmF222, (dctB17::Tn5-233, Dct$^+$, phoA$^-$) | This work |
| RmF642 | Homogenote from pTH24::Tn5 insert #14, dctA14::Tn5 | This work |
| RmF643 | Homogenote from pTH24::Tn5 insert #20, dctB20::Tn5 | This work |
| RmF644 | Homogenote from pTH24::Tn5 insert #22, dctB22::Tn5 | This work |
| RmF646 | Homogenote from pTH24::Tn5 insert #25, dctB25::Tn5 | This work |
| RmF647 | Homogenote from pTH24::TnphoA insert #2C1, dctA26::TnphoA | This work |
| RmF648 | Homogenote from pTH24::TnphoA insert #2C6, dctA27::TnphoA | This work |
| RmF649 | Homogenote from pTH24::TnphoA insert #2C4, dctA28::TnphoA | This work |
| RmF651 | Homogenote from pTH31::Tn5 insert #19, dctA19::Tn5 | This work |
| RmF652 | Homogenote from pTH31::Tn5 insert #21, dctB21::Tn5 | This work |
| RmF653 | Homogenote from pTH31::Tn5 insert #23, dctB23::Tn5 | This work |
| RmF654 | Homogenote from pTH31::Tn5 insert #31, dctD31::Tn5 | This work |
| RmF655 | Homogenote from pTH31::Tn5 insert #29, dctD29::Tn5 | This work |
| RmF656 | Homogenote from pTH31::Tn5 insert #30, dctD30::Tn5 | This work |
| RmF664 | Tn5 replacement of Rm5421 (dctB17::Tn5-233) | This work |
| RmF680 | ΔΩ5085-5061::Tn5-233 | T. Charles |
| RmF693 | ΔΩ5085-5142::Tn5-233 | T. Charles |
| RmF726 | ΔΩ5079-5149::Tn5-233 | T. Charles |
| RmF728 | ΔΩ5056-5177::Tn5 | T. Charles |
| RmF738 | dctB18::Tn5Ω5085::Tn5-233 | This work |
| RmF740 | dctD16::Tn5Ω5085::Tn5-233 | This work |
| RmF746 | RmF642 x RmF222, (dctA14::Tn5, phoA$^-$) | This work |
| RmF747 | RmF651 x RmF222, (dctA19::Tn5, phoA$^-$) | This work |
| RmF748 | RmF664 x RmF222, (dctB17::Tn5, phoA$^-$) | This work |
| RmF865 | Tn5-233 replacement of RmF746 (dctA14::Tn5-233) | This work |
| RmF881 | RmF121 x RmF865, (phoA$^-$, dctD16::Tn5, dctA14::Tn5-233) | This work |
| RmF883 | RmF332 x RmF865, (phoA$^-$, dctB18::Tn5, dctA14::Tn5-233) | This work |

*Rhizobium leguminosarum*

| | | |
|---|---|---|
| GF160 | Wild-type, $Sm^r$ | Finan et al. 1981 |
| GF252 | GF160 $Dct^-$ Tn*5* | Finan et al. 1981 |
| GF31 | GF160 $Dct^-$ Tn*5* | Finan et al. 1983 |

[1] All *R. meliloti* strains are in the Rm1021 background unless otherwise indicated.

TABLE 2: BACTERIAL PLASMIDS

| Plasmid | Characteristics | Reference |
|---|---|---|
| pRK2013 | ColE1 replicon, $Nm^r$, $Tra^+$ helper plasmid | Figurski & Helinski 1979 |
| pRK600 | pRK2013 *npt*::Tn*9*, $Cm^r$, $Nm^s$ | Finan et al. 1986 |
| pRK607 | pRK2013 Ω::Tn*5*-233 | T. M. Finan |
| pRK7813 | 11.5 kbp RK2 derivative costramid vector containing pUC9 polylinker, IncP1, *cos*, $Tc^r$ | Jones & Gutterson 1987 |
| pRK7813-1 | Spontaneous deletion derivative of pRK7813, approx 10.5 kbp | This work |
| pRmT100 | pLAFR1 clone complementing group I $Dct^-$ mutants | Finan et al. 1988 |
| pSF1 | pRK7813 containing 3.5 kbp *Bam*HI-*Eco*RI *ntrA* subclone from pRmT100 | J. Stanley |
| pSF2 | *Bam*HI-*Pst*I subclone of pSF1 in pRK7813 | J. Stanley |
| pSF3 | *Pst*I-*Eco*RI subclone of pSF1 in pRK7813 | J. Stanley |
| pRmT1 | pLAFR1 clone complementing *thi502*::Tn*5*-11 | Finan et al. 1986 |
| pRmT8 | pLAFR1 clone complementing *thi502*::Tn*5*-11 and group V $Dct^-$ mutants | Finan et al. 1988 |
| pTH24 | 6.0 kbp *Hind*III subclone containing *dct* in pRK7813 | This work |
| pTH31 | 5.1 kbp *Eco*RI subclone of *dct* from pTH24 into pRK7813-1 | This work |
| pTH32 | 2.2 kbp *Hind*III-*Eco*RI *dct* subclone from pTH24 into pRK7813-1 | This work |
| pTH33 | 2.9 kbp *Eco*RI *dct* subclone from pTH24 into pRK7813-1 | This work |
| pTH2A | pTH32 *dctA*::Tn*phoA* | This work |
| pTH2C6 | pTH24 *dctA*::Tn*phoA* *dctB*$^+$ *dctD*$^+$ | This work |
| pPH1JI | IncP1, $GmSp^r$, used in homogenotizations | Ruvkun & Ausubel 1981 |

## Media

All media was autoclaved prior to use. Escherichia coli strains were grown at 37°C on LB agar (Miller 1972) containing 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 mM NaOH, and 15 g Difco Nobel agar per litre of water. For liquid cultures of E. coli, agar was omitted and NaOH concentration was increased to 4 mM. Rhizobium meliloti was grown on LB agar or in LB broth supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ after autoclaving. Optimum temperature for growth was 30°C.

Complex media for Rhizobium leguminosarum was TY containing 5 g tryptone, 3 g yeast extract, 3 mM $CaCl_2$ and, when required, 15 g of Difco Nobel agar per litre of water. TY broth supplemented with 15 mM succinate was used to grow up cultures of R. meliloti and R. leguminosarum for succinate uptake assays.

To examine utilization of carbon sources R. meliloti strains were grown in minimal M9 salts media (Miller 1972) containing, per litre, 5.8 g $Na_2HPO_4$, 3.0 g $KH_2PO_4$, 0.5 g NaCl, 1.0 g $NH_4Cl$ and, when required, 15 g agar. After autoclaving, $MgSO_4$, $CaCl_2$, and biotin were added to give a final concentration of 1 mM, 0.25 mM and 0.5 mg/l respectively. Carbon sources such as glucose or succinate were added at a concentration of 15 mM. For analysis of nitrate utilization, $NH_4Cl$ was replaced with either 5 mM or 0.5 mM $KNO_3$.

Minimal media for studies of complementation of R. leguminosarum mutants was Basal Salts (BS) agar (Jordan 1984) containing 0.2 mM $CaCO_3$, 0.1 mM NaCl, 2.0 mM $KH_2PO_4$, 6.0 mM $K_2HPO_4$, 1X Trace elements solution, 20 mM $NH_4Cl$ and 15 g Difco Nobel agar per litre. After autoclaving the media, $MgSO_4$, appropriate carbon source, and 2 M MOPS in 1 M KOH were added to final concentrations of 4 mM, 15 mM and 20 mM, respectively.

Media for generalized transduction (1/2 LB 1/2 M9) consisted of combining 150 ml of LB agar containing twice the usual amount of agar with 150 ml of M9 salts.

Water agar plates for the germination of alfalfa seeds contained 15 g Difco Nobel agar per litre. 2X Jensen's media (Jensen 1942) for plant nodulations contained, per litre, 2.0 g $CaHPO_4$, 0.4 g $K_2HPO_4$, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.4 g NaCl, 0.2 g $FeCl_3$, and 2.0 ml of 1000X Trace elements solution. 2X Jensen's media was made to pH 7.0 prior to diluting 1:1 with water and adding to pots. 1000X Trace elements solution consisted of 1.0 g $H_3BO_3$, 1.0 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $CuSO_4 \cdot 5H_2O$, 0.5 g $MnCl_2 \cdot 4H_2O$, 1.0 g $NaMoO_4 \cdot 2H_2O$, 10 g EDTA, 2.0 g NaFe·EDTA, and 0.4 g biotin per litre.

## Antibiotics and indicators

For selection of Tn5 and its derivative transposons the following antibiotic concentrations were added to the

media: 10 μg/ml tetracycline (Tc), 0.35-0.5 μg/ml oxytetracycline (Ot), 200 μg/ml neomycin (Nm), 200 μg/ml streptomycin (Sm), 5 μg/ml gentamycin (Gm), 100 μg/ml spectinomycin (Sp), 20 μg/ml chloramphenical (Cm), 20 μg/ml kanamycin (Km), and 50 μg/ml ampicillin (Am).

For selection of complementing fragments cloned into the polylinker contained in the lacα fragment of pRK7813, media was supplemented with 20 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). For Tn*phoA* mutagenesis 20 μg/ml of 5-bromo-4-chloro-3-indolyl phosphate (X-Phos) was added to the media.

## Chemicals

Antibiotics used were obtained from Sigma or Boehringer Mannheim. Restriction enzymes and T4 DNA ligase were obtained from Boehringer Mannheim or from BRL Life Technologies, Inc. Sodium succinate was purchased from Sigma, and X-Phos was obtained from Diagnostic Chemicals Ltd. X-Gal and 4-nitrophenyl phosphate (NPP) were purchased from Boehringer Mannheim. [$\alpha$-$^{32}$P]- deoxycytidine 5'-triphosphate (830 Ci/mmole) was obtained from ICN Biomedicals Canada Ltd., while [2,3-$^{14}$C]- succinic acid (56.0 mCi/mmol) was purchased from New England Nuclear Research Products. Nick Translation Reagent Kit was from BRL Life Technologies, Inc. Sephadex G-25 was obtained from Pharmacia Fine Chemicals AB. Other chemicals were reagent

grade and were obtained from Fisher Scientific Company, Difco Laboratories, Bio-Rad Laboratories, BDH Chemicals, or Sigma.

## Equipment

For spectrophotometric analysis a table-top Bausch & Lomb Spectronic 20 was used. A Beckman GPR Tabletop centrifuge, Beckman ultracentifuge and Sorvall centrifuges were used to pellet bacterial cells and DNA. Succinate uptake assays were carried out on an Amicon manifold vacuum apparatus.

# CHAPTER 3

# METHODOLOGY

## Rhizobium total DNA extraction

*Rhizobium* strains were grown up overnight in 2.5 ml of $LB^{mc}$ broth. Cells were pelleted at 4000 rpm, washed in 5 ml of cold 0.85% NaCl followed by 5 ml of cold TES (10 mM Tris·HCl pH 8.0, 25 mM EDTA pH 8.0, 150 mM NaCl). Cells were resuspended in 2.5 ml of cold $T_{10}E_{25}$ (10 mM Tris·HCl, 25 mM EDTA, pH 8.0) to which was added 0.25 ml of lysozyme (2 mg/ml in $T_{10}E_{25}$ pH 8.0). The tubes were incubated at 37°C for 15 min. To this was added 0.3 ml of Sarkosyl-protease (10% Sarkosyl, 5 mg/ml of pronase E in $T_{10}E_{25}$) and the mixture incubated at 37°C for 60 min. Phenol, phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) extractions were each carried out twice. DNA was precipitated by adding 5 M ammonium acetate to 0.3 M (about 0.15 ml) and dropwise adding 0.54 volumes of isopropanol (about 1.5 ml) while gently swirling until a white string of DNA was evident. The DNA precipitate was collected with a pipette and dissolved in 0.2 ml of $T_{10}E_1$ pH 8.0 for 30 min at 37°C.

## Plasmid preparation

### Small scale boiling preparation

*E. coli* strains were grown overnight in 5 ml of LB supplemented with the appropriate antibiotic. 1.5 ml was transferred to an eppendorf tube and centrifuged for 20 sec. The pellet was resuspended in 0.35 ml of STET (8% sucrose, 0.5% Triton X-100, 50 mM EDTA pH 8.0, 50 mM Tris·HCl pH 8.0) to which was added 10 $\mu$l of freshly prepared lysozyme solution (30 mg/ml in 10 mM Tris·HCl pH 8.0). The tubes were placed into a boiling water bath, the heat was shut off, and the tubes were allowed to stand for 3 min. The mixture was centrifuged immediately and the pellet debris removed. 330 $\mu$l of isopropanol was added to the supernatant and the DNA was precipitated for 15 min at -80°C. The plasmid DNA was centrifuged for 10 min at 4°C, washed in 70% ethanol and resuspended in 50 $\mu$l of $T_{10}E_1$. To dissolve the DNA the tube was placed in a 65°C water bath for 15 min. 8 $\mu$l of DNA was used per restriction mix.

### Alkaline lysis preparation

For large-scale DNA preparations, *E. coli* strains were grown up in 250 ml LB cultures supplemented with the appropriate antibiotic and pelleted in centrifuge bottles at 6000 rpm for 15 min at 4°C. Pellets were resuspended in 20 ml of TEG (50 mM Tris·HCl pH 7.8, 20 mM EDTA pH 8.0, 1% glucose) to which was added 10 mg of lysozyme. 40 ml of ALS (0.2 M NaOH, 1% SDS) was added to the bottles and mixed.

This was followed by 90 ml of sterile $dH_2O$ and 30 ml of high salts solution (HSS) (3 M potassium acetate, pH 4.8). The mixture was left at -70°C for 15 min, spun at 6000 rpm for 10 min at 4°C, and the supernatant passed through cheesecloth into new centrifuge bottles. 90 ml of isopropanol was added to precipitate the DNA at -20°C for 20 min. The pellet was collected by centrifuging at 6000 rpm for 10 min. After washing the pellet with 95% ethanol, the DNA was resuspended in 9.0 ml of $T_{50}E_{20}$ pH 7.8 in which was dissolved 9.9 g of cesium chloride. The solution was transferred to a 13 ml Beckman centrifuge tube containing 0.5 ml of 10 mg/ml ethidium bromide, topped off with paraffin oil, and sealed. The tubes were centrifuged in a Beckman 65.1 centrifuge rotor at 55,000 rpm, 10°C for 18 hours. The plasmid band was removed using a syringe. Isopropanol saturated with CsCl in $dH_2O$ was used to extract the ethidium bromide out of the DNA until no further pink colour was obtained in the aqueous DNA layer. Two volumes of $dH_2O$ and six volumes of 95% ethanol were added to precipitate the DNA solution overnight at -20°C. After pelleting the plasmid DNA at 8000 rpm for 15 min at 4°C, the DNA was resuspended in 400 $\mu$l of $T_{50}E_{20}$ pH 8.0 and reprecipitated with 10 $\mu$l 5 M NaCl and with 800 $\mu$l of 95% ethanol. The pellet was collected, dried and resuspended in 200 $\mu$l of $T_{20}E_1$ pH 8.0. One $\mu$l of plasmid DNA was run on a minigel.

Alternately, the large-scale preparation was carried out until the ethanol wash step was completed and the pellet had been dried and resuspended in 2.0 ml of $T_{10}E_1$ pH 8.0. Ten $\mu$l of a 10 mg/ml RNAase solution was added and the mixture incubated for 15 min at 37°C. Two ml of 13% PEG 8000 1.6 M NaCl solution was added and the tubes were stored on ice for 30 min. The DNA was spun down and resuspended in 1.0 ml $T_{20}E_1$ pH 8.0 and phenol, phenol/chloroform, and chloroform extracted. After ethanol precipitation with 2.0 volumes of 95% ethanol, the DNA was dried and resuspended in 200 $\mu$l of $T_{20}E_1$ pH 8.0.

A small-scale version of this preparation was also carried out using a 10 ml culture of cells and reagent volumes subsequently scaled down. The DNA was resuspended in 50 $\mu$l and 5 $\mu$l used per restriction analysis.

## DNA restriction analysis

For the determination of restriction enzyme cleavage sites and resulting DNA fragment lengths, 100-500 ng of plasmid DNA contained within 0.5-3.0 $\mu$l of $T_{10}E_1$ was cut with 1-3 units of appropriate restriction endonuclease in a total mix volume of 20-30 $\mu$l of solution. The restriction mix contained the appropriate 10X salt buffer diluted to 1X in the total volume as recommended by protocols from Boehringer Mannheim or BRL.

Restriction mixes were incubated for 1-4 hours in a

37°C water bath. 10X loading buffer (0.25% bromophenol blue, 25% Ficoll (type 400) in $dH_2O$; Maniatis et al. 1982) was added to a final concentration of 1X. DNA fragments were separated on an 0.8%-1.2% agarose gel by electrophoresis at 10-50 volts. Running buffers used were either 1X TAE (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 8.0 with glacial acetic acid; G. De Vos, personal communication), or 1X TBE (89 mM Tris·borate, 89 mM boric acid, 2 mM EDTA pH 8.0; Maniatis et al. 1982).

Restricted bands were detected by staining the gel with ethidium bromide solution (0.5 μg/ml) and visualizing by ultra-violet light.

Purification of DNA fragments from agarose gels

Five μg of plasmid DNA to be gel purified was thoroughly restricted with appropriate restriction endonucleases and fragments separated by electrophoresis in 1.0-1.2% large agarose gels utilizing 1X TBE running buffer. One-twentieth of the restriction mix was loaded into a well designated for ethidium bromide staining and UV visualization of fragment location. The remainder of the restriction mix was distributed into two to four adjacent wells. After fragment separation appropriate bands were isolated from the gel and the finely chopped gel corresponding to those fragments was distributed among several eppendorfs. After adding 0.5 ml of phenol to each

tube and vortexing, the eppendorfs were frozen in liquid nitrogen for 15 min and centrifuged for 15 min. The DNA-containing aqueous layer was phenol and phenol/chloroform extracted twice, and chloroform extracted once. DNA was precipitated with 1/10 volume of 5 M ammonium acetate and 2 volumes of isopropanol. After pelleting, the DNA was resuspended in 1/2 of the original restriction volume and quantified by mini-gel electrophoresis.

## Ligation reactions

Each ligation mix contained: 2 μg of restricted vector DNA, 750 ng of the DNA fragments to be cloned, 1 unit of T4 DNA ligase, 6 μl of 5X ligase reaction buffer (final concentration 50 mM Tris·HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 5% (w/v) polyethylene glycol-8000) and sterile $dH_2O$ to 30 μl total volume. The DNA was ligated overnight at 15°C, precipitated with 3 μl 5 M ammonium acetate and 60 μl 95% ethanol, and resuspended in a final volume of 30 μl. To confirm ligation, 5 μl of the ligation mix was examined on a minigel. For transformation into competent cells, 5 μl of the ligation mix was used.

## Transformation of E. coli cells

An 8.0 ml culture (OD 675nm 0.2-0.4) of E. coli cells to be transformed was chilled on ice for at least 10 min in screw-top centrifuge tubes. Cells were spun down at 5000 rpm for 5 min in a chilled rotor, resuspended in 2.0 ml of chilled 100 mM $MgCl_2$ (in 0.85% NaCl), and incubated on ice for 10 min. After pelleting at 4000 rpm, 10 min, 4°C, cells were resuspended in 4 ml of chilled 100 mM $CaCl_2$ (in 0.85% NaCl) and incubated on ice for one hour. After a final spin (as previously) pellets were resuspended in 0.8 ml 100 mM $CaCl_2$.

For each transformation, 1-2 $\mu$l of plasmid DNA was combined with 50 $\mu$l of chilled 100 mM $CaCl_2$ (in 0.85% NaCl) and added to 200 $\mu$l of bacterial cells contained in an eppendorf tube. After incubating on ice for 30 min, the mixture was placed at 42°C for 90 sec and again on ice for 2 min. One ml of LB broth was added and phenotypic expression allowed for at least one hour at 37°C. Cells were spun down and pellets were resuspended in 0.5 ml 0.85% NaCl; 0.1 ml was plated onto antibiotic media selecting for plasmid transformation. Transformed colonies grew up overnight at 37°C.

## Complementation analysis

For complementation of R. meliloti Dct$^-$ mutations, donor strains (E. coli containing the constructed pRK7813-derived plasmid), MT616 (containing pRK600 with the

mobilizing genes), and recipient R. meliloti mutant strains were grown in 5 ml overnight LB cultures supplemented with appropriate antibiotics. Cultures were spun down and pellets resuspended in 5 ml saline. Donor, mobilizer and recipient strains were spotted 1:1:1 onto LB agar. Mating was allowed to occur overnight at 30°C. Mating spots were restruck onto media selecting for transfer of the plasmid into the R. meliloti mutant strain (LB Tc Sm), and carbon phenotypes of the resulting strains were examined on M9 media containing the required carbon source (15 mM succinate or 15 mM glucose).

For complementation of R. leguminosarum Dct$^-$ mutations the procedure was modified as follows. Cells from plates of recipient R. leguminosarum strains were inoculated into the liquid spots containing the donor and mobilizer strains on TY media. Matings were allowed to occur overnight at 30°C. Spots were restruck onto TY Tc (3 μg/ml) Sm (100 μg/ml) to select for plasmid transfer. Carbon phenotypes of the resulting strains were examined on BS media containing Sm and 15 mM of the appropriate carbon source.

## Tn5 mutagenesis

pRK7813-derived plasmids containing a region of DNA complementing the mutants were transformed into competent MT614 cells (refer to Transformation of E. coli cells).

MT614 is an E. coli MT607-derivative containing a chromosomal Tn5 insert. Plasmids containing an inserted Tn5 element were mobilized from MT614 into the E. coli polA1 strain MT609 using the mobilizing function of pRK600 found in MT616 by carrying out the spot-mating technique outlined previously. Mating spots were resuspended in 1.0 ml of 0.85% NaCl and 0.1 ml was plated onto LB agar containing 100 μg/ml Sp, 20 μg/ml Km, and 2 μg/ml of Tc. Resultant colonies were used to grow up cells for plasmid preparations to examine insertion locations within the plasmid.

## TnphoA mutagenesis

The plasmid to be mutagenized (pTH24 or its derivatives) was transformed into E. coli CC202, a strain containing an F' TnphoA insertion. To select for TnphoA transposition a triparental mating using CC202/pTH24 as the donor strain, MT616 as mobilizing strain, and RmF222 (a phoA$^-$ derivative of Rm1021) as recipient strain was carried out as outlined previously; mating spots were plated on M9 agar containing X-P (5-bromo-4-chloro-3-indolyl-phosphate ) (20 mg/l), Sm (200 μg/ml), Nm (200 μg/ml), 15 mM succinate, and 1% casamino acids. Transconjugants were obtained after three days of growth at 30°C. Blue colonies indicating fusion protein TnphoA expression were purified and moved, via a triparental mating, back into an E. coli background, such as MT609, for storage purposes. To examine Dct the

TnphoA insertion-containing plasmid was moved, using triparental matings, into R. meliloti phoA$^-$ backgrounds with the required dct, ntrA, or second-site mutations.

## Homogenotizations

Homogenotizations were carried out similarly to that described by Ruvkin and Ausubel (1981). R. meliloti transconjugant colonies, containing Tn5-mutagenized plasmid DNA to be homogenotized into R. meliloti chromosomal or megaplasmid DNA, were grown up in 2.0 ml LB broth supplemented with Tc. E. coli 2174 (J53/pPH1JI) was grown up in 5.0 ml of LB, and 0.5 ml of R. meliloti culture was mixed with 0.1 ml of E. coli cells, pelleted and resuspended in 0.1 ml LB. 25 $\mu$l of the mixture was spotted onto LB agar and grown overnight at 30°C. Spots were resuspended in 0.5 ml 0.85% NaCl and plated onto LB agar containing 250 $\mu$g/ml Sm, 70 $\mu$g/ml Gm, and 150 $\mu$g/ml Nm. R. meliloti colonies grew up in three days and were purified on LB Gm Nm.

To eliminate pPH1JI, the purified homogenotized strain was used to prepare a phage lysate, and generalized transduction into Rm1021 was carried out selecting for the Tn5 resistance marker (refer to Generalized transduction).

## Revertant selection

To select for revertants of mutant strains to the wild type phenotype ($Dct^+$) independent colonies of R. meliloti $Dct^-$ strains were grown overnight in 5 ml $LB^{mc}$ cultures. Cells were spun down at 4000 rpm, washed twice in 0.85% saline, and resuspended in 5 ml saline. 0.1 ml of each culture was plated separately onto M9 media containing 15 mM succinate. Viable counts were determined by carrying out serial dilutions and plating $10^{-5}$, $10^{-6}$, and $10^{-7}$ per strain on LB plates. One week was allowed for revertant growth.

Revertants were screened for loss of the transposon resistance by patching colonies on M9 plates with 4.5 mM succinate as the carbon source and containing the appropriate antibiotic in concentrations given previously.

## Generalized transduction

Phage lysates were prepared by inoculating 5 ml $LB^{mc}$ cultures of required R. meliloti strains (OD 675nm 0.3-0.4) with 0.1 ml of phage Φ(Rm1021). Cultures were incubated for six hours at 30°C or until lysis was complete. 0.1 ml of chloroform was added and dead cells were spun down. Phage lysate was transferred to a screw-top tube and stored at 4°C.

For generalized transductions, 1.0 ml of phage lysate diluted 1/30-1/20 in $LB^{mc}$ was mixed with 1.0 ml of

the recipient culture previously grown overnight in $LB^{mc}$. The mixture was incubated for 20 min after which time cells were washed three times in 0.85% NaCl. Pellets were resuspended in 0.5 ml 0.85% NaCl, and 0.1 ml was plated onto 1/2 LB media containing appropriate antibiotics selecting for the phage resistance marker. Resistant colonies that grew up after three to four days of incubation at 30°C were patched onto M9 media containing 15 mM succinate to verify the Dct phenotypes of the resulting colonies and onto LB supplemented with antibiotic to determine the transductional linkage. Distances of transductional linkage in kb were calculated according to Wu (1966) using a phage size of 160 kb.

## Utilization of nitrogen sources

To examine R. meliloti nitrogen source utilization media used was M9 agar, made up without a nitrogen source, and supplemented with biotin, magnesium sulfate, calcium chloride, and glucose as previously indicated. Nitrogen sources were either 5 mM ammonium chloride or 5 mM potassium nitrate. R. meliloti mutant strains were streaked out and growth patterns examined after four days of growth. R. leguminosarum strains were similarly tested utilizing BS agar, made up without a nitrogen source, and supplemented with biotin, magnesium sulfate, MOPS, glucose, and either 5 mM ammonium chloride or 5 mM potassium nitrate.

Media used for growth curves was M9 broth made up without ammonium chloride. After autoclaving, the broth was supplemented with biotin, magnesium sulfate, calcium chloride and glucose as previously indicated. Nitrogen sources added to the media were: 5 mM ammonium chloride, 5 mM potassium nitrate, or 0.5 mM potassium nitrate. R. meliloti strains were grown up overnight in 5.0 ml of $LB^{mc}$. Cells were spun down and washed twice in 0.85% NaCl and pellets were resuspended in 2.0 ml of saline. Tubes were inoculated in triplicate per nitrogen source per strain to an initial OD 675 nm of approximately 0.05. Cultures were grown on a rotating wheel at 30°C and the OD 675 nm recorded at a variety of time intervals. Growth curves were not carried out with R. leguminosarum strains.

## Utilization of carbon sources

Media used was M9 agar supplemented with biotin, magnesium sulfate and calcium chloride as described previously. Required carbon sources were added in concentrations of 15 mM. R. meliloti mutant strains were streaked out and growth patterns examined after four days of growth.

For broth cultures method used was as for determination of nitrogen utilization. Media used for the growth curves was M9 broth which, after autoclaving, was supplemented with biotin, magnesium sulfate and calcium

chloride as outlined previously. Carbon sources added were 15 mM succinate or 15 mM glucose.

## Southern transfer

R. meliloti total DNA was cleaved with desired restriction endonucleases and resulting fragments separated on a 1.2% agarose gel in 1X TAE running buffer. After staining with ethidium bromide, the gel was washed twice for 5 min in 0.25 M HCl, twice for 30 min in 1.5 M NaCl/0.5 M NaOH, and twice for 30 min in 1.0 M Tris·HCl, pH 8.0/0.6 M NaCl. Nitrocellulose paper was prepared to the required size and prewetted in 2X SSC. 20X SSC was prepared according to Maniatis et al. (1982): one liter contained 175.3 g of NaCl, 88.2 g of sodium citrate, and adjusted to pH 7.0 with HCl. Nitrocellulose was applied to the gel and blotted for 4-14 hours with 10X SSC (Southern 1975, Maniatis et al. 1982). The blotted nitrocellulose paper was soaked in 6X SSC for 5 min, drained, dried at 37°C and baked at 80°C for 1-2 hours.

## Preparation of radiolabelled probe

Nick translation of plasmid DNA used for radioactive probing was carried out according to the kit and protocol of BRL Life Technologies, Inc. To an eppendorf were added the following: 5 $\mu$l of nucleotide mix A2 (containing dATP, dGTP, and dTTP), 1 $\mu$l of plasmid DNA, 5 $\mu$l of DNA polymerase I/

DNAase I, 156 pmol deoxycytidine 5'-[$\alpha$-$^{32}$P]triphosphate, and $dH_2O$ to 50 $\mu$l. Reaction mix was incubated at 15°C for one hour and the reaction terminated with 5 $\mu$l of 300 mM $Na_2EDTA$, pH 8.0. Volume was brought up to 105 $\mu$l with STE (10 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl), and the mix applied through a spin column (Maniatis et al. 1982) to eliminate unincorporated nucleotides.

## Hybridization of nitrocellulose blots

Heat sealable bags were utilized for both the prehybridization and hybridization reactions.

DNA blots were prehybridized at 42°C for at least 15 min in the following solution: 5X SSC, 50 mM phosphate buffer pH 7.0, 50% deionized formamide, 1X Denhardt's, and 100 $\mu$g/ml herring or salmon sperm DNA. Four ml of prehybridization solution was added per 100 $cm^2$ of blot. 20X SSC and 100X Denhardt's were prepared according to Maniatis et al. (1982). After prehybridization the solution was removed from the blots.

25 $\mu$l of radioactive probe was added to 100 $\mu$l of herring or salmon sperm DNA; the DNA was denatured by adding 0.1 volume of 1 M NaOH, and allowing denaturation for 10 min at room temperature. 0.1 volume of 1 M HCl was added to neutralize the mixture. Hybridization solution (consisting of prehybridization solution minus the sperm DNA) was added to the blots in a concentration of 2 ml per 100 $cm^2$ of blot.

The radioactive probe solution was added to the hybridization solution prior to its addition to the blots. Hybridization was carried out at 42°C for 24 hours.

Nitrocellulose blots were washed twice for 15 min at RT in 2X SSC/1X Denhardt's, twice for 20 min at RT in 2X SSC/0.1% SDS, and three times for 20 min at 65°C in 0.1X SSC/0.1% SDS. Blots were wrapped in plastic wrap and exposed to X-ray film at -70°C.

## Alkaline phosphatase assays

R. meliloti strains to be examined for alkaline phosphatase expression were grown overnight at 30°C in 5 ml cultures of LB broth and in 5 ml cultures of M9 media supplemented with magnesium sulfate, biotin, and calcium chloride (amounts as previously indicated) and 15 mM glucose as carbon source.

Assay method was according to Brickman and Beckwith (1975). Strains were spun down at 4000 rpm, washed three times with saline, and resuspended in 2 ml of M9 broth containing $CaCl_2$, $MgSO_4$, and biotin. Cells of strains grown in LB broth (an inducer of dicarboxylate transport) were used to inoculate 5 ml of M9 broth (no carbon source) to an OD 600 nm of 0.4 and immediately assayed as reported further. Cells of strains grown in M9 broth supplemented with glucose as a carbon source were used to inoculate 5 ml of M9 broth containing 15 mM glucose as well as a second

tube of 5 ml of M9 broth containing 15 mM succinate, both to an OD 600 nm of 0.4. The cells in M9 glucose were assayed immediately; the cells transferred to the succinate-containing media were Dct-induced for four hours at 30°C prior to assaying.

For the assay (Brinkman and Beckwith 1975) 1 ml in triplicate was transferred from each media tube to a small test tube and pelleted at 4000 rpm. The cells were resuspended in 4.5 ml Tris·HCl pH 8.0 and equilibrated in a 37°C water bath. The assay was commenced with the addition of 0.5 ml NPP (p-nitro-phenyl-phosphate), the tube was vortexed, and placed at 37°C. Incubation time varied from 30 to 60 min; this was later used in the calculation of alkaline phosphatase activity. To terminate the reaction 0.6 ml of 1 M $KH_2PO_4$ was added to the tubes and OD readings at 420 and 600 mn were recorded for each tube. To calculate phosphatase specific activity the following equation was used:

$$1000 \times \frac{(\text{OD 420 nm} - 1.5 \times \text{OD 600 nm})}{(\text{time (min)} \times \text{OD 600 nm})}$$

Assuming a molar extinction coefficient of 16,000 for p-nitrophenyl, 1 unit is equivalent to 0.062 nmoles of p-nitrophenyl phosphate hydrolysed per min at a cell optical density at 600 nm of 1.

## $C_4$-dicarboxylic acid transport assays

Assays for the uptake of [2,3-$^{14}$C] succinate was carried out as described by Finan et al. (1981). Free-living cells to be assayed for succinate transport activity were grown as 5 ml overnight cultures in TY succinate cultures and used to inoculate 50 ml TY cultures also containing succinate. Alternatively, cultures were grown in M9 minimal media supplemented with $CaCl_2$, $MgSO_4$, biotin, and the required carbon source in concentrations described earlier. Cells were pelleted at 4000 rpm for 10 min at 4°C and resuspended in TAS solution (40 mM MOPS pH 7.0, 20 mM KOH, 4 mM $MgSO_4$, 20 mM $NH_4Cl$, 0.2 mM $CaCO_3$, 0.1 mM NaCl, 1.2 mM $K_2HPO_4$, and 0.4 mM $KH_2PO_4$). Pellets were washed three times, resuspended in 1-2 ml of TAS solution to give a protein concentration of about 2 mg/ml and maintained on ice.

For assays, 30 $\mu$l of cell suspension was added to 445 $\mu$l of TAS solution in a 10 x 75 mm test tube and incubated at 30°C for 5 min. 25 $\mu$l of 1 mM [2,3-$^{14}$C] succinate (50 $\mu$Ci/umole) was added and the mixture was vortexed. 100 $\mu$l samples were removed at 15 or 30 sec intervals (up to 3 min total), filtered through Millipore 0.45 $\mu$m nitrocellulose filters, and immediately washed with TAS solution. Filters were dried under a heat lamp and placed into vials containing 10 ml of scintillation fluid (4 g 2,5-diphenyloxazole and 0.1 g 1,4-bis-[2-(4-methyl-5-

phenyloxazolyl)]-benzene per litre of toluene). For background counts wild-type cells were incubated with 2% toluene for 30 min at 30°C; 30 µl was removed into TAS solution and assayed as above.

## Protein determination

Protein determination was according to Lowry et al. (1951). Cell cultures used for the transport assay were diluted 1:1 in 1 N NaOH and solubilized by boiling in a screw-top centrifuge tube for 15 min. Dilutions (1:10 - 1:15) were further carried out with 1 N NaOH. For the assay 0.075 ml of 1 N NaOH was added to 0.075 ml of cells and 1.35 ml of $dH_2O$; to this was added 1.5 ml of Reagent A (2 ml 2% $CuSO_4$, 2 ml 4% Na·K·tartrate and 96 ml 6% $Na_2CO_3$), and test tubes were allowed to stand for 10 min prior to the addition of 0.15 ml of Folin reagent (diluted to 1 N with $dH_2O$). Colour development was allowed to occur for 15 min and optical density recorded at 750 nm. Bovine serum albumin (100 µg/ ml) was used as the protein control.

## Preparation and maintenance of inoculated alfalfa seedlings

### Preparation of Leonard pots

Nodulation pots were prepared according to a modification of Leonard (1943). Plastic pots for the top unit of the assembly contained a 1.0 cm diameter hole in the bottom for drainage. Rolled cotton batting (approximately

10 cm in length) was inserted through the hole leaving about 8 cm extending away from the pot. A wad of cotton was also placed in the bottom of the jar. The pot was placed inside a 250 ml beaker and filled to within 2 cm of the top with 1:1 quartz sand:vermiculite mixture, which was then soaked with 200-250 ml of 1X Jensen's media (2X Jensen's diluted with $dH_2O$). The pot top and the pot-beaker connection were sealed with aluminium foil, and the unit autoclaved for two hours.

Preparation of seedlings

For an estimation of seed number, 1 g of Medicago sativa var. Iroquois corresponded to approximately 400 seeds. This was sufficient for 40 pots as 10 alfalfa seedlings were planted per pot. Seeds were soaked in a 3.5% v/v Chlorox solution for 20 minutes and rinsed with sterile $dH_2O$ repeatedly over an hour. Using a sterile spatula, seeds were spread on water agar plates and allowed to germinate in the dark at room temperature for two days. Sterile technique was used to plant seedlings 1-2 cm below the vermiculite surface. Seedlings were subsequently maintained under the following conditions: 16 hr days at 25°C and 8 hr nights at 19°C.

## Inoculation of seedlings

Each R. meliloti strain was inoculated into triplicate pots. Strains for inoculation of four day-old seedlings were grown overnight in triplicate in 2 ml of $LB^{mc}$, spun down, and resuspended in 10-20 ml of sterile $dH_2O$. Each tube was used to inoculate one pot. Seedlings were watered with sterile $dH_2O$ every four days, or as required.

## Acetylene reduction assays

Acetylene reduction assays were performed on seedlings 28-32 days after inoculation with R. meliloti cultures. Assays were performed on a gas chromatograph. For each strain tested plants were removed from the pots and separated out. Nine representative plants were distributed among three assay test tubes (18 mm x 150 mm). Prior to commencement of the assay stem tops were removed and test tubes were stopped up with rubber stoppers. Acetylene gas (3 ml) was injected per tube, and the reaction allowed to proceed for 15 min. After 15 min, 0.3 ml of gas was removed from the tube and injected into the analyzer unit of the gas chromatograph. Three peaks were evident on the recorder with the units of the second peak correlating with the amount of ethylene produced. For a standard measurement, 3.2 nmol of ethylene (in nitrogen) was injected into the analyzer unit, and corresponding units on the recorder used

to calculate ethylene production (in nmoles) by R. meliloti bacteroids. For the calculation of mean nmols of ethylene produced per hour per plant the following equation was applied:

$$\frac{(a \text{ nmol}/15 \text{ min}) \times 53 \times 4}{b}$$

where a is total nmol obtained per three test tubes of the strain per 15 min, b represents the total number of plants assayed (usually nine), and the dilution factor (53.3) represents 0.3 ml out of a total test tube volume of 16 ml.

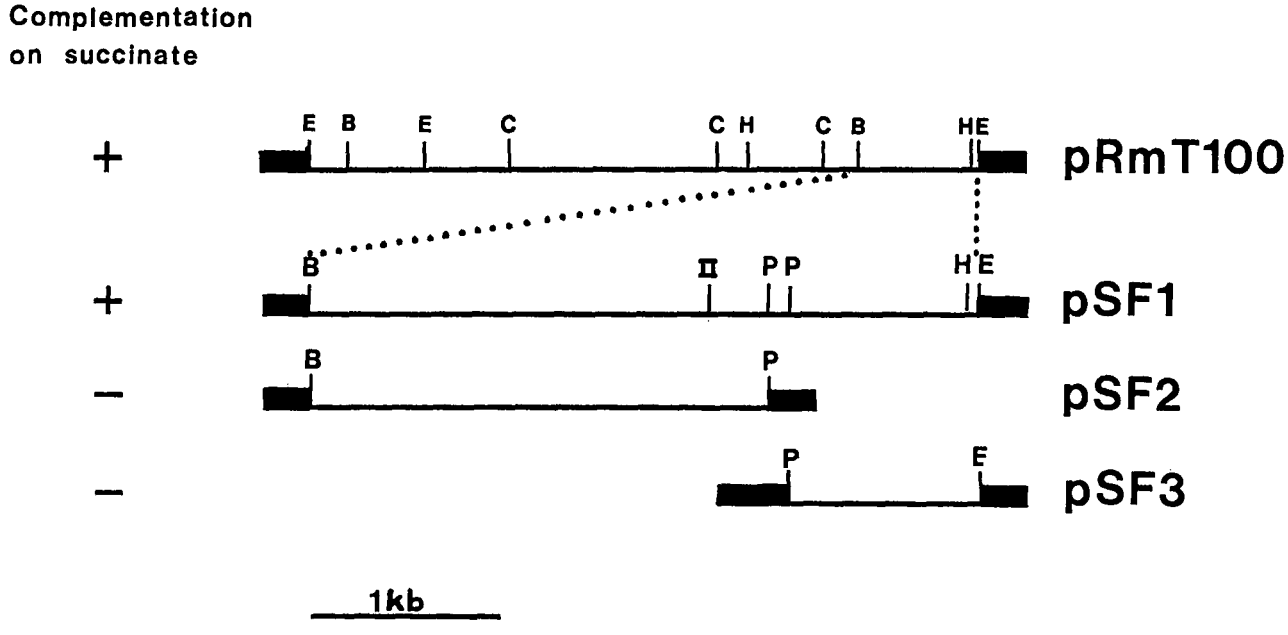
# CHAPTER 4

# RESULTS

## GROUP I MUTANT ANALYSIS

### Complementation of group I mutants

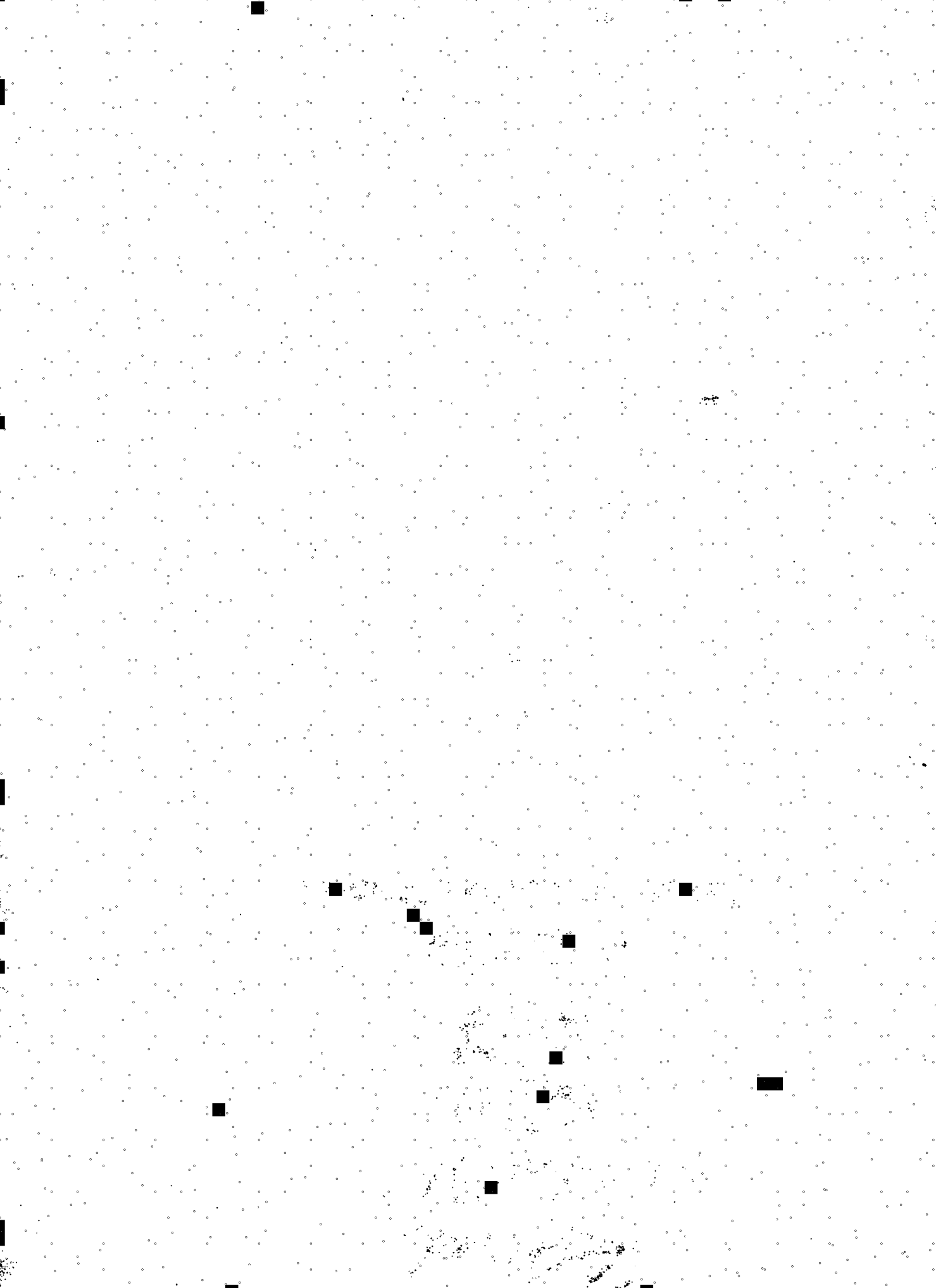
Previous research by other members of this lab (Finan et al. 1988, T.M. Finan unpublished) indicated that the group I mutants could be complemented by a pLAFR1 cosmid clone, pRmT100, containing an 18 kbp R. meliloti EcoRI fragment. Deletion analysis narrowed the region containing the gene(s) corresponding to the group I mutations to a 3.5 kbp BamHI-EcoRI fragment. Plasmid pSF1, constructed by John Stanley (University of Geneva), and containing the BamHI-EcoRI fragment cloned into the pRK7813 pUC9 polylinker region, was conjugated into the group I mutants. The resultant strains were struck out on M9 media containing 15 mM succinate as sole carbon source. Growth on this media indicated complementation of the mutation (Figure 2). Two subclones from pSF1 (also constructed by J.Stanley): pSF2, containing the 2.2 kbp BamHI-PstI fragment, and pSF3, containing the 1.0 kbp PstI-EcoRI fragment, failed to complement all group I mutant strains (Figure 2). Restriction analysis of the complementing region contained within pSF1 revealed unique BamHI, EcoRI, BglII, and HindIII sites as well as two closely

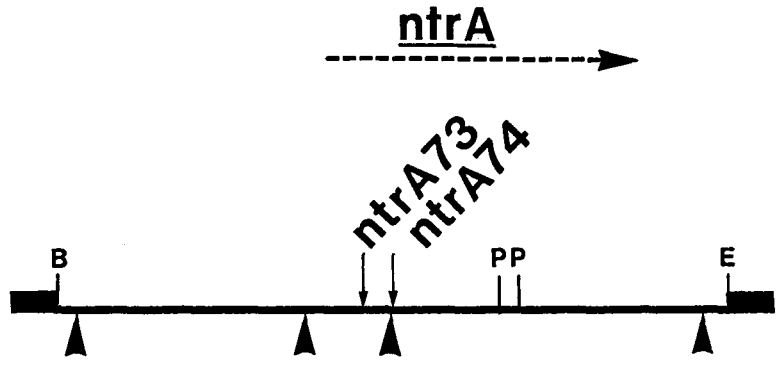

Complementation
on succinate
+
+
−
−
E B E C C H C B HE
pRmT100
B II P P H E
pSF1
B P
pSF2
P E
pSF3
1kb

positioned PstI sites (Figure 2). While this work was in progress, Ronson et al. (1987b) reported the identification and sequence of the ntrA gene of R. meliloti. A comparison of restriction patterns and fragment sizes indicated that the region contained within pSF1 encoded an ntrA-like gene. The following experiments provided further evidence for this.

## Tn5 mutagenesis of pSF1

pSF1 was transformed into E. coli MT614 which carries a Tn5 insert in its chromosome. Selection for Tn5 mutagenesis was carried out by mobilizing plasmid pSF1 into E. coli strain MT609, and selecting both for Tc resistance carried by the plasmid and Km resistance of transposon Tn5. The positions of the Tn5 inserts was established with restriction enzymes BamHI, EcoRI, and BglII. Four inserts were obtained within the BamHI-EcoRI region (Figure 3). Using triparental matings, the inserts were examined for complementation of the group I mutation. Plasmid pSF1 carrying inserts #4, #6, and #20, continued to complement all group I mutants for growth on succinate-containing minimal media. pSF1 carrying insert #5 failed to complement the mutants. After conjugating the plasmids into R. meliloti wild type strain Rm1021, all four inserts were further recombined into the genome (see Methodology). RmF288 (ntrA76::Tn5), resulting from a homogenotization of insert






| Tn5 insert | 20 | 6 | 5 | 4 |
|---|---|---|---|---|
| Complementation | + | + | − | + |
| $N_2$ Fixation | ND | ND | − | ND |

1 kb

#5, failed to grow on succinate minimal media while the remaining inserts were Dct$^+$.

Southern analysis of the group I ntrA mutants

To define the position of transposon insertion in ntrA mutant strains RmF288 (ntrA76::Tn5) (a site-specific Tn5 insertion), RmF250 (ntrA74::Tn5-233), and RmF122 (ntrA73::TnV), total DNA was digested with the restriction enzyme HindIII, separated by gel electrophoresis, and blotted onto nitrocellulose. Blots were probed with a $^{32}$P-labelled pBR322::Tn5 probe. Previous restriction analysis by T.M. Finan (personal communication) demonstrated the region contained within pSF1 to be part of a 7.5 kbp HindIII fragment (Figure 2). Analysis of HindIII fragments hybridizing to Tn5 indicated that the transposon inserts were in the region corresponding to the ntrA gene as determined by Ronson et al. (1987b). The positions of three Group I mutant alleles are presented in Figure 3.

Selection for ntrA revertants

All ntrA mutations studied resulted from transposon insertions. Experiments were undertaken to obtain colonies reverting to the wild-type Dct$^+$ phenotype by spreading $10^8$ cells on succinate minimal media. One should be able to isolate true revertants that have lost the transposon antibiotic resistance mutation, and antibiotic resistant revertants in which a probable secondary mutation had

occurred. Group I mutant strains Rm5422, Rm5419, RmF288, and Rm5011 were grown up in $LB^{mc}$ culture, washed, and plated onto M9 media containing 15 mM succinate as carbon source. Thus, selection was made for variants able to utilize succinate. Revertant colonies, obtained at a rate of approximately 1 x $10^{-7}$ - 1 x $10^{-8}$ per CFU, were screened on succinate minimal media containing Nm for loss of the mutation-causing transposon Tn5 or TnV insertion. For Rm5422 one out of twelve $suc^+$ colonies screened had maintained the transposon insertion; 10 out of 33 colonies of Rm5419, and three out of 10 colonies of RmF288 were also antibiotic resistant (Table 3). One revertant colony of strain Rm5422, and two independent revertant colonies of each of strains Rm5419 and

TABLE 3: ISOLATION OF GROUP I REVERTANTS

| Strain | Allele | Colonies screened | $Nm^r$ | $Nm^s$ | $Nm^r$ Strains |
|---|---|---|---|---|---|
| Rm5419 | ntrA74 | 33 | 10 | 23 | RmF193, RmF194 |
| Rm5422 | ntrA75 | 12 | 1 | 11 | RmF188 |
| RmF288 | ntrA76 | 10 | 3 | 7 | RmF365, RmF367 |

RmF288 retaining the transposon insertion were purified. No resistant revertants were obtained with strain Rm5011. Neomycin resistant revertant strains were designated as RmF188, RmF193 and RmF194, and RmF365 and RmF367, respectively (Table 3). The retention of the transposon insertion suggested a mutation occurring elsewhere

(secondary-site) that was able to overcome or suppress the Dct$^-$ phenotype of the ntrA mutants.

## Transductional mapping of second-site mutation

Phage lysates of the neomycin resistant Dct$^+$ revertant strains (RmF188, RmF193, RmF194, and RmF365) were used to transduce neomycin resistance into wild-type Rm1021 cells. Fifty to one hundred colonies were screened from each cross; all of the transductants examined failed to grow on succinate minimal media but grew on glucose minimal media (Table 4b). Thus, the Nm resistance of the Tn5 insertion was 100% cotransducible with the suc$^-$ phenotype. Transduction of GmSp$^r$ from strain RmF250 (ntrA74::Tn5-233) into strains RmF188, RmF193, and RmF194 resulted in a 100% loss of neomycin resistance while growth on succinate minimal media

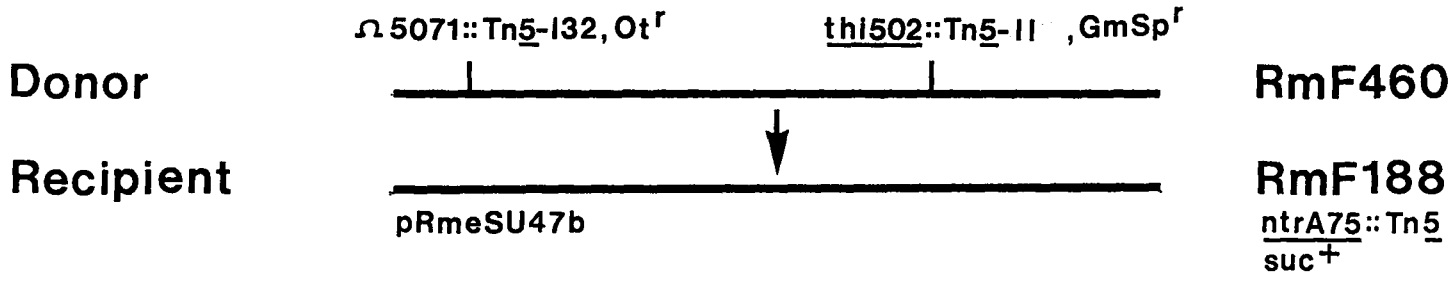
TABLE 4: TRANSDUCTIONAL ANALYSIS OF SECOND-SITE MUTATIONS

| | Lysate | Recipient | Comments |
|---|---|---|---|
| a. | RmF250 (ntrA74::Tn5-233) | RmF188<br>RmF193<br>RmF194 | Loss of Nm$^r$ by all transductants screened |
| b. | RmF188<br>RmF193<br>RmF194<br>RmF365 (ntrA::Tn5, Dct$^+$) | Rm1021 | Loss of Dct by all transductants screened |
| c. | RmF155 (Ω5072::Tn5-132) | RmF188 | 34 colonies were Dct$^+$, 4 colonies were Dct$^-$, all colonies were Nm$^r$ |

was maintained (Table 4a). Over 200 colonies were screened in each transductional cross. These results showed that the neomycin resistant revertant strains retained their ntrA::Tn5 mutation. Presumably the revertants carry $Dct^+$ second-site mutations.

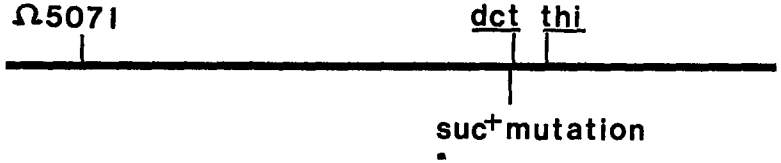
In other work a second Dct region was mapped adjacent to thi502 on megaplasmid pRmeSU47b (Finan et al. 1988). This region was examined for the presence of the second-site mutation that resulted in $Dct^+$ in the revertant strains. Two Tn5-132 inserts, Ω5069, and Ω5072 (2%, and 9% linked to the thi502::Tn5-11 locus on megaplasmid pRmeSU47b, respectively; A. Bottacin and T.M. Finan, personal communication) were transduced into the $Nm^r$ revertant strains to check for linkage to the second-site mutation. All Ω5069::Tn5-132 transductant colonies retained the $suc^+$ phenotype and $Nm^r$. The transduction of $Ot^r$ from RmF155 (Ω5072::Tn5-132) into strain RmF188 resulted in transductant colonies with two phenotypes: 34 colonies were $suc^+$ $Nm^r$ $Ot^r$ while 4 colonies were $suc^-$ $Nm^r$ $Ot^r$ (Table 4c). Thus, Ω5072::Tn5-132 was approximately 10% linked to the second-site $Dct^+$ mutation.

Three factor transductional crosses were undertaken to localize the second-site $Dct^+$ mutation. First, a double insertion strain was constructed (RmF460) containing Ω5071::Tn5-132 and thi502::Tn5-11. These inserts are about 30% linked in transduction (A. Bottacin and T.M. Finan, personal communication). A lysate of RmF460 was used to




| GmSp$^r$ colony phenotype (200 colonies screened) | % |
|---|---|
| suc$^+$ Ot$^r$ Nm$^r$ | 0 |
| suc$^+$ Ot$^s$ Nm$^r$ | 10.5 |
| suc$^-$ Ot$^r$ Nm$^r$ | 27.5 |
| suc$^-$ Ot$^s$ Nm$^r$ | 62.0 |

∴ second-site mutation is 89.5% linked to thi502

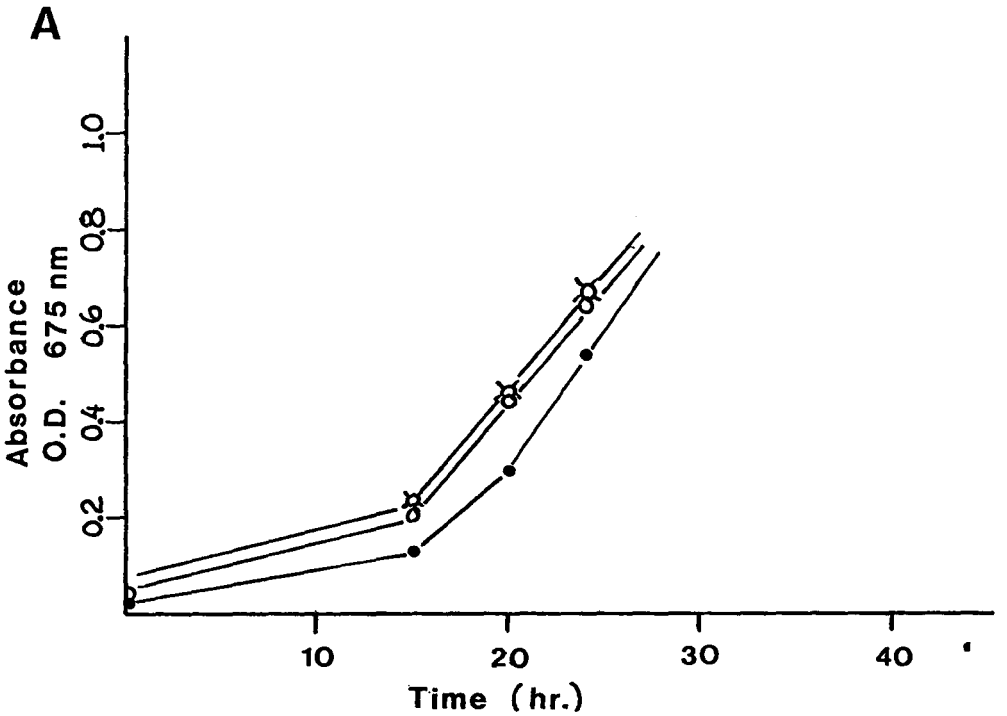


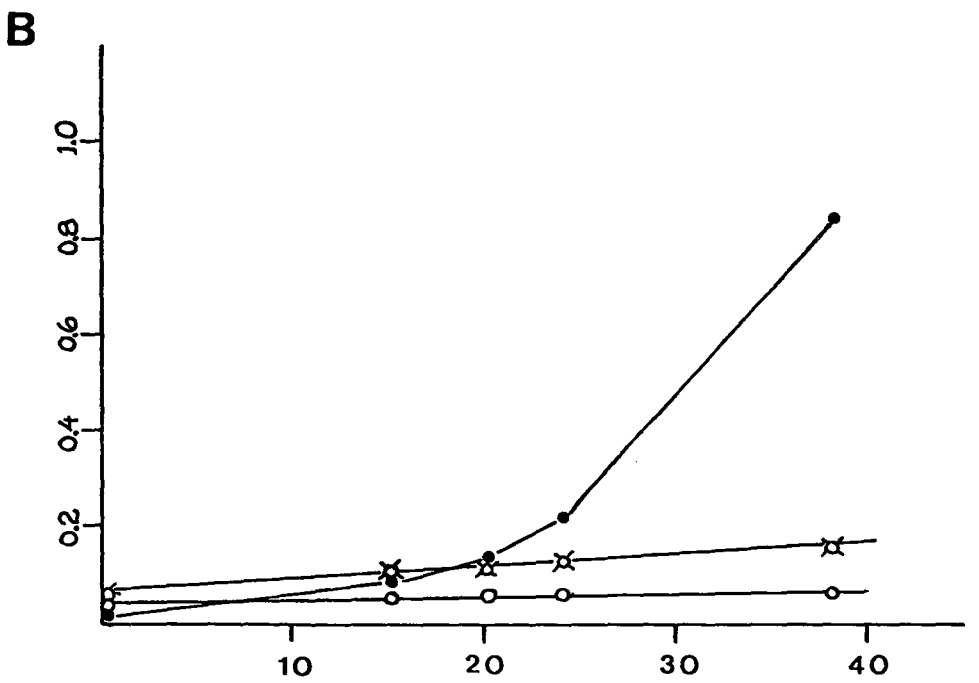
transduce $GmSp^r$ into the $Nm^r$ second-site revertant strains RmF188, RmF365, and RmF367. The transduction into RmF188 is presented graphically in Figure 4. GmSp resistant transductant colonies were screened for succinate phenotype, Nm resistance, and Ot resistance. The $suc^+$ phenotype was lost in about 90% of the colonies; this reflected the loss of the $Dct^+$ ntrA-independent mutation, and indicated that the second-site mutation was 90% linked to thi502. The second-site mutations in RmF367 and RmF365 were found to be 92% and 90% linked, respectively, and lay between thi and Ω5072::Tn5-132.

## Growth in media containing 5 mM $KNO_3$

One of the functions of the NTRA protein is the regulation of the nitrogen assimilation system in E. coli (Magasanik and Neidhardt 1984). Rm1021 and all group I mutants were examined for growth in minimal media supplemented with 15 mM glucose as carbon source, and 5 mM potassium nitrate or ammonium chloride as nitrogen source. Optical density was recorded at a wavelength of 675 nm at various time intervals up to forty hours of incubation. Results are presented in Figure 5 for growth patterns of Rm1021, mutant strain RmF122, and second-site revertant strain RmF188 in A) control media containing 5 mM ammonium chloride, and B) media containing 5 mM potassium nitrate as nitrogen sources.

Figure 5: Nitrogen utilization by *ntrA* mutants and second-site revertants. A. Growth with 5 mM ammonium chloride as nitrogen source. B. Growth with 5 mM potassium nitrate as nitrogen source. Strains were grown in triplicate at 30'C. Strains examined were: Rm1021 (wild-type), ● ; RmF122 (*ntrA73*::Tn*V*), o ; RmF188 (*ntrA75*:: Tn*5*, $Dct^+$), ¤ . Optical density was recorded at 675 nm.


A
Absorbance
O.D. 675 nm
0.2
0.4
0.6
0.8
1.0
10
20
30
40
Time (hr.)


B
0.2
0.4
0.6
0.8
1.0
10
20
30
40

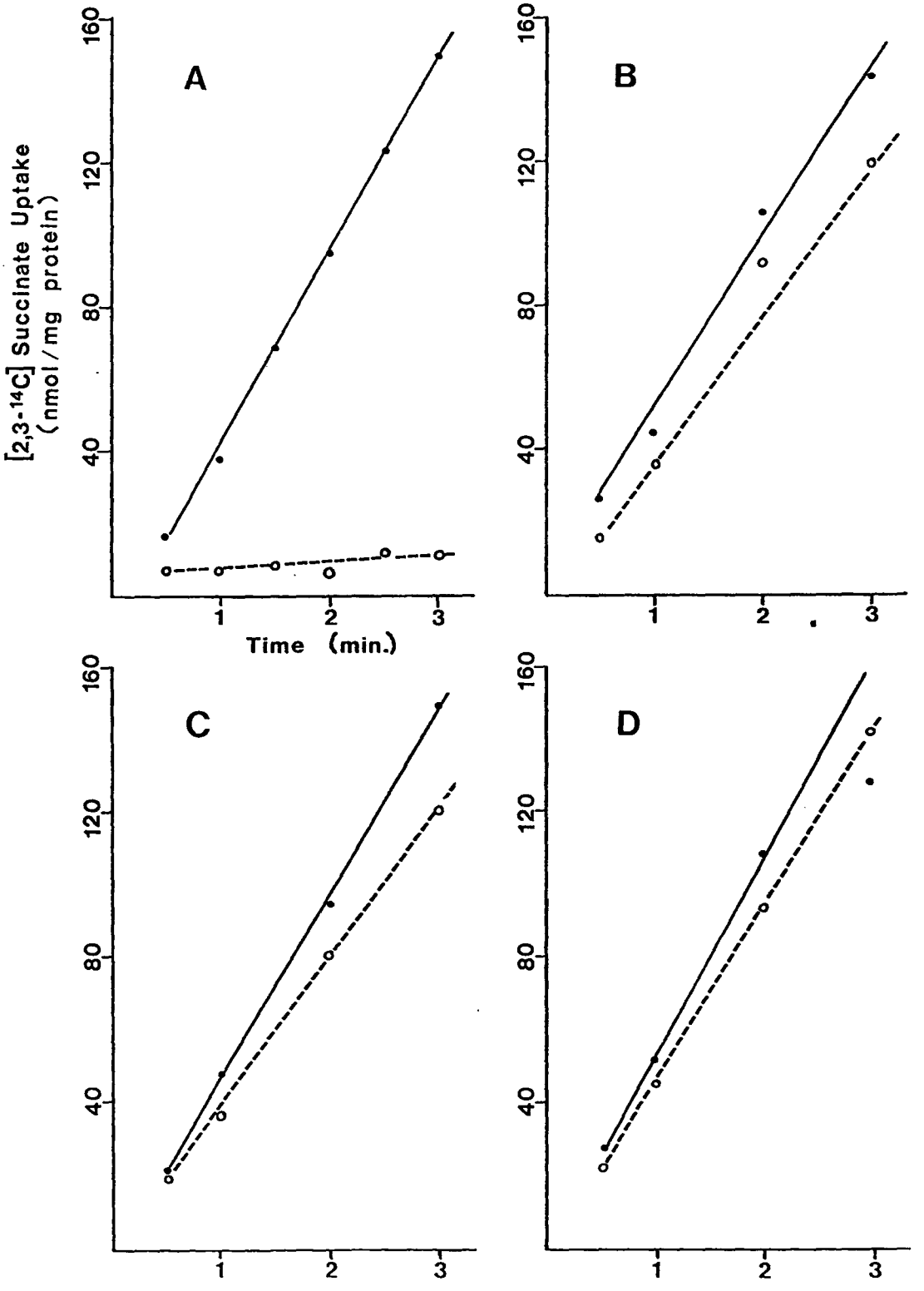
For growth with ammonium chloride as nitrogen source (graph A) all strains exhibited similar growth rates with doubling times of ten hours for the cell densities. With potassium nitrate as nitrogen source (graph B) a longer lag period was followed by a slower growth rate by Rm1021 as compared with graph A. Strains RmF122 and RmF188 demonstrated no growth with minimal change in optical density. This suggested that, like the previously described ntrA mutants (Ronson et al. 1987b), the group I mutants were defective for nitrate utilization as well as for $C_4$-dicarboxylic acid transport. The presence of the second-site $Dct^+$ mutation was unable to restore the wild-type nitrate phenotype consistent with the second-site mutation being Dct specific.

Succinate uptake by ntrA-independent $Dct^+$ strains

Rm1021 and the three second-site mutation strains were grown in flasks containing M9 broth with either 15 mM glucose or 15 mM succinate as carbon sources. Cells were centrifuged, washed, and assayed for succinate transport activity as described in Methodology.

Figure 6, graph A shows that Rm1021 cells grown in M9-succinate demonstrated a steady rate of succinate uptake whereas Rm1021 cells grown in M9·glucose showed minimal transport activity. Thus, in wild type cells $C_4$-dicarboxylate transport is induced in media containing

Figure 6: [2,3-$^{14}$C] succinate uptake by *ntrA* second-site revertant strains. Rm1021 and three *ntrA* second-site revertant strains were examined for *dct* induction after growth in M9-succinate (●), or in M9-glucose (○). Graphs presented are: A, Rm1021 (wild-type); B, RmF188 (*ntrA75*::Tn*5*, Dct$^+$); C, RmF193 (*ntrA74*::Tn*5*, Dct$^+$); D, RmF365 (*ntrA76*::Tn*5*, Dct$^+$).


[2,3-14C] Succinate Uptake
(nmol/mg protein)
A
B
C
D
160
120
80
40
1
2
3
Time (min.)

succinate. Graphs B,C, and D demonstrate transport activities by the second-site revertant strains RmF188, RmF193, and RmF365,respectively. Cells grown in succinate-containing media showed similar transport rates to wild-type cells. However, unlike the wild-type Rm1021, all three revertant strains showed induced succinate transport rates after growth in glucose-containing media. The rates were slightly lower than those shown by cells grown in succinate-containing minimal media. Patterns of succinate uptake in the revertant strains show that the second-site mutation resulted in a Dct constitutive phenotype.

Symbiotic properties of group I mutants and revertants

To examine the symbiotic properties of the ntrA mutants, and the $Dct^+$ second-site revertants, alfalfa seedlings were inoculated with cultures of the strains and grown under regulated conditions for 28 days. Inoculation of wild-type Rm1021 onto alfalfa plants resulted in healthy plants with an extensive tap root system. Nodules formed on roots were cylindrical in shape, large, and pink in colour indicating active heme production. Nitrogenase activity was measured by the acetylene reduction technique. Nitrogenase activity by Rm1021 was calculated as 476.7 nmol of ethylene per hour per plant (Table 5). Control alfalfa plants that had not been infected with R. meliloti were assayed to have acetylene reducing activity about 1% that obtained in the

wild-type strain. This residual activity was probably due to the detection of ethylene naturally produced by the plants'

TABLE 5: SYMBIOTIC PROPERTIES OF ntrA DERIVATIVES

| Allele | | nmol ethylene $hr^{-1}$ $plant^{-1}$ | % of wild type |
|---|---|---|---|
| wild type | (Rm1021) | 476.7 ± 75.0 | 100.0 % |
| ntrA71 | (Rm5011) | 5.0 ± 0.6 | 1.1 % |
| ntrA72 | (Rm5074) | 5.8 ± 0.7 | 1.2 % |
| ntrA74 | (Rm5419) | 5.4 ± 1.0 | 1.1 % |
| ntrA74, $suc^+$ | (RmF188) | 5.6 ± 1.1 | 1.2 % |
| ntrA75 | (Rm5422) | 4.7 ± 0.7 | 1.0 % |
| ntrA75, $suc^+$ | (RmF193) | 5.9 ± 0.6 | 1.2 % |
| ntrA76 | (RmF288) | 5.8 ± 0.6 | 1.2 % |
| ntrA76, $suc^+$ | (RmF365) | 5.2 ± 0.8 | 1.1 % |
| RmF502 | | 354.0 ± 25.5 | 74.3 % |
| RmF504 | | 339.7 ± 76.5 | 71.2 % |
| RmF506 | | 417.5 ± 91.4 | 87.6 % |
| uninoculated plants | | 3.0 ± 0.6 | 0.6 % |

Acetylene reduction assays were performed 28 days after inoculation. Values represent the mean of three or more assays.

root system.

Plants inoculated by the group I mutants were stunted and chlorotic in appearance, comparable to the uninoculated control alfalfa plants. A minimal secondary root system existed on which few nodules were found. The nodules were smaller than wild-type nodules and were white indicating no heme production. The acetylene reducing activity of the

mutant strains was comparable to control uninoculated plants.

Nodules of plants inoculated with strains RmF188, RmF193, or RmF365 ($Dct^+$ second-site revertant strains carrying alleles ntrA75, ntrA74, and ntrA76, respectively) had nitrogenase levels comparable to group I mutants. Plants remained small and chlorotic while nodules were round and white. This indicated that the wild-type ntrA gene was required for nitrogen fixation.

Strains carrying the wild-type ntrA gene along with the second-site $Dct^+$ mutation were inoculated onto alfalfa seedlings to examine the effect of the $Dct^+$ second-site mutation alone on nitrogenase activity. As shown in Table 5, strains RmF502, RmF504, and RmF506 were all able to fix nitrogen though at a slightly lower rate than wild-type bacteroids. This confirmed that the mutation in ntrA was responsible for the $Fix^-$ phenotype in the second site revertant strains RmF188, RmF193, and RmF365.

# CHAPTER 5

# RESULTS

# ANALYSIS OF GROUP V MUTANTS

## Complementation of group V mutants

pLAFR1 cosmid clones were isolated from an R. meliloti gene bank to complement group V mutants (Finan et al. 1988). As with group I mutants, complementation was determined by growth on M9 minimal media containing 15 mM succinate as carbon source. Plasmid pRmT8, previously found to complement thi502::Tn5-11 (Finan et al. 1986), complemented all group V mutations. A second cosmid clone, pRmT1, failed to complement the group V mutations though it complemented thi502::Tn5-11, and shared some restriction fragment homology with pRmT8. After a comparison of restriction fragments, a 6 kbp HindIII fragment was subcloned from pRmT8 into the polylinker region of pRK7813 to give plasmid pTH24 (Figure 7). Restriction fragments from the 6 kbp region were further subcloned into pRK7813-1 to give derivative plasmids pTH31, pTH32, and pTH33 (Figure 7). The plasmids were examined for complementation of the group V mutations. As shown in Figure 7, both pTH24 and pTH31 complemented all mutants. pTH32 complemented one subgroup of mutants, later identified as dctA, and pTH33 demonstrated

Figure 7: Plasmid complementation of dct mutants. Complementation of the dct mutations on M9-succinate is defined as (+) complementation, (+/-) partial complementation, or (-) no complementation. Restriction enzyme sites presented are: E, EcoRI; H, HindIII.

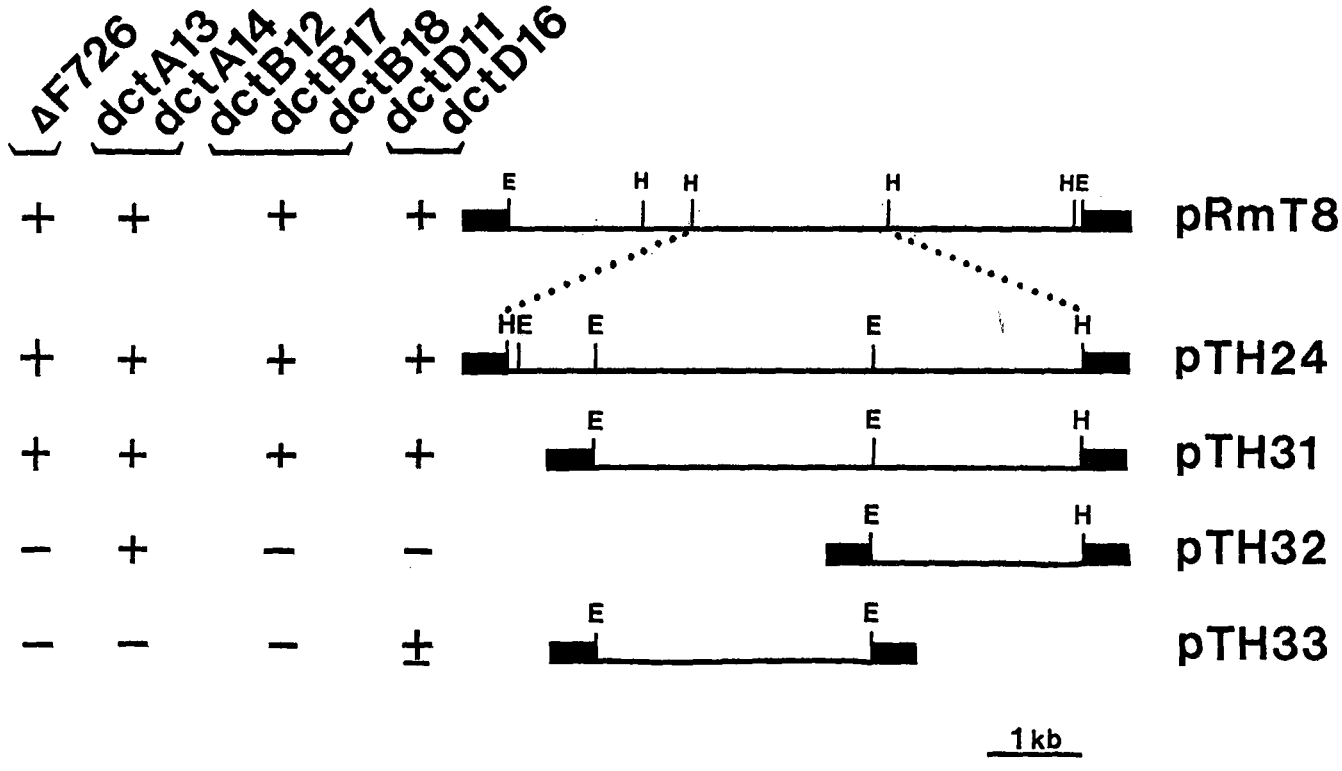
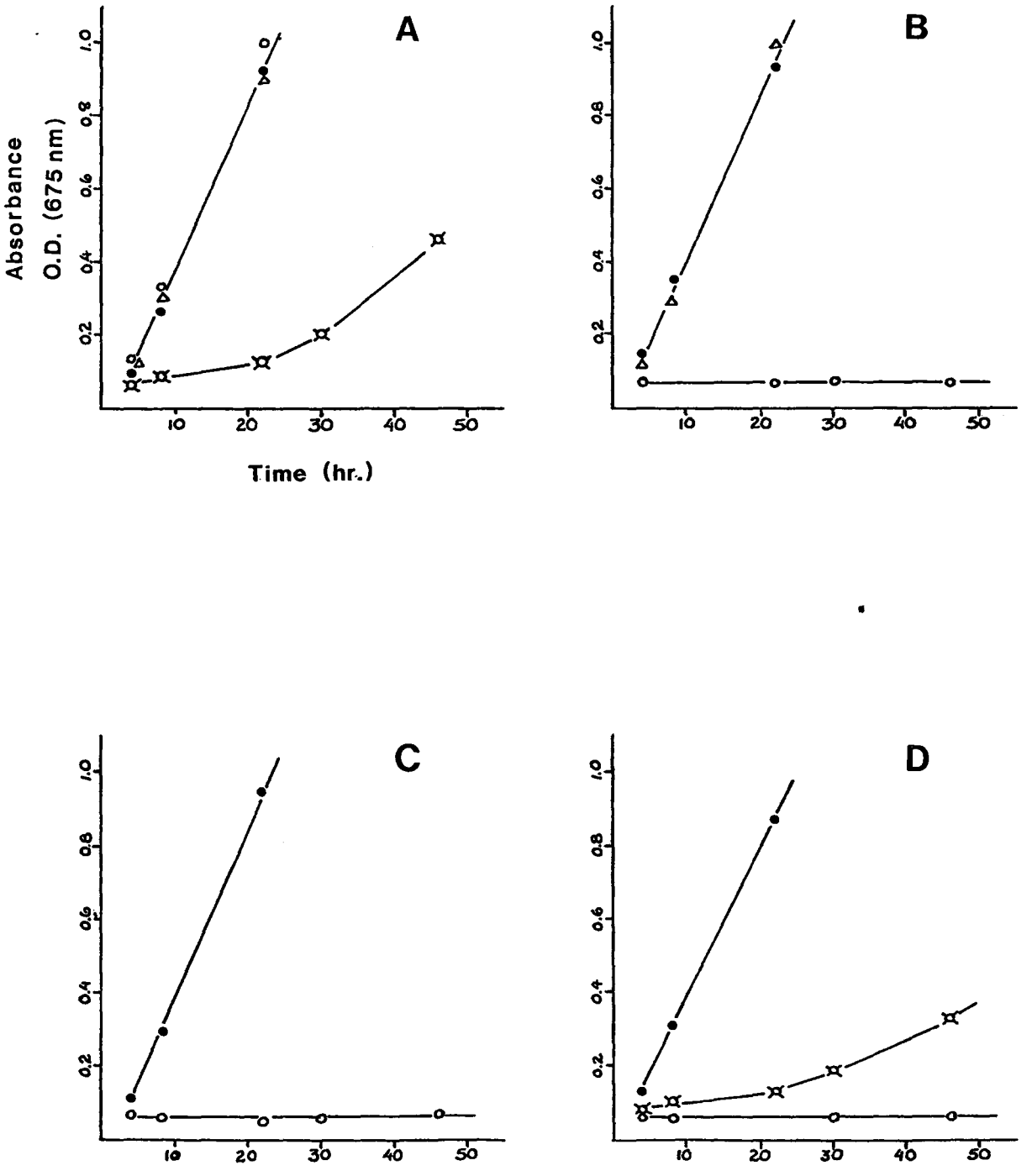

ΔF726
dctA13
dctA14
dctB12
dctB17
dctB18
dctD11
dctD16
+ + + + pRmT8
+ + + + pTH24
+ + + + pTH31
− + − − pTH32
− − − ± pTH33
E
H
HE
1 kb

Figure 8: Plasmid complementation of dct mutants: growth analysis in M9-succinate (15 mM). Optical density was recorded at 675 nm. Strains were examined on their own (o), in the presence of pTH31 (●), pTH32 (▲), or pTH33 (¤). Graphs represent: A, Rm1021 (wild-type); B, RmF642 (dctA14::Tn5); C, RmF332 (dctB18::Tn5); D, RmF121 (dctD16::Tn5).


A
B
C
D
Absorbance
O.D. (675 nm)
Time (hr.)
0.2
0.4
0.6
0.8
1.0
10
20
30
40
50

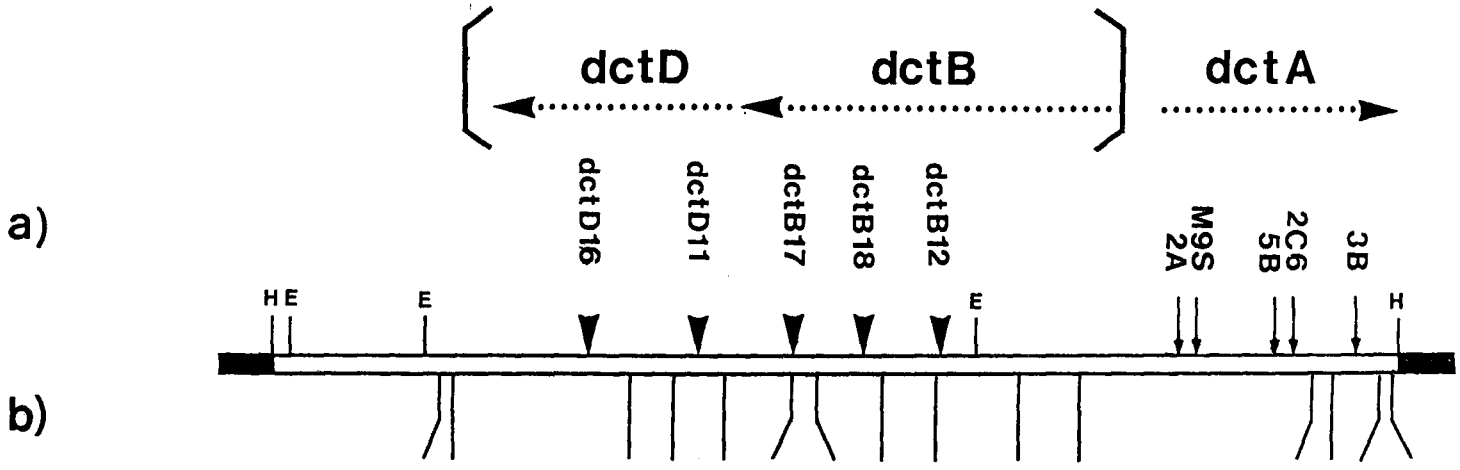
partial complementation of a second group, the dctD mutants. The remaining dct mutants (dctB) were not complemented by either of the two smaller plasmids.

To obtain quantitative data with respect to the above plasmid complementation experiments we monitored growth (as determined by optical density at 675 nm) of various strains growing in M9 broth containing 15 mM glucose or 15 mM succinate. Results are presented in Figure 8. Graph A demonstrates that wild-type strain Rm1021, by itself or in the presence of pTH31 or pTH32, reached late-log phase growth after 22 hours growth in M9-succinate. The presence of pTH33 retards Rm1021 growth in M9-succinate. RmF642 (dctA14::Tn5) (Graph B) was unable to utilize succinate unless pTH31 or pTH32 were present at which point the growth rate was similar to wild-type. In Graph C, RmF332 (dctB18::Tn5) was complemented by pTH31 to wild-type levels of growth by 22 hours. RmF121 (dctD16::Tn5) (Graph D) was fully complemented in the presence of pTH31. The presence of pTH33 resulted only in partial complementation of the dctD mutation. This was also observed on solid media. The growth rate for all strains in M9- 15 mM glucose (results not shown) was a log curve reaching late-log phase at approximately 24 hours.

## Tn5 mutagenesis of pTH24 and pTH31

Plasmids pTH24 and pTH31, complementing all group V mutants, were transformed into Tn5-containing E. coli strain

Figure 9: Tn*5* and Tn*phoA* inserts, and complementation of *dct* mutants. A. Positions of *dct* alleles as determined by hybridization studies are designated with large arrow-head. Positions of Tn*phoA* inserts as designated with small arrow. B. Positions of Tn*5* inserts as designated with vertical lines. C. Complementation of *dct* mutations and the *dct* deletion-containing strain RmF726. Complementation is defined as: (+) complementation, (+/-) partial complementation, (-) no complementation on M9-succinate (15 mM)




| INSERT DESIGNATION | 82 | 81 | 30 | 29 | 31 | 25 | 24 | 23 | 22 | 21 | 20 | 19 | 14 | 15 | 32 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| c) dctA13 | + | + | + | + | + | ± | + | + | + | + | + | − | − | − | + |
| dctA14 | + | + | + | + | + | ± | + | + | + | + | + | − | − | − | + |
| dctA19 | + | + | + | + | + | ± | + | + | + | + | + | − | − | − | + |
| dctB12 | + | + | + | + | + | − | − | − | − | − | − | + | + | + | + |
| dctB17 | + | + | + | + | + | − | − | − | − | − | − | + | + | + | + |
| dctB18 | + | + | + | + | + | − | − | − | − | − | − | + | + | + | + |
| dctD11 | + | + | − | − | + | + | + | + | + | + | + | + | + | + | + |
| dctD16 | + | + | − | − | + | + | + | + | + | + | + | + | + | + | + |
| △F726 | + | + | − | − | + | ± | − | − | − | − | − | − | − | − | + |

MT614. Selection for mutagenesis was carried out by conjugating into MT609 and plating onto media containing Tc and Km as outlined in the Methodology section. Twelve independent conjugations were undertaken and mini plasmid preparations performed on two colonies per conjugation. Restriction analysis with enzymes EcoRI, and HindIII was carried out to determine the positions of the inserts. The inserts are presented in Figure 9b where inserts are positioned with vertical lines; assigned Tn5 insert numbers were later utilized for allele designations. Fifteen Tn5 inserts were obtained that mapped either to the 2.9 kbp EcoRI fragment, or to the 2.2 kbp EcoRI-HindIII fragment.

## Complementation by Tn5 inserts

Plasmids pTH24 and pTH31 carrying defined Tn5 inserts were conjugated into the R. meliloti dct mutant strains as well as into RmF726 by selecting for $Tc^r$ transfer. Transconjugants were then examined for complementation by streaking onto M9-succinate (15 mM) and, as a control, M9-glucose (15 mM) media. RmF726 contains a 200 kb deletion of megaplasmid pRmeSU47b encompassing the dct and the thi region (T. Charles, personal communication). Complementation data is presented in Figure 9c. The dct mutants were classified into one of three groups. Mutant alleles dctA14::Tn5, dctA13::TnphoA, and dctA19::Tn5 failed to be complemented by Tn5 inserts 14, 15, and 19. Mutant alleles dctB17::Tn5-233,

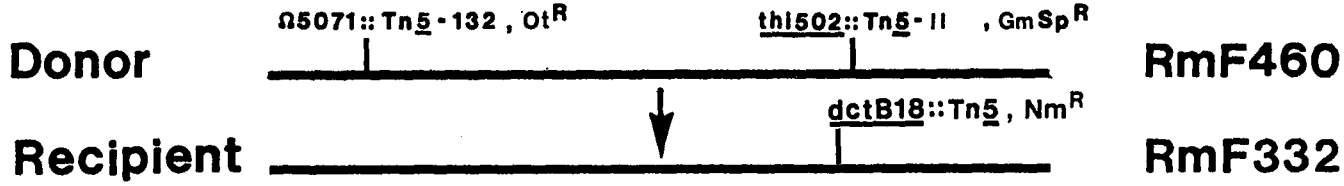
dctB18::Tn5, and dctB12::Tn5-132 were not complemented by inserts 20 through 25. Inserts 29 and 30 failed to complement dctD16::Tn5 and dctD11::Tn5-lac. Inserts 31, 32, 81, and 82 complemented all mutants. This suggested that all four of these inserts were outside the region corresponding to the dct genes. Later analysis of the dctA sequence (communicated by B.T. Nixon) demonstrated that Tn5 insert 32 was located within the termination sequences of the dctA transcript, and restriction analysis suggested that insert 31 was located between the dctB and dctD coding regions. Interestingly plasmid pTH24 carrying insert 25 failed to complement dctB mutants and only showed partial complementation of dctA mutations.

## Homogenotization of Tn5 inserts

Plasmids containing Tn5 inserts 14, 19 through 25, and 29 through 31 were conjugated into Rm1021 using the mobilizing plasmid pRK600. Neomycin resistant homogenotes were then selected with the aid of the pRK7813-incompatible plasmid pPH1JI (see Methodology). Strains obtained were examined for the Dct phenotype. The results showed that all inserts retained the same Dct phenotypes that were previously observed when mutagenized plasmids were conjugated into the dct deletion strain RmF726 (Figure 9c).

Figure 10: Transductional linkage of dct to thi502 on pRmeSU47b. a. Transduction of RmF460 into RmF121 (dctD16::Tn5). b. Linkage of dct to thi502. Antibiotic resistances presented are: Ot, oxytetracycline; GmSp, gentamycin/spectinomycin; Nm, neomycin.

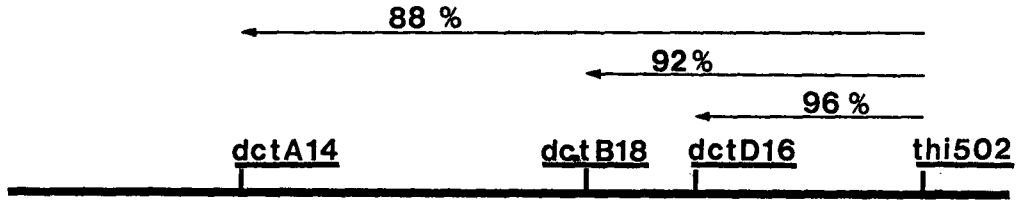
a)




| GmSp$^R$ colony phenotype (200 colonies screened) | % |
|---|---|
| suc$^+$ Nm$^S$ Ot$^R$ | 26.6 |
| suc$^+$ Nm$^S$ Ot$^S$ | 65.3 |
| suc$^-$ Nm$^R$ Ot$^R$ | 0.0 |
| suc$^-$ Nm$^R$ Ot$^S$ | 7.0 |

∴ linkage of dctB to thi502 is 92 %

b)



## Transductional mapping of the dct locus

Previously, it was described that the dct mutants were complemented by cosmid clone pRmT8, and that the clone could also complement mutations in the thi502 allele. This indicated a close proximaty of the dct gene locus to the thi gene. The use of RmF460 in a series of three factor crosses enabled the mapping of the dct locus with respect to the thi locus. RmF460, used previously to map the ntrA second-site mutation, contains a Tn5-132 insert Ω5071 linked to the thi502::Tn5-11 allele (Figure 10). Φ(RmF460) was used to infect cells of RmF642 (dctA14::Tn5), RmF332 (dctB18::Tn5), and RmF121 (dctD16::Tn5), and transductants were selected by plating on media supplemented with GmSp. Two hundred colonies from each transduction were screened on the appropriate media.

The cross of RmF460 into RmF642 yielded 87.9% of colonies recombining to a $suc^+$ $Nm^s$ $GmSp^r$ phenotype indicating that dctA14::Tn5 was located about 7 kb from thi502. Screening the remaining crosses indicated that dctB18::Tn5 was 92%, or about 3 kb, linked to thi502, whereas dctD16::Tn5 was 96%, or about 2 kb, linked to thi502. Data for the mapping of dctB18::Tn5 is presented in Figure 10a. The order of the genes is dctA-dctB-dctD-thi (Figure 10b).

To facilitate the construction of dct double mutant strains the Tn5 in strain RmF746 (dctA14::Tn5, $phoA^-$) was replaced with the $GmSp^r$ Tn5 derivative Tn5-233. The

resulting strain, RmF865, was dctA14::Tn5-233 and phoA$^-$. Phage lysates of RmF121 and of RmF332 were used to transduce RmF865 selecting on media containing Nm. About 300 transductant colonies were screened in each cross. Transducing dctD16::Tn5 into dctA::Tn5-233 yielded 295 suc$^-$ GmSp$^s$ Nm$^r$ and five suc$^-$ GmSp$^r$ Nm$^r$ colonies indicating a 98.3% linkage between the mutant alleles. Transducing in dctB18::Tn5 resulted in 279 suc$^-$ GmSp$^s$ Nm$^r$ and three suc$^-$ GmSp$^r$ Nm$^r$ colonies indicating a 98.9% linkage. Colonies carrying both antibiotic resistances, containing transposon mutations in both alleles, were purified for regulation studies to be described further on; these strains were identified as RmF881 (dctA14::Tn5-233 dctD16::Tn5), and RmF883 (dctA14::Tn5-233 dctB18::Tn5). The structure of the dct region in the double mutants was subsequently confirmed by Southern hybridization (Appendix 3).

## Southern analysis of the dct region

Total DNA prepared from the dct mutants was restricted with enzyme HindIII, separated by electrophoresis, and blotted onto nitrocellulose. Blots were probed with $^{32}$P-labelled pTH24. Restriction patterns were analyzed to determine the positions of the transposon inserts in the dct region. Insert positions of the dct mutants are presented in Figure 9. The restriction map of transposon Tn5 is presented in Appendix 1. Restriction analysis from the hybridizations

is presented in Appendix 3.

## Isolation of group V revertants

All *dct* revertants were isolated as outlined in METHODOLOGY. *R. meliloti* strains RmF651 (*dctA19*::Tn*5*) and RmF642 (*dctA14*::Tn*5*) were utilized to select for *dctA* revertant colonies. Few colonies were obtained (three for RmF651 and one for RmF642) after two separate platings. Screening the resultant colonies on appropriate media indicated that these were true revertants having lost the transposon Tn*5* insert ($Nm^S$) (Table 6).

Screening of revertant colonies from Rm5421 (*dctB17*::Tn*5*-233) showed that a very high percentage of colonies were $GmSp^r$ indicating the presence of a second-site $Dct^+$ mutation (Table 6). Five independently isolated $GmSp^r$ colonies were purified and designated RmF173 through RmF177. Characterization of these second-site revertants is presented further on. Very few true revertants of Rm5421 or its derivative strain RmF664 (*dctB17*::Tn*5*) were obtained. In addition, revertants of RmF644 (*dctB22*::Tn*5*) and of RmF646 (*dctB25*::Tn*5*) were also selected. In five independent platings of strain RmF644 71 colonies were found to be true revertants, whereas eight colonies retained the transposon insertion. Of 275 revertants of RmF646 only 30 were true revertants as indicated by their $Nm^S$ phenotype.

TABLE 6: ISOLATION OF GROUP V REVERTANTS

| Strain | Allele | Colonies Screened | Loss of Transposon | Transposon Maintained |
|---|---|---|---|---|
| RmF642 | dctA14 | 1 | 1 | 0 |
| RmF651 | dctA19 | 3 | 3 | 0 |
| Rm5421 | dctB17 | 100 | 0 | 100 |
| RmF644 | dctB22 | 79 | 71 | 8 |
| RmF646 | dctB25 | 275 | 30 | 245 |

Second-site Dct$^+$ revertant strains of RmF121 (dctD16::Tn5), designated RmF283, and RmF284, and of Rm5314 (dctD16::Tn5, RCR2011 background), designated strain RmF208, were previously isolated by Ivan Oresnik.

Analysis of dctB17 second-site Dct$^+$ revertants

GmSp$^r$ was transduced from the putative second-site revertant strains RmF173 through RmF177 into wild-type Rm1021. Resistant colonies were screened on M9 succinate. Out of 107 transductants of RmF175 into Rm1021, 103 remained Dct$^+$. The transduction of Φ(RmF176) into Rm1021 resulted in 124 out of 126 colonies retaining a Dct$^+$ phenotype. Thus, the second-site Dct$^+$ mutation is closely linked to dctB17::Tn5-233 (about 1 kb). Similar linkages were obtained with the second site mutations contained within RmF173, RmF174, and RmF177 (Table 7).

Lysate Φ(RmF288) was transduced into all suppressor strains (RmF173 through RmF177) to determine whether the Dct$^+$

TABLE 7: TRANSDUCTIONAL ANALYSIS OF *dctB17* SECOND-SITE MUTATIONS

| Lysate | | Recipient | Comment |
|---|---|---|---|
| a) RmF173<br>RmF174<br>RmF175<br>RmF176<br>RmF177 | (*dctB17*::Tn*5*-233, suc$^+$) | Rm1021 | 103/107 transductants suc$^+$<br>124/126 transductants suc$^+$ |
| b) RmF288 | (*ntrA76*::Tn*5*) | RmF173<br>RmF174<br>RmF175 | 200 transductants screened<br>All were suc$^-$ |

activity of the second-site mutations required the NTRA protein. In each case two hundred colonies were screened and all showed a suc$^-$ Nm$^r$ GmSp$^r$ phenotype (Table 7). This indicated that the second-site mutations in all five revertants were dependent on an active *ntrA* gene product.

## Characterization of dctD16::Tn5 second-site revertant strains RmF283 and RmF284

To determine whether the putative second-site Dct$^+$ mutation was linked to the *dct* region, ΦM12 lysates prepared on RmF283 and RmF284 were used to transduce Rm5421 cells (*dctB17*::Tn*5*-233) to Nm resistance (Table 8). All Nm$^r$ transductants from RmF283 were suc$^-$ GmSp$^s$ Nm$^r$ indicating that the second-site mutation was not co-transducible with the *dctD16* mutation, and also indicating that the revertant

TABLE 8: TRANSDUCTIONAL ANALYSIS OF *dctD16* SECOND-SITE MUTATIONS

| Lysate | Recipient | Comment |
|---|---|---|
| a) RmF283 | Rm5421 (*dctB17*) | 200 transductants $suc^-$ |
| RmF284 | | 200 transductants $suc^+$ |
| b) RmF250 (*ntrA74*::Tn*5*-233) | RmF283 | All transductants $suc^-$ |
| | RmF284 | All transductants $suc^+$ |

strain RmF283 still carried the *dctD16*::Tn*5* mutation. Transducing from RmF284 yielded colonies that were $suc^+$ $GmSp^s$ $Nm^r$. Since the $suc^+$ phenotype was retained in all ninety colonies screened the second-site mutation appeared to be located in the proximity of the *dctD16*::Tn*5* insertion.

To examine whether the secondary mutation was dependent on a functional *ntrA* gene for the *dctD* suppressing activity, $GmSp^r$ was transduced from RmF250 (*ntrA74*::Tn*5*-233) into RmF283 and RmF284. All RmF283 transductants failed to grow on M9-succinate, and were $Nm^r$ and $GmSp^r$. Thus, the second-site $Dct^+$ mutation in RmF283 required a functional NTRA protein. Conversely transduction of $GmSp^r$ from RmF250 into RmF284 yielded transductant colonies that still grew on M9-succinate and were $Nm^r$. This indicated that RmF284 carried an *ntrA*-independent $Dct^+$ second-site mutation.

To characterize the suppressing activity in a liquid culture, cultures of wild-type, *dctD16*::Tn*5*, and both suppressor strains were set up in M9 broth supplemented with

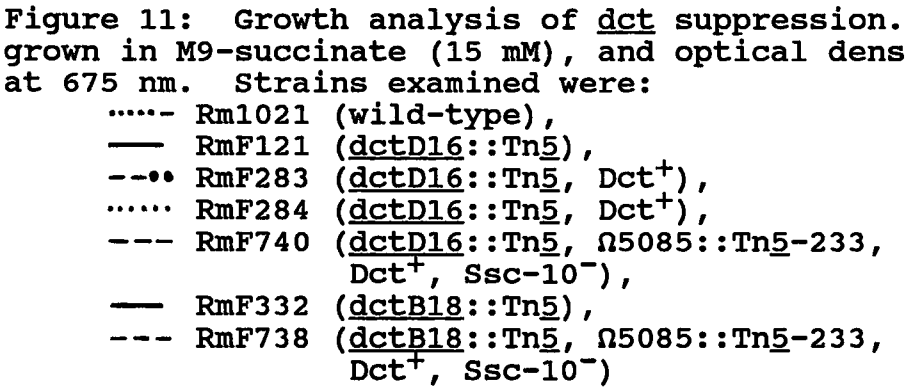
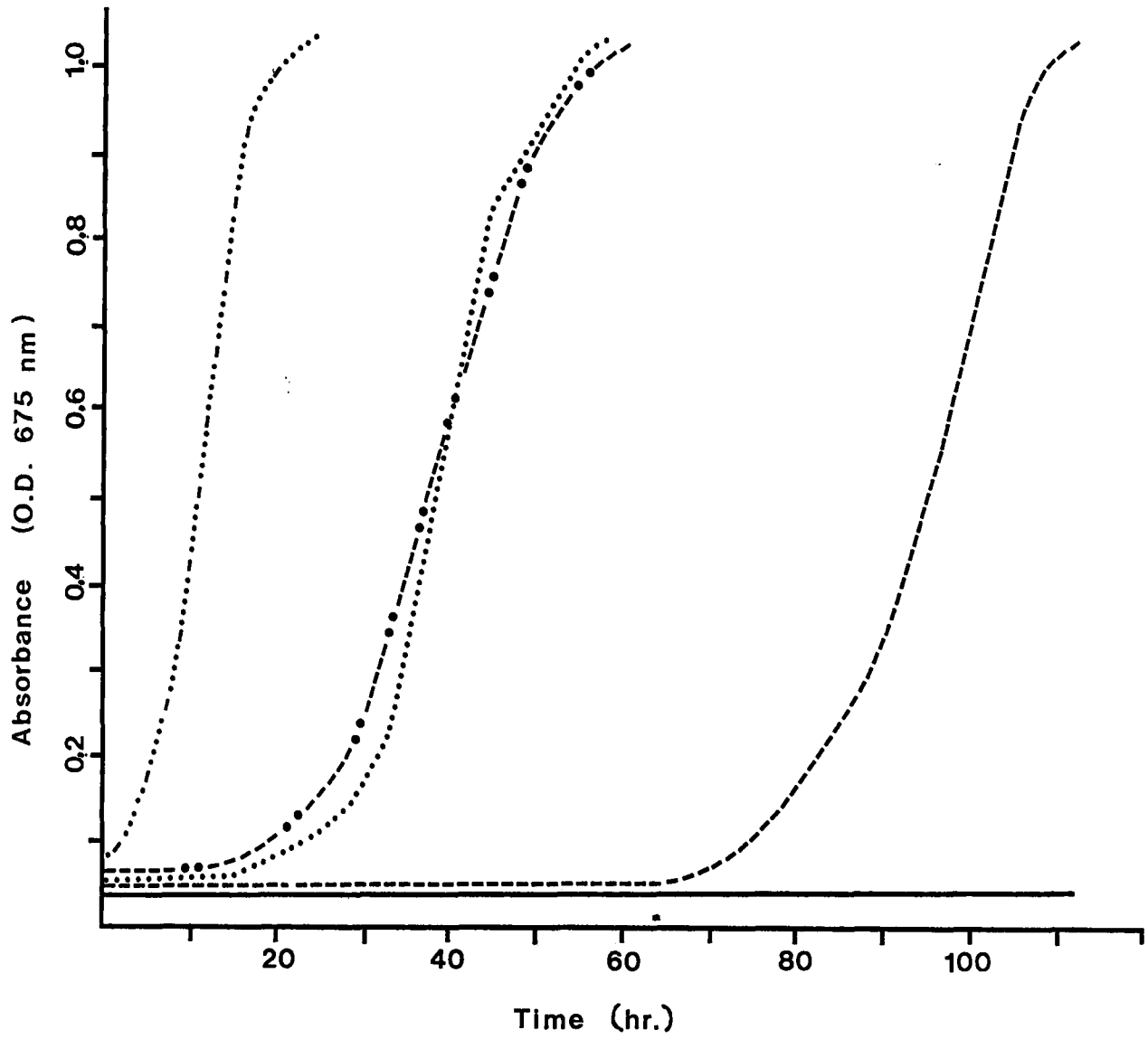


Figure 11: Growth analysis of *dct* suppression. Strains were grown in M9-succinate (15 mM), and optical density recorded at 675 nm. Strains examined were:

- ·····- RmF1021 (wild-type),
- —— RmF121 (*dctD16*::Tn*5*),
- --•• RmF283 (*dctD16*::Tn*5*, Dct$^+$),
- ····· RmF284 (*dctD16*::Tn*5*, Dct$^+$),
- --- RmF740 (*dctD16*::Tn*5*, Ω5085::Tn*5*-233, Dct$^+$, Ssc-10$^-$),
- —— RmF332 (*dctB18*::Tn*5*),
- --- RmF738 (*dctB18*::Tn*5*, Ω5085::Tn*5*-233, Dct$^+$, Ssc-10$^-$)


Absorbance (O.D. 675 nm)
0.2
0.4
0.6
0.8
1.0
20
40
60
80
100
Time (hr.)

succinate. Optical density was recorded at a wavelength of 675 nm. Results are presented in Figure 11. Wild-type strain Rm1021 reached saturation after 20 hours of incubation while the mutant strain, dctD16::Tn5, remained at the inoculating OD throughout the incubation period. The dctD16::Tn5 mutation, in the presence of the suppressors (RmF283 or RmF284), reached saturation after 50 hours of incubation with similar growth kinetics to Rm1021.

## Characterization of dctD16::Tn5 second-site revertant mutation Ssc-10$^-$

An insert, Ω5085::Tn5-233, was previously isolated by I. Oresnik, and found to be 93% linked to a second-site Dct$^+$ mutation in strain RmF208. RmF208 was obtained as a Dct$^+$ Nm$^r$ revertant after plating Rm5314 (dctD16::Tn5, RCR2011 background) onto M9-succinate plates. The second-site dctD suppressor allele was designated Ssc-10$^-$ and strain RmF314 carries this allele linked to Ω5085::Tn5-233. A series of transductions utilizing Ssc-10$^-$ were carried out to examine the effect of its presence on other dct mutants. Results are presented in Table 9. Ssc-10$^-$ did not suppress the Dct$^-$ phenotype of the mutant alleles dctA13, dctA14, dctA19, dctB12, and dctB17. Transduction of the Ssc-10$^-$ mutation into strains containing alleles dctB18, dctD11, and dctD16 enabled these mutants to grow on succinate i.e. 90% of the transductants screened could grow on M9-succinate. Thus, the Ssc-10$^-$ allele appeared to suppress dctD mutations but was

dctB allele specific, requiring the production of an active DCTB protein.

Growth curves in M9-succinate (15 mM) broth were set up to quantitate the suppressing activity as well as the length of time required to activate it. Results of the following suppressor combinations are presented in Figure 11: wild-type strain Rm1021, dctB18::Tn5, dctD16::Tn5, dctB18::Tn5 + Ssc-10$^-$, and dctD16::Tn5 + Ssc-10$^-$. The wild-type strain achieved late-log phase growth by 20 hours of incubation whereas mutants dctB18::Tn5 and dctD16::Tn5

TABLE 9: SUPPRESSING EFFECT OF Ssc-10$^-$ ON THE dct MUTANTS

DONOR LYSATE: (RmF314) = 5085::Tn5-233, 93 % linked to Ssc-10$^-$

| RECIPIENT | COLONIES SCREENED | COLONY PHENOTYPE |
|---|---|---|
| dctA13::TnphoA | 90 | 90 suc$^-$ Nm$^r$ GmSp$^r$ |
| dctA14::Tn5 | 90 | 90 suc$^-$ Nm$^r$ GmSp$^r$ |
| dctA19::Tn5 | 90 | 90 suc$^-$ Nm$^r$ GmSp$^r$ |
| dctB12::Tn5-132 | 90 | 90 suc$^-$ Ot$^r$ GmSp$^r$ |
| dctB17::Tn5 | 90 | 90 suc$^-$ Nm$^r$ GmSp$^r$ |
| dctB18::Tn5 | 90 | 89 suc$^+$ Nm$^r$ GmSp$^r$ |
| | | 1 suc$^-$ Nm$^r$ GmSp$^r$ |
| dctD11::Tn5-lac | 90 | 83 suc$^+$ Nm$^r$ GmSp$^r$ |
| | | 7 suc$^-$ Nm$^r$ GmSp$^r$ |
| dctD16::Tn5 | 89 | 85 suc$^+$ Nm$^r$ GmSp$^r$ |
| | | 4 suc$^-$ Nm$^r$ GmSp$^r$ |

showed no growth and remained at the inoculating optical density. Both mutants, in the presence of Ssc-10$^-$, demonstrated good growth only after 60 hours of incubation, and reached late-log phase after about 100 hours. These

strains appeared unimpaired in growth in M9-glucose (data not shown). Thus, it appears that the dct suppression exhibited by the Ssc-10$^-$ is dependent on environmental conditions such that good suppression is obtained on M9-succinate agar but not in liquid media.

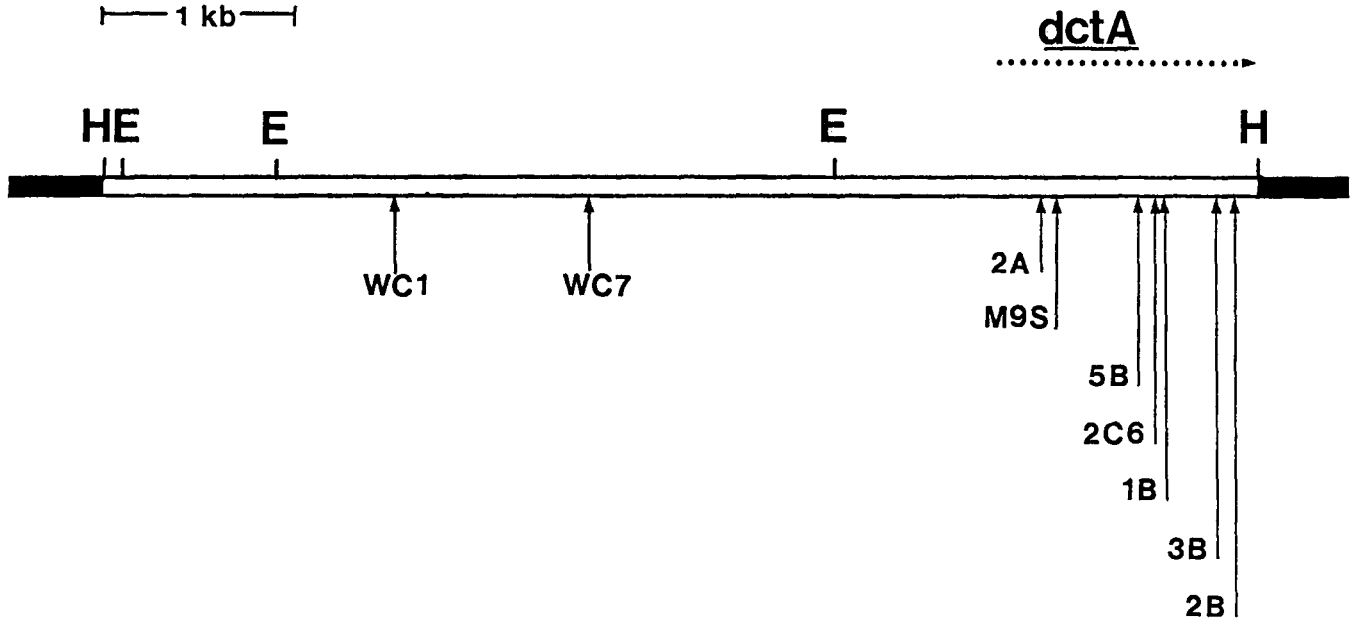
To examine the ntrA-dependence of the Ssc-10$^-$ suppressing activity, a lysate of ntrA76::Tn5 (RmF288) was transduced into RmF314. One hundred transductant colonies were screened; all were suc$^-$ GmSp$^r$ Nm$^r$ indicating that the suppressing activity was ntrA-dependent.

Two strains deleted of the Ssc-10$^-$ region (T.Charles, personal communication) were used to further characterize the suppressing activity. RmF680 (Rm1021ΔΩ5085-5061::Tn5-233), and RmF693 (Rm1021ΔΩ5085-5142::Tn5-233) carry 480 kb and 200 kb deletions, respectively, that delete the Ssc-10$^-$ region but not the dct region. RmF651 (dctA19::Tn5), RmF332 (dctB18::Tn5), and RmF121 (dctD16::Tn5) were transduced into both RmF680, and into RmF693. Ninety colonies were screened for each cross. All were suc$^-$ Nm$^r$ GmSp$^r$. This suggests that a gene product is required for the suppressing activity. Moreover strains RmF680 and RmF693 grew normally on M9 succinate suggesting that the wild-type suppressor allele was not involved in $C_4$-dicarboxylate transport.

## TnphoA mutagenesis and strain construction

To identify dct transmembrane proteins involved in the detection and active transport of $C_4$-dicarboxylates across the peribacteroid membrane dct::TnphoA insertions were isolated. TnphoA, a Tn5 derivative, contains DNA coding for the alkaline phosphatase gene (Manoil and Beckwith 1985) (Appendix 1). The expression of alkaline phosphatase is dependent on an in-frame TnphoA insertion, and can be detected by cleavage of the substrate 5-bromo-4-chloro-3-indolyl-phosphate resulting in blue colony colour. TnphoA insertions were isolated in plasmids pTH24, pTH31, and pTH32. The positions and orientations of insertions were determined by restriction analysis with BamHI, EcoRI, and XhoI (Figure 12). Alkaline phosphatase activity of potential plasmid insertions was assayed in a phoA$^-$ background (Rm8002; Long et al. 1988). Seventeen independently isolated blue inserts mapped in the same orientation within the dctA transcriptional unit of the 2.2 kbp EcoRI-HindIII fragment. All, when transferred to the dctA mutant strains RmF642 (dctA14::Tn5) and RmF651 (dctA19::Tn5), failed to complement the dctA mutations. Plasmids from independent white colonies were also analysed: the inserts mapped to either vector DNA or to the 2.9 kbp EcoRI fragment. This suggests that the dctA gene product is the only highly-expressed dct protein in the membrane, and is consistent with the model of dctA encoding a dicarboxylate transport protein. Two TnphoA

Figure 12: Tn*phoA* inserts in plasmids pTH24, pTH31, and pTH32.


1 kb
dctA
HE
E
E
H
WC1
WC7
2A
M9S
5B
2C6
1B
3B
2B

inserts were employed to study the regulation of dctA gene expression. Thus, insert 2C6 in pTH24 (designated pTH2C6) resulted in a dctA::TnphoA, dctB$^+$, dctD$^+$ plasmid. Insert 2A in pTH32 (pTH2A) resulted in a dctA::TnphoA plasmid lacking most of dctB and all of the dctD coding sequence.

Regulation of dct utilizing dctA::TnphoA fusions

Regulation of the dctA::TnphoA fusions was assayed by measuring the alkaline phosphatase activity in wild-type and mutant background strains. Lysates of strains containing dct or ntrA mutations were transduced into RmF222 to construct mutant strains in the phoA$^-$ background. The double mutant strains RmF881 (dctA14::Tn5-233 dctD16::Tn5) and RmF883 (dctA14::Tn5-233 dctB18::Tn5) had been constructed in the phoA$^-$ background. Plasmids pTH2C6 and pTH2A were mobilized into the phoA$^-$ background (RmF222), and into the dct and ntrA mutant derivatives of RmF222. These constructs were utilized in the study of dct regulation. Results are presented in Table 10. Strains were incubated in minimal media supplemented with a) 15 mM succinate, b) 15 mM glucose and 15 mM succinate, or c) 15 mM glucose, or incubated in LB, and were assayed for alkaline phosphatase activity.

The expression of dctA::TnphoA from plasmids pTH2C6 and pTH2A in a wild-type background was induced in succinate-grown cells compared to glucose-grown cells. In the ntrA::Tn5 background basal levels of dctA::TnphoA expression

was obtained from both plasmids. This confirmed the requirement of NTRA for dctA transcription. Expression of plasmid pTH2C6 in dctB or dctD backgrounds was similar to that in the wild-type background: high levels in the presence of succinate and no induction when glucose alone was added to the media. This result was expected as copies of dctB and dctD were present on pTH2C6. Plasmid pTH2A was not expressed in either dctB or dctD backgrounds indicating a requirement for these gene products. Both plasmids were expressed in a dctA background when grown in either carbon source indicating constitutive deregulated dctA expression.

Double mutant strains were constructed to determine whether dctB or dctD were required for dctA::TnphoA expression in a dctA background. Double mutants containing plasmid pTH2A demonstrated a lack of alkaline phosphatase activity. Double mutants containing pTH2C6 were measured with constitutive dctA::TnphoA expression when cells were grown in media containing glucose. These fusions demonstrated the requirement for ntrA, dctB, and dctD for dctA::TnphoA expression.

## Succinate uptake by dctD16::Tn5 and second-site revertant strains

Strains utilized for succinate uptake assays were grown as cultures in TY broth supplemented with 15 mM succinate. Assays were carried out as outlined in

TABLE 10: ALKALINE PHOSPHATASE ACTIVITY FROM *dctA*::TnphoA FUSION IN VARIOUS MUTANT BACKGROUNDS

| Chromosomal background | Fusion plasmid | Alkaline phosphatase specific activity for cells grown in: Suc | Suc + Glu | Glu | LB |
|---|---|---|---|---|---|
| wild-type | none | 22 | 33 | 24 | 11 |
| | pTH2A | 573 | 388 | 63 | 194 |
| | pTH2C6 | 772 | 571 | 51 | 158 |
| *ntrA76* | pTH2A | ND | 24 | 29 | 11 |
| | pTH2C6 | ND | 44 | 31 | 8 |
| *dctB18* | pTH2A | ND | 25 | 29 | 0 |
| | pTH2C6 | 1632 | 645 | 54 | 201 |
| *dctD16* | pTH2A | ND | 24 | 30 | 5 |
| | pTH2C6 | 1947 | 728 | 71 | 178 |
| *dctA14* | pTH2A | ND | 1015 | 496 | 390 |
| | pTH2C6 | ND | 402 | 580 | 416 |
| *dctA dctB* | pTH2A | ND | 28 | 27 | 9 |
| | pTH2C6 | ND | 173 | 632 | 561 |
| *dctA dctD* | pTH2A | ND | 47 | 28 | 26 |
| | pTH2C6 | ND | 283 | 287 | 395 |

Plasmid pTH2A is pTH32 *dctA*::TnphoA; plasmid pTH2C6 is pTH24 *dctA*::TnphoA *dctB*$^{+}$ *dctD*$^{+}$. All assays were carried out in the Rm8002 background (Rm1021, *phoA*$^{-}$). One unit of alkaline phosphatase is defined as the amount required to hydrolyse 0.062 nmoles of p-nitrophenyl phosphate per min at a cell optical density at 600 nm of 1. Abbreviations Glu or Suc refer to M9 broth supplemented with 15 mM glucose or 15 mM succinate, respectively.

Methodology with 50 $\mu$M [2,3-$^{14}$C] succinate (1 $\mu$Ci/$\mu$mole) as the substrate. Results are presented in graphic form in Figure 13.

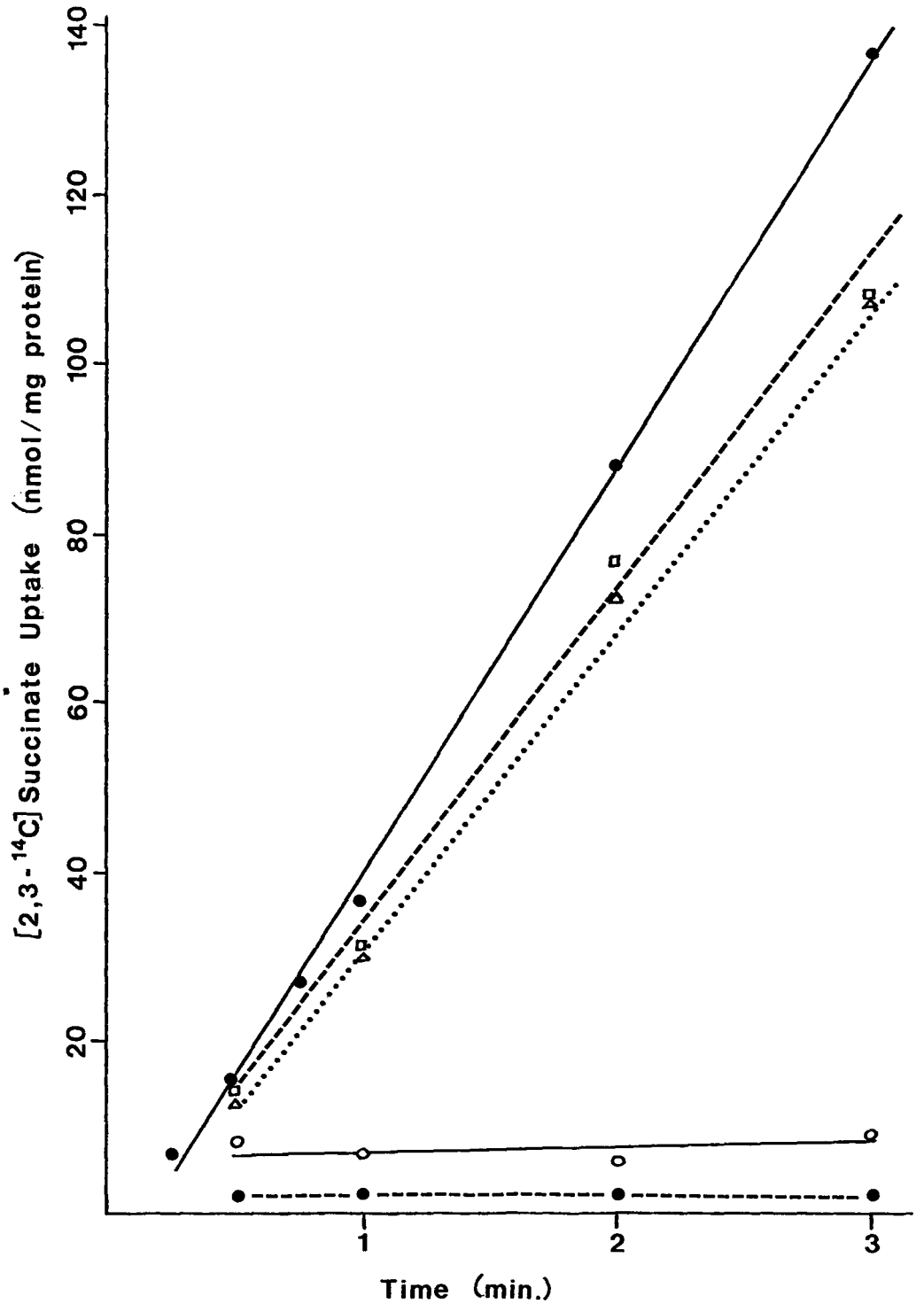
Wild-type Rm1021 showed linear uptake of succinate with time and, after three minutes of incubation, had transported 135 nmol of labelled succinate per mg of cellular protein. Mutant strain RmF121 (dctD16::Tn5) failed to transport succinate and remained at a minimal level of uptake of 2 nmol succinate/mg protein. The dctD16::Tn5 mutation in the presence of the Ssc-10$^-$ suppressor also demonstrated minimal transport accumulating 9 nmol succinate per mg of protein. This further suggests that the dct suppressing activity demonstrated by Ssc-10$^-$ is dependent on particular environmental conditions. Strains RmF283 and RmF284 contain both the dctD16::Tn5 mutation as well as independently isolated second-site mutations. Both strains show significant transport with rates of uptake after three minutes of incubation of 112 and 106 nmol succinate/mg protein, respectively, slightly lower than those seen with Rm1021.

## Symbiotic properties of group V mutants and second-site revertant strains

Inoculation of the group V dct mutants onto alfalfa seedlings resulted in variable levels of nitrogenase activity (Table 11) by the resulting nodules. Plants inoculated with

Figure 13: [2,3-$^{14}$C] succinate uptake in dctD16 and second-site revertant strains. Strains were examined for dct induction after growth in TY containing 15 mM succinate. Strains examined were:

—●— Rm1021 (wild-type),
-●- RmF121 (dctD16::Tn5),
—○— RmF740 (dctD16::Tn5, Ssc-10$^-$),
-□- RmF283 (dctD16::Tn5, Dct$^+$),
···△··· RmF284 (dctD16::Tn5, Dct$^+$),


[2,3-14C] Succinate Uptake (nmol/mg protein)
140
120
100
80
60
40
20
1
2
3
Time (min.)

the dctA mutants showed minimal nitrogenase activity comparable to uninoculated control plants. Partial nitrogenase activity was detected in nodules containing bacteroids mutated in dctB and dctD genes with lowest and highest levels at 13% and 85% of wild-type values. Plants were smaller than wild-type, and nodules varied from small, round, and white to cylindrical and actively producing heme.

An interesting observation was that there appeared to be a pattern of decreasing nitrogenase activity with Tn5 insert positions further into the dctB gene region. Visual observations were carried out twice with homogenotized inserts dctB22::Tn5 (RmF644), dctB24::Tn5 (RmF645), and dctB25::Tn5 (RmF656) (Figure 9), and control mutant allele dctB18::Tn5 (RmF332). Inoculation of dctB18::Tn5 and dctB22::Tn5 resulted in plants similar in appearance and smaller in height to wild-type plants. Plants inoculated with dctB24::Tn5 were smaller than dctB18::Tn5-containing plants and leaves showed some yellowing. The inoculation of the dctB25::Tn5 strain resulted in plants with no appearance of nitrogen fixation and very close resemblance to uninoculated plants.

As shown in Table 11, inoculation of strains containing second-site mutations that suppress the dctB or dctD mutation resulted in plants with higher levels of acetylene reduction than strains carrying only dctB or dctD mutations. Levels of nitrogenase activity by the bacteroids

ranged from 48% to 96% of wild-type levels and no suppressor-containing strain gave higher nitrogenase activity than nodules containing wild-type bacteroids.

TABLE 11: SYMBIOTIC PROPERTIES OF *R. meliloti* *dct* DERIVATIVES

| Allele | | nmol ethylene $hr^{-1}$ $plant^{-1}$ | % of wild type |
|---|---|---|---|
| wild type | (Rm1021) | 451.0 ± 120 | 100.0 % |
| *dctA13* | (RmF481) | 7.9 ± 1 | 1.7 % |
| *dctA14* | (RmF642) | 7.3 ± 2 | 1.6 % |
| *dctB12* | (RmF153) | 267.8 ± 33 | 59.2 % |
| *dctB18* | (RmF332) | 353.0 ± 118 | 78.3 % |
| *dctB17* | (Rm5421) | 60.0 ± 24 | 13.3 % |
| *dctB17* $suc^+$ | (RmF173) | 236.8 ± 71 | 49.0 % |
| *dctD11* | (Rm5312 | 404.1 ± 41 | 89.6 % |
| *dctD16* | (RmF121 | 330.5 ± 69 | 73.4 % |
| *dctD16* $suc^+$ | (RmF208) | 220.0 ± 7 | 48.8 % |
| *dctD16* $suc^+$ | (RmF283) | 455.5 ± 99 | 101.0 % |
| *dctD16* $suc^+$ | (RmF284) | 364.2 ± 93 | 80.7 % |

Acetylene reductions were performed 28 days after inoculation. Values represent the mean of three or more assays.

Analysis of R. leguminosarum Dct$^-$ mutants

Finan et al. (1981, 1983) isolated Dct$^-$ mutants of Rhizobium leguminosarum wild-type strain GF160 using Tn5 mutagenesis. Plasmids complementing R. meliloti ntrA and dct mutations were conjugated into the control strain GF160, and into two R. leguminosarum Dct$^-$ strains GF252 and GF31. The Dct$^-$ phenotypes of GF252 and GF31 were complemented by plasmids pTH24 and pTH31, but no complementation was observed by the ntrA-containing plasmid pSF1. Finally, pTH24 and pTH31 plasmids containing defined Tn5 inserts in dctA, dctB, or dctD genes were conjugated into GF252. Inserts 14 and 19 in the dctA gene or inserts 21 and 22 in the dctB gene did not hinder complementation of GF252. Transfer of plasmids containing inserts 29 and 30 in the dctD gene resulted in no complementation of the Dct$^-$ phenotype of GF252. Collectively these data suggested that strain GF252 carried a dctD mutation.

# CHAPTER 6

# DISCUSSION

## Group I mutations correspond to the ntrA gene

The first group of Dct$^-$ mutants appeared identical to ntrA mutants of R. meliloti identified by Ronson et al. (1987b). This conclusion is based on (1) physical analysis of the DNA region, and (2) the Dct$^-$, Fix$^-$, and $NO_3^-$ phenotypes of group I and ntrA mutants. The restriction sites contained within the BamHI-EcoRI fragment in pSF1 (Figure 2) are similar to those present in a restriction map of the R. meliloti ntrA region (Ronson et al. 1987b). pSF1 containing Tn5 inserts #4, #6, and #20 complemented the group I mutation (Figure 3). Comparison of restriction data to the published sequence confirmed these inserts to be located outside the ntrA transcriptional unit. pSF1 Tn5 insert #5 failed to complement, and was mapped to within the ntrA coding region. This insert was exchanged into the wild-type Rm1021 genome resulting in strain RmF288 (ntrA76::Tn5). Southern blot analysis of RmF288, RmF122 (ntrA73::TnV), and RmF250 (ntrA74::Tn5-233) confirmed these insertions to be localized in the 3.5 kbp BamHI-EcoRI fragment in the ntrA gene (Figure 3). Previous work by Finan et al. (1988) had

mapped the group I mutation adjacent to the trp-33 locus on the R. meliloti chromosome.

The ntrA mutation in group I resulted in three phenotypes: (1) an inability to fix $N_2$ symbiotically, (2) an inability to use $KNO_3$ as a nitrogen source, and (3) an inability to transport $C_4$-dicarboxylic acids. It has been demonstrated that Klebsiella pneumoniae nif and R. leguminosarum dctA genes have the NTRA consensus promoter sequence (Ausubel 1984, Buck 1986, Ronson et al. 1987a). Mutations in the ntrA gene of Klebsiella pneumoniae and Azotobacter vinelandii resulted in $Fix^-$ and $NO_3^-$ phenotypes (Santero et al. 1986, Kennedy and Toukdarian 1987). NTRA and NIFA are required for transcription of other nif genes (Ausubel 1984, Hirschman et al. 1985, Hunt and Magasanik 1985, Gussin et al. 1986). Furthermore, NTRA is required for the regulatory role of NTRC ($NR_I$) in the activation of nitrogen-regulated (Ntr) operons (Magasanik and Neidhardt 1987, Ninfa and Magasanik 1987). Thus, the phenotypes for the group I mutation are consistent with reported phenotypes of R. meliloti ntrA mutants (Ronson et al. 1987b), and with the characterized roles of NTRA in the bacterial cell.

Independent $Dct^+$ second-site revertant strains of the ntrA mutation were isolated. These revertant strains remained $Fix^-$, and failed to utilize nitrate as a nitrogen source (Table 5, Figure 5). Three-factor transductional crosses mapped the second-site mutation to the dct locus

(Figure 4) on megaplasmid pRmeSU47b adjacent to the previously described thi locus (Finan et al. 1986). The second-site mutation in dct resulted in constitutive Dct expression independent of functional NTRA. This suggests that alternative sigma factors can replace NTRA to enable RNA polymerase to initiate transcription at dctA; thus, dctA transcription is no longer dependent on the presence of $C_4$-dicarboxylates.

Group V mutations correspond to the dct genes

The second group of $Dct^-$ mutants corresponds to the dct genes (dctA, dctB, and dctD) involved in aspects of $C_4$-dicarboxylic acid transport. Evidence supporting this is based on: (1) physical analysis of the DNA region and comparison to other R. meliloti and R. leguminosarum work, (2) Tn5 mutagenesis of complementing clones and analysis, (3) regulation studies of dct::TnphoA fusions, and (4) symbiotic analysis.

A 6 kbp HindIII subclone (pTH24) complemented all group V mutants (Figure 7). Restriction analysis indicated that the EcoRI and HindIII fragments corresponded to that reported for other R. meliloti work (Watson et al. 1988, B. T. Nixon personal communication). Complementation with subclones pTH31, pTH32, and pTH33 indicated the presence of three transcriptional units in the dct region (Figure 7). These were identified as dctA, dctB, and dctD on the basis of

sequencing studies provided by B. T. Nixon (personal communication). Plasmid pTH31 appears to contain the complete *dct* region as it was able to fully complement all *dct* mutant strains. The 2.2 kbp *Eco*RI-*Hind*III fragment carried in plasmid pTH32 complemented mutations arising in the *dctA* region, and is thus likely to carry the *dctA* promoter. The 2.9 kbp *Eco*RI fragment in pTH33 demonstrated only partial complementation of *dctD* mutants. Furthermore, the introduction of pTH33 decreased the growth rate of wild-type Rm1021 in minimal media containing succinate (Figure 8). Growth in glucose minimal media was not affected. This observation may be the result of having the additional copy of *dctD* present on pTH33 whereas only one complete copy of *dctB* is present in the cell. An insufficient amount of translated DCTB to activated the additional DCTD produced from pTH33 and from pRmeSU47b may result in the inactive DCTD repressing *dctA* transcription. Thus, only a partial $Dct^+$ phenotype is expected.

Tn*5* mutagenesis of plasmids pTH24 and pTH31, and examination of insert phenotypes in mutant backgrounds (Figure 9) provided an insertional complementation map of the *dct* locus. Comparison of the transcriptional units with a complete sequence of the *R. meliloti dct* locus (Nixon personal communication) verified the genotypic assignment, and the localization and extent of the genes. The *dctA* promoter region was found to contain the NTRA consensus

promoter sequence; dctA was transcribed divergently from dctB and dctD. Furthermore, Nixon compared the DNA sequence of the dct genes in both R. meliloti and in R. leguminosarum and found 92% homology between the dctA genes, 75% homology for dctB, and 85% homology for dctD.

Several Tn5 inserts in pTH24 and pTH31 demonstrated unusual Dct phenotypes (Figure 9). Insert 32, although located prior to the termination sequences of the dctA gene, continued to complement the dctA mutations. This indicated that approximately the last 100 bp of dctA were not essential for the production and localization of an active and effective transmembrane protein. Insert 25, in the terminal region of dctB, demonstrated only partial complementation of dctA mutations. It failed to complement dctB mutants, and fully complemented dctD mutants. A partial Dct phenotype was observed in the deletion background. In the model (Ronson et al. 1987a) the DCTA component of the DCTA-DCTB complex, in the presence of $C_4$-dicarboxylates, releases DCTB to activate DCTD. Several explanations are suggested for the partial DCT phenotype obtained: the truncated DCTB may be inefficiently released by DCTA, or, if released, may inefficiently activate DCTD to promote dctA transcription. Insert 31, exhibiting a $Dct^+$ phenotype, was mapped to the region between dctB and dctD. According to Nixon only four bp separate dctB termination and dctD initiation sites for transcription, thus the probability of a Tn5 insert in this region is very low.

Furthermore one would expect a requirement for an intact dctD promoter region for effective dctD transcription. A possible explanation may be that an alternate start codon for transcription is utilized. Further studies sequencing the area of insertion and examining sequence requirements for transcription and translation are required.

Fusions of dct::TnphoA indicated that DCTA was the only dct protein containing transmembrane regions, supporting its assigned role as a $C_4$-dicarboxylate carrier protein (Ronson et al. 1987a). It was shown that, in free-living cells, dctA::TnphoA expression was ntrA, dctB, and dctD dependent (Table 10). In dctA mutant backgrounds the expression of the dctA::TnphoA was constitutive. A similar observation has been reported for dctA-lacZ fusions in R. leguminosarum (Ronson and Astwood 1985). Furthermore, assays of the dctA::TnphoA fusions in double dct mutant backgrounds demonstrated that the constitutive dctA::TnphoA expression in a dctA mutant background was dependent on dctB and dctD (Table 10). In the model, in the presence of $C_4$-dicarboxylates DCTB is activated; in turn DCTD is activated and together with NTRA activates transcription at dctA. The dctA::TnphoA fusion did not result in functional DCTA. As a result, both DCTB and DCTD remained activated and the dctA::TnphoA fusion was constitutively expressed. We suggest a modification to the model whereby DCTA, not DCTB, senses

$C_4$-dicarboxylates, and DCTB recognizes the resulting conformation change to DCTA.

Analysis of dctB and dctD second-site mutations

Second-site revertant strains were isolated for transposon mutations in both dctB and dctD that restored the Dct and Fix phenotypes to near wild-type levels.

A high percentage of second-site mutations were obtained for dctB25::Tn5 (RmF646), and dctB17::Tn5-233 (Rm5421). The Fix$^-$ phenotype of these alleles was discussed in the previous section. The second-site mutation was mapped to be tightly linked to the dctB gene suggesting the mutation was in the dctD gene. The mutation would alleviate the requirement for active DCTB by causing DCTD to be translated in an active form. As was expected, the second-site revertant strains remained NTRA-dependent. The second-site mutation may also map to the dctA promoter altering the requirement for activated DctD but remaining NTRA-dependent.

Three second-site mutation strains of dctD16::Tn5 (RmF208, RmF283, and RmF284) were obtained and further characterized. The presence of secondary mutations increased nitrogen fixation to almost wild-type levels. The second-site mutation in strain RmF284 was demonstrated to be closely linked to the dctD16::Tn5 allele. The secondary mutation was not dependent on functional NTRA suggesting a mutation in the dctA promoter region altering its requirement for NTRA.

Thus, in this strain dctA transcription is probably initiated by an alternate sigma factor binding RNA polymerase and, thus, is not regulated by the presence or absence of $C_4$-dicarboxylates. These results are similar to those observed with the second-site revertants of mutations in ntrA.

The secondary mutation in RmF208 (designated Ssc-10$^-$) was mapped 150 kb from the dct region on pRmeSU47b (I. Oresnik personal communication). Although the Dct phenotype was restored on M9-succinate agar, [$^{14}$C]-succinate uptake assays on cells grown in TY succinate indicated an inactive transport system (Figure 13). Furthermore, little growth (or suppression of the dctD16 mutation) was obtained in M9-succinate broth (Figure 11). This is suggestive of environmental growth conditions affecting Ssc-10$^-$. As strains deleted of the region to which Ssc-10$^-$ map have a wild-type succinate phenotype, the wild-type allele does not appear to play a primary role in the regulation of the dct locus. The normal function of the gene remains to be identified. The NTRA-dependence of the mutation suggests the protein is similar in function to DCTD, replacing DCTD to interact with NTRA in initiating transcription of dctA.

The secondary mutation with RmF283 is 100% linked to ntrC::Tn5 (Szeto et al. 1987), and thus is likely to be allelic with ntrC (Finan and Charles, personal communication). As outlined earlier, NTRC and NTRB play an important role in nitrogen regulation (McFarland et al. 1981,

Magasanik 1982, Keener and Kustu 1988). NTRC has been demonstrated to activate several R. meliloti nif genes (nifH, fixA and others) in free-living bacteria but not in bacteroids (Szeto et al. 1987). NTRB-NTRC and DCTB-DCTD have been identified as members of two-component regulatory systems (Nixon et al. 1986, Ronson et al. 1987a and 1987c). Sensor or modulator proteins such as DCTB and NTRB contain homologous regions in both amino and carboxy terminal regions of the protein and are postulated to act as protein kinases in the activation of regulator proteins. Regulator or effector proteins such as DCTD and NTRC contain homologous regions in the amino terminal regions of the protein, contain possible DNA-binding regions, and act at transcriptional promoters through an interaction with NTRA. Recently "crosstalk" or cross-specificity has been reported (Ninfa et al. 1988): the protein kinases CHEA (regulation of chemotaxis) and NTRB (nitrogen assimilation) can phosphorylate NTRC and CHEY, respectively, suggesting a common protein phosphotransfer mechanism. It is probable that, in the dctD16 suppressing strain RmF283, the mutation in ntrC permits very efficient recognition of the dctA promoter by NTRC in the presence of NTRA resulting in "cross-communication". Similarly, the suppressing action of Ssc-10$^-$ is probably the result of another, as of yet, unidentified regulator protein of a two-component system.

## Effective symbiosis requires dctA transcription

Symbiotic phenotypes of the dct mutants were determined. The Fix$^-$ phenotype of dctA mutants supports the model of direct DCTA involvement in $C_4$-dicarboxylate transport, and the requirement of Dct for effective symbiosis. This result has also been reported for R. leguminosarum dctA mutants (Ronson and Astwood 1985). Two groups of Fix phenotypes (partially effective and ineffective) were obtained for dctB and dctD mutants. A partially effective Fix phenotype was reported by other researchers. Finan et al. (1981 and 1983) obtained an R. leguminosarum Dct$^-$ mutant, GF252; bacteroids isolated from mutant nodules were Dct$^+$ and partially Fix$^+$. Ronson and Astwood (1985) isolated R. leguminosarum dctB and dctD mutants that gave a partially effective Fix phenotype. Engelke et al. (1988) obtained R. meliloti Dct$^-$ mutants; bacteroids purified from the mutant nodules could transport $C_4$-dicarboxylates, and were partially Fix$^+$. The partially effective and ineffective Fix phenotypes will be addressed separately.

The partial nitrogen fixation exhibited by inserts in dctB and dctD suggests these genes are not absolutely required for $N_2$-fixation. We used R. meliloti dct-containing plasmids to identify a dctD::Tn5 mutation in R. leguminosarum GF252. It had been demonstrated that Dct occurred in the partially Fix$^+$ GF252 bacteroids (Finan et al. 1983). An

attempt was made to assay our dctB and dctD bacteroids for succinate uptake; this proved to be unsuccessful even for wild-type bacteroids (results not shown). Based on the GF252 result, we would expect to obtain transport by dctB and dctD bacteroids. We presume that the $Fix^+$ phenotype demonstrated by these mutants indicates dctA expression and $C_4$-dicarboxylic acid transport are maintained in the bacteroid independent of dctB and dctD expression. This may involve proteins in a regulatory crosstalk role or may reflect bacteroid dct specific regulation.

Inserts corresponding to the carboxy-terminal region of DCTB had very low rates of nitrogen fixation. Based on sequence homology in this region and interactions in other systems it was suggested that the DCTB carboxy-terminal region was involved in DCTD activation (Ronson et al. 1987a). The truncated DCTB produced as a result of insertions dctB17::Tn5-233 (Rm5421) and dctB25::Tn5 (RmF646) may interfere with DCTD or other regulatory proteins that are required to activate dctA transcription, or may interfere with the transport of $C_4$-dicarboxylates by DCTA.

The results indicate that, in the bacteroid, only DCTA of the DCT proteins is required for effective $C_4$-dicarboxylic acid transport and nitrogen fixation. How is transcription of dctA initiated in the bacteroid? An example of crosstalk was demonstrated between the chemotaxis and the nitrogen regulation systems (Ninfa et al. 1988). We have

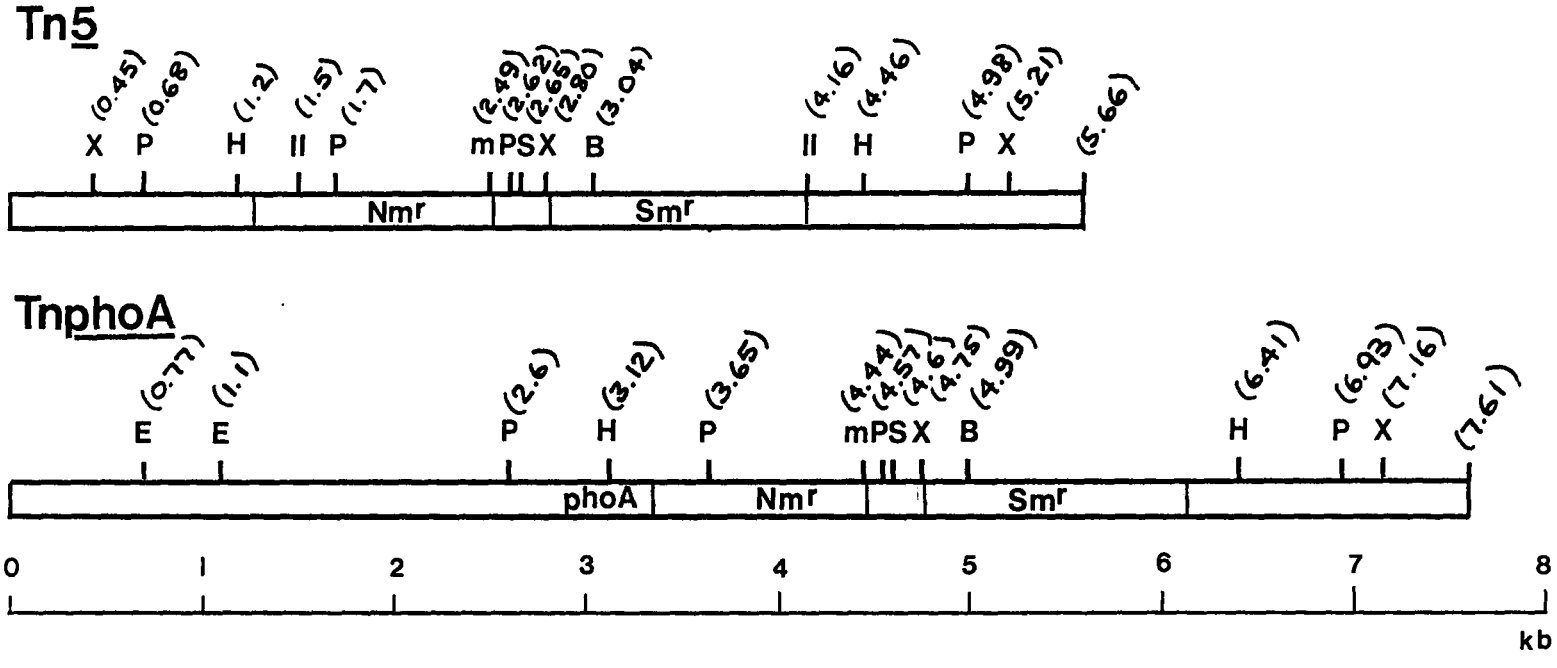
obtained second-site mutations that map to other regulatory systems (eg. to the *ntrC* locus in the revertant strain RmF283). Further work may demonstrate that crosstalk does occur and may be responsible for activation of *dctA* transcription, or that there exist other *dct*-specific regulatory mechanisms in the bacteroid.

## APPENDIX 1

### RESTRICTION MAP OF TRANSPOSONS Tn*5* AND Tn*phoA*

A map of insertion transposons Tn*5* and Tn*phoA* indicating key restriction enzyme sites. Symbols for restriction enzymes are as follows:

| | | | |
|---|---|---|---|
| B | *Bam*HI | II | *Bgl*II |
| E | *Eco*RI | H | *Hind*III |
| P | *Pst*I | S | *Sal*I |
| m | *Sma*I | X | *Xho*I |


Tn5
(0.45)
(0.68)
(1.2)
(1.5)
(1.7)
(2.49)
(2.62)
(2.65)
(2.80)
(3.04)
(4.16)
(4.46)
(4.98)
(5.21)
(5.66)
X P H II P mPSX B II H P X
Nmr
Smr
TnphoA
(0.77)
(1.1)
(2.6)
(3.12)
(3.65)
(4.44)
(4.57)
(4.61)
(4.75)
(4.99)
(6.41)
(6.93)
(7.16)
(7.61)
E E P H P mPSX B H P X
phoA
Nmr
Smr
0 1 2 3 4 5 6 7 8
kb

## APPENDIX 2

### ACETYLENE REDUCTION BY *dct* MUTANTS IN THE PRESENCE OF COMPLEMENTING PLASMIDS

| Allele | Strain | Plasmid | nmol ethylene $hr^{-1}$ $plant^{-1}$ | % of wild type |
|---|---|---|---|---|
| wild-type | Rm1021 | none | 434 ± 190 | 100 % |
| *dctA14* | RmF642 | none | 7 ± 2 | 2 % |
| *dctA14* | | pTH32 | 226 ± 19 | 52 % |
| *dctB18* | RmF332 | none | 259 ± 60 | 60 % |
| *dctB18* | | pTH31 | 274 ± 39 | 63 % |
| *dctD16* | RmF121 | none | 346 ± 84 | 80 % |
| *dctD16* | | pTH31 | 380 ± 121 | 88 % |
| *dctD16* | | pTH33 | 230 ± 11 | 53 % |

Acetylene reductions were carried out 28 days after inoculation. Values reported are based on the assaying of nine plants in groups of three.

## APPENDIX 3

## SOUTHERN ANALYSIS OF SINGLE AND DOUBLE *dct* MUTANTS

Analysis of hybridizations obtained to plasmid pTH24 containing a 6 kbp *Hin*dIII fragment corresponding to the *dct* region. Transposons Tn*5* and Tn*5*-233 contain *Hin*dIII sites 1.2 kb into each of the *IS50* regions. A map of pTH24 is presented in Figure 7, pg 69.

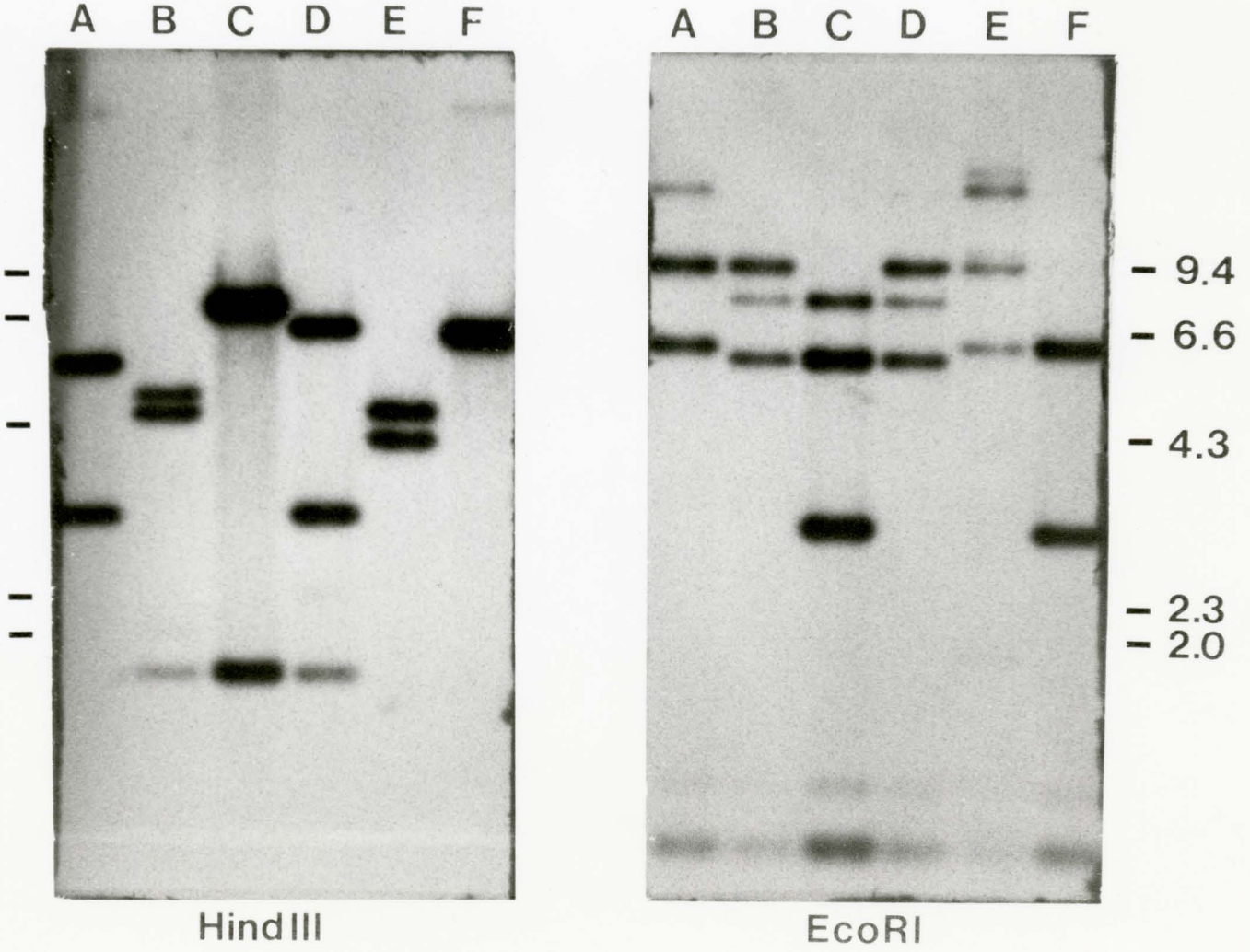
*Hin*dIII restriction of total *dct* DNA

Lane A: RmF121 (*dctD16*::Tn*5*). Two bands obtained. Top band at 4.9 cm corresponds to a 5.3 kbp fragment. Bottom band at 7.2 cm corresponds to a 3 kbp fragment. Subtracting the IS*50* *Hin*dIII regions in Tn*5* results in the mapping of this allele at 4.1 and 1.8 kbp from the vector *Hin*dIII sites.
Lane B: RmF883 (*dctA14*::Tn*5*-233, *dctB18*::Tn*5*, *phoA*$^-$). Three bands obtained. The 5.55 cm band is as described for RmF612. The 9.6 cm band is as described for RmF865. The top band at 5.3 cm corresponds to a 4.8 kbp fragment. As this fragment included regions of Tn*5* from both inserts the distance between the alleles was calculated to be 2.4 kbp.
Lane C: RmF865 (*dctA14*::Tn*5*-233, *phoA*$^-$). Two bands obtained. Top band at 4 cm corresponds to a 6.9 kbp fragment. Bottom band at 9.6 cm corresponds to a 1.65 kbp fragment. Calculating for IS*50* *Hin*dIII regions in Tn*5*-233 results in the mapping of this allele 5.7 and 0.45 kbp from the vector *Hin*dIII sites.
Lane D: RmF881 (*dctA14*::Tn*5*-233, *dctD16*::Tn*5*, *phoA*$^-$). Three bands obtained. The 7.2 cm band is as described for RmF121. The 9.6 cm band is as described for RmF865. The top band at 4.3 cm corresponds to a 6.25 kbp fragment. As this fragment included regions of Tn*5* from both inserts the distance between the inserts was calculated to be 3.85 kbp.
Lane E: RmF612 (*dctB18*::Tn*5*, *phoA*$^-$). Two bands obtained. The 5.55 cm band corresponds to a 4.45 kbp fragment. The 6 cm band corresponds to a 4 kbp fragment. Calculating for IS*50* *Hin*dIII regions in Tn*5* results in the mapping of this allele 3.25 and 2.8 kbp from the vector *Hin*dIII sites.
Lane F: Rm1021 (wild-type DNA). One band at 4.35 cm corresponds to the 6 kbp *Hin*dIII fragment containing the Dct genes.

EcoRI restriction of total DNA

Analysis of hybridizations to plasmid pTH24 by DNA restricted with EcoRI. Shift of bands confirms presence of a Tn5 or Tn5-233 insertion in the EcoRI fragment.

Lane A: RmF121 (dctD16::Tn5)
Lane B: RmF883 (dctA14::Tn5-233, dctB18::Tn5, phoA$^-$)
Lane C: RmF865 (dctA14::Tn5-233, phoA$^-$)
Lane D: RmF881 (dctA14::Tn5-233, dctD16::Tn5, phoA$^-$)
Lane E: RmF612 (dctB18::Tn5, phoA$^-$)
Lane F: Rm1021 (wild-type)


A B C D E F
A B C D E F
9.4
6.6
4.3
2.3
2.0
HindIII
EcoRI

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