INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Tellurite Resistance of *Proteus mirabilis*

by

Anna A. Toptchieva

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
October, 1998

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Tellurite Resistance of *Proteus mirabilis*"

by Anna Toptchieva

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dated: November 30, 1998

External Examiner

Research Supervisor

Examining Committee

DALHOUSIE UNIVERSITY

DATE November 30, 1998

AUTHOR Anna Alexandria Toptchieva

TITLE Tellurite Resistance of *Proteus mirabilis.*

Department or School Microbiology and Immunology

Degree Ph. D. Convocation May Year 1999

Permission is herewith granted to Dalhousie University to circulate and to have copied for non-commercial purposes, at its discretion, the above title upon the request of individuals or instructions.

_____

Signature of Author

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Proteus mirabilis, a Gram-negative urinary tract pathogen, displays a form of developmental behaviour on solid medium, termed swarming, that includes cycles of outward migration interspersed with periods of consolidation. To identify developmentally regulated genes, I used transposon mutagenesis to generate promoter-reporter gene fusions. One of the fusions exhibited negative regulation of the reporter gene expression during swarmer cell differentiation (namely, the short vegetative cells showed four-fold increase in the reporter-gene activity as compared to the swarmer cells), and was studied further by subcloning flanking DNA fragments into pBR322. The mini-transposon (Tn5-*lacZ*) insertion was located within an open reading frame (ORF) termed *terC*, a gene associated with tellurite resistance in enteric bacteria. Further analysis of flanking *terC* sequences revealed six open reading frames designated *terZ, -A, -B, -C, -D* and *–E*. All of the putative proteins encoded by these genes showed extensive amino acid sequence similarity with the previously characterized gene products of the IncH12 tellurite resistance (Te^r) operons from plasmids pR478 and pMER610 found in Gram-negative bacteria. A screen of 31 clinical isolates of *P. mirabilis* and 4 strains of *Proteus vulgaris* indicated that resistance is a common feature of this genus. Insertion of the mini-transposon into *terC* reduced the level of tellurite resistance by more than 50%. The pR478-derived *ter* locus restored wild type levels of tellurite resistance in the mutant strain. Northern blot analysis revealed several transcripts that were detectable only when the wild type bacteria were grown in the presence of tellurite. The 5 kb transcript was disrupted by the mini-

transposon insertion in the *terC* of the mutant. These studies suggest that the *ter* locus may be negatively regulated during swarmer cell development, but is positively regulated by the presence of tellurite. This thesis further suggests that *Proteus mirabilis* may be the evolutionary origin of the plasmid-borne *ter* loci.

ABBREVIATIONS

APS	Ammonium persulfate
DEPC	Diethyl pyrocarbonate
DTT	Dithiothreitol
IM	Inner membrane
LPS	Lipopolysaccharide
MIC	Minimal inhibitory concentration
OM	Outer membrane
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	Tetramethylethylenediamide
UTI	Urinary tract infection
WT	Wild type

INTRODUCTION

The opportunistic urinary tract pathogen *Proteus mirabilis* is found in water, soil and in the intestinal tracts of mammals. *P. mirabilis* is a motile Gram-negative bacterium that produces a variety of virulence factors (urease, protease, hemolysin etc.). The ability to undergo a cyclical developmental process, termed swarmer cell differentiation, is considered to be one of the virulence factors of *P. mirabilis*. The swarming phenomenon, previously believed to be unique to the *Proteae,* has now been described for many bacterial species (Harshey and Matsuyama, 1994; Rózalski *et al.*, 1997). Among these microorganisms, *P. mirabilis* deserves particular attention. The *Proteus* bacilli have integrated swarmer cell differentiation and coordinate regulation of virulence factors into a multi-layered developmental program that is fine-tuned to facilitate colonization of the urinary tract (see the following sections on swarming and virulence). The program controls many morphogenetic activities, including flagellum and cell-wall biosynthesis, DNA replication, synthesis of polysaccharide and virulence determinants, and the down-regulation of genes associated with cell division and a large number of other genes, such as the *ter* locus (tellurite resistance) described in this study. The molecular analysis of the *ter* gene cluster described in this study is the first example of a repressed (down-regulated) locus in swarmer cells.

The *ter* gene cluster includes six genes, which have sequences highly similar to the characterized IncH plasmid-borne *ter* gene clusters of Gram-

negative bacteria. Typically, plasmid-borne *ter* loci contain seven genes (*terZ*, *terABCDEF*), which encode three phenotypes: phage-inhibition (Phi), pore- (*terZ*, *terABCDEF*) which encode three phenotypes: phage inhibition (Phi), pore-forming colicin resistance (PacB) and tellurite resistance (Te^r) (Whelan *et al.*, 1995). However, the underlying biochemical mechanisms remain elusive despite much research (Whelan *et al.*, 1995; 1997).

This study describes the use of transposon mutagenesis to generate promoter-reporter gene fusions, one of which disrupted the *terC* coding region in the chromosome of *P. mirabilis*. Insertional inactivation of *terC* resulted in reduced levels of potassium tellurite resistance in the mutant strain. Northern blot and primer extension analysis provided the evidence that the expression of the *ter* gene cluster is activated by potassium tellurite. This work further demonstrates that resistance to tellurite is both chromosomally encoded and ubiquitous among *Proteus* spp. Based on these observations, it is suggested that *P. mirabilis* may be the evolutionary origin of the plasmid-borne *ter* loci among enteric bacteria.

LITERATURE REVIEW

In 1885 Gustav Hauser made the first description of putrefying dimorphic rods that he aptly named *Proteus* (Hauser, 1885). The bacterium was named after the marine deity character in Homer's *Odyssey* who "has the power of assuming different shapes to escape being questioned" (Williams and Schwarzhoff, 1978). A new genus, *Proteus,* was established and included two species, *P. vulgaris* and *P. mirabilis.* Hauser was also the first to suggest a potential role for these bacteria in pathogenesis. Wenner and Rettger proved Hauser's suggestion to be correct, and established the *Proteeae* as opportunistic pathogens of higher animals (Wenner and Rettger, 1919). *Proteus* spp. belong to the family *Enterobacteriaceae*, and are very similar in many aspects of physiology and biochemistry to other members of this family, such as *Escherichia coli* (Brenner *et al.*, 1978; Penner, 1989; 1992). However, the *Proteeae* bacilli are unique among *Enterobacteriaceae* in possessing phenylalanine deaminase (Sultan, 1969). Additionally, *Proteus* rods overall are significantly less virulent than pathogenic *E. coli* strains (Rozalski *et al.*, 1997). The *Proteus* spp. can be easily distinguished from other members of the family by their swarming behaviour (Belas, 1992; Mobley and Belas, 1995; Williams and Schwarzhoff, 1978). According to *Bergey's Manual* (Penner, 1989), *Proteus* comprises three species: *Proteus vulgaris*, *P. mirabilis*, and *P. myxofaciens.* Only the first two species are implicated in pathogenesis in higher animals.

General characteristics of *Proteus* spp. and the role in pathogenesis

Proteus mirabilis rods are motile, lactose-negative, indole-negative bacteria (Ewing, 1986). They are proteolytic under aerobic and anaerobic conditions, capable of oxidative deamination of amino acids (phenylalanine), hydrolyze urea to ammonia and carbon dioxide, and produce abundant hydrogen sulfide (Mobley, 1996).

Proteus spp. are ubiquitous in natural environments. They can be easily isolated from polluted water, soil and manure, where they play an active role in decomposition of organic matter. In addition, these bacteria, especially *Proteus mirabilis*, colonize the intestinal tracts of humans and other animals. The significance of *P. mirabilis* as an opportunistic pathogen can be explained by its high carriage rate (25%) in human intestines (Chow *et al.*, 1979). Under favorable conditions, *Proteus* rods can colonize and cause nosocomial infections in geriatric, psychiatric, and paraplegic patients, as well as in individuals with structural abnormalities of the urinary tract, and in immuno-compromised and catheterized patients in intensive-care units (Chow *et al.*, 1979; Scott, 1960; Sogaard *et al.*, 1974).

Proteus cells progressively colonize the introitus, urethra, bladder, ureter, and in the end, the kidneys (Rubin *et al.*, 1986). *P. mirabilis* was localized to the kidney at a frequency far greater than that for the most common urinary tract pathogen, *E. coli,* in humans and in mouse model studies. These studies also demonstrated high concentrations of *P. mirabilis* in kidney versus bladder (Fairley *et al.*, 1971; Kohnle *et al.*, 1975). Warren demonstrated that *P. mirabilis*,

after *E. coli* and *Klebsiella pneumonia*, is the third most common cause of complicated urinary tract infections, and the second most common (after *Providencia stuartii)* cause of catheter-associated bacteriuria in long-term catheterized patients (Warren, 1996). Interestingly, *Proteus* spp. preferentially cause urinary tract infections in young boys and older men, while *E. coli* infections are more common in girls and women (Larsson *et al.*, 1978). These data establish the role of *P. mirabilis* as an opportunistic urinary tract pathogen that plays a particularly prominent role in ascending urinary tract infections.

In addition to the marked predilection for the urinary tract, *Proteus* bacilli can infect many other organs and tissues in the human host. When an opportunity presents itself, *P. mirabilis* and, to lesser extent, *P. vulgaris* can cause infections of the respiratory tract, skin, eyes, nose, ears and throat, as well as establish secondary infections of skin burns. In addition, consumption of meat or other food contaminated with these bacteria can cause gastroenteritis (Cooper *et al.*, 1971; Penner, 1992). A possible role for *P. mirabilis* in rheumatoid arthritis was suggested, based on the detection of specific anti-*Proteus* antibodies in patients with active rheumatoid arthritis (Deighton *et al.*, 1992; Ebringer *et al.*, 1989; Wilson *et al.*, 1990).

Swarmer cell differentiation

Proteus bacilli, when grown on solid media, exhibit a form of developmental behavior called swarming. Henrichsen defined swarming as an organized surface translocation that is dependent on extensive flagellation and

cell-to-cell contact (Henrichsen, 1972). Swarming was first described for the genus *Proteus* over a century ago, but the phenomenon has been reported in a growing numbers of Gram-positive and Gram-negative bacteria, including *Vibrio* spp., *E. coli*, *Salmonella typhimurium*, *Serratia* spp., *Yersinia* spp., *Aeromonas* spp., *Rhodospirillum centenum*, *Bacillus* spp. and *Clostridium* spp. (Alberti and Harshey, 1990; Harshey and Matsuyama, 1994; Harshey, 1994 and references therein; Henrichsen, 1972; McCarter and Silverman, 1989). The ubiquitous occurrence of swarming among eubacteria suggests that this type of surface translocation may play an important role during colonization of natural environments (Harshey, 1994).

When inoculated on solid rich medium (e.g., Luria-Bertani agar), *Proteus mirabilis* cells initially grow as short vegetative rods, "swimmer cells" with 6 to 10 flagella per cell. After a period of growth, the swimmer cells undergo elongation as the result of inhibition of cell septation, but with continuation of DNA replication. The resultant filamentation is concurrent with an overproduction of additional flagella, and results in a biochemically and physiologically new type of cell termed the swarmer cell. A swarmer cell no longer possesses rod-like surface organelles termed fimbriae that mediate adherence of bacterial cells to uroepithelial cells (Silverblatt, 1974), but can bear between 500 and 5000 flagella (Hoeniger, 1965). The swarmer cells align themselves along side of each other in "rafts" that rapidly move outward from the site of the initial inoculation, forming a swarm band around the initial "mother" colony. The movement of an individual swarmer cell is retarded, while cells moving in larger groups do so much more

effectively (Morrison and Scott, 1966). Eventually this movement stops, cell septation and division take place, and swarmer cells revert to swimmer cells, a process termed consolidation. The cycle is repeated until the entire agar surface is covered with concentric rings of swarm bands and consolidation zones, giving the plate a characteristic appearance of a bull's eye. The occurrence of swarmer cells has been demonstrated *in vivo*, leading to the conclusion that the differentiated cells are the virulent forms of *P. mirabilis* (Allison *et al.*, 1994).

It has been postulated that a new genetic program is turned on to produce swarmer cells (Allison *et al.*, 1992a; Harshey, 1994). Swarmer cell differentiation culminates in the appearance of elongated hyperflagellated cells which have the same DNA/length ratio as the short swimmer cells due to DNA replication in the absence of septation (Hoeniger, 1964; Jones and Park, 1967). This type of cell is uniquely adapted to life on surfaces and in viscous environments, such as the urinary epithelial mucosa (Belas, 1996). Some striking physiological and biochemical changes accompany swarmer cell morphogenesis. The polyploid, hyper-flagellated and elongated nature of the swarmer cells, together with the known involvement of the cell envelope and flagella in cell division, led Armitage and co-workers to hypothesize that marked differences might exist between the cell envelopes of swarmer cells and swimmer cells (Armitage *et al.*, 1975, 1979; Armitage and Smith, 1978). A variety of direct and indirect evidence confirmed this suspicion. Namely, the membrane of the swarmer cell has increased permeability, demonstrated by leakage of intracellular amino acids and pentose sugars (Armitage *et al.*, 1975). The results of freeze-fracture electron

microscopy and spin-label electron paramagnetic resonance studies demonstrated that the outer membrane (OM) of swarmers is more fluid than the OM of swimmers, and regions of lipid bilayer appear in the OM during swarmer cell differentiation (Armitage, 1982). Changes in the outer membrane protein and in the cytochromes of the inner membrane (Falkinham and Hoffman, 1984), as well as changes in the actual composition of the lipopolysaccharide (LPS) layer (Armitage *et al.*, 1979), provided direct evidence for the hypothesis of Armitage and co-workers. Swarmer cells contain predominantly LPS with long O-antigenic side chains, while swimmer cells contain LPS with both short and long O-chains (Sidorczyc and Zych, 1986). Falkinham and Hoffman (1984) demonstrated the inducible nature of phenylalanine deaminase and urease in swarmer cells, thus indicating that swarmers possess functional transcription and translation machinery. However, neither transcription nor translation are required for the persistence of migration, for once initiated, swarming continues even in the presence of the transcriptional and translational inhibitors rifampin and chloramphenicol (Falkinham and Hoffman, 1984). Furthermore, no exogenous energy source is required for migration, because swarmer cells will continue to swarm when transferred to non-nutrient agar (Williams *et al.*, 1976). These observations led to the hypothesis that swarming motility utilizes stored energy, and that the periodicity of swarming could be related to the depletion and regeneration of energy stores (VanderMolen and Williams, 1977; Williams and Schwarzhoff, 1978).

Transcript analysis by Allison and co-workers (1992a), using 27 random non-overlapping DNA probes and RNA isolated from swarmers and swimmers, demonstrated that the majority of *P. mirabilis* genes are specifically repressed during swarmer cell differentiation. At the same time, a small number of genes show increased expression in swarmer cells. Since the synthesis of virulence factors is concomitant with swarmer cell differentiation, it is likely that these induced genes are either associated with flagellum biosynthesis or encode virulence factors (Allison *et al.*, 1992a). The synthesis and operation of flagella are very energetically expensive (Macnab, 1992). Therefore, the vast hyperflagellation of swarmers may require a concurrent repression of non-essential loci. Indeed, a number of enzymes (e.g., tryptophanase [Hoffman and Falkinham, 1981]) display reduced activity in swarmer cells. The pigmented swarming bacteria *Serratia* and *Chromobacterium* provide the best examples of such frugal behavior of swarm cells: vegetative cells contain a bright orange pigment, which is absent in the colorless swarmer cells (Allison and Hughes, 1991). Based on the measurements of "global" levels of transcription by Allison *et al.* (1992a), it is reasonable to propose that the *P. mirabilis* genome contains a global regulon of genes associated with swarmer cell differentiation and virulence. An alternative explanation is based on transcript stability, and does not invoke the existence of a global regulon. Thus, the selective activity of virulence-associated and flagellar biosynthesis genes during swarming may be due to greater stability of the corresponding mRNAs; silencing of the remaining genes could be achieved by the degradation of their mRNAs. The generation of

mutants defective in swarmer cell development, using transposon mutagenesis, showed that at least 45 loci are involved in swarmer cell differentiation by *P. mirabilis* (Belas, 1992; Belas *et al.*, 1991, 1991a). The expression of these loci is likely to be up-regulated during the developmental program. The identity and function of only a few of these genes are currently established (see below).

The factors responsible for inducing morphogenesis and the mechanisms involved still remain largely a mystery. However, the exciting and innovative research published in the last two decades has provided a glimpse of the fascinating developmental program that elicits this behavior. Several investigators reported the inhibition of swarming by electrolyte-deficient media, but the mechanism of swarming stimulation by sodium chloride (and other compounds) remains unknown (Williams and Schwarzhoff, 1978). Examination of the periodic morphogenesis observed during growth of a *Proteus* colony on solid medium led Rauprich and co-workers (1996) to conclude that swarming is initiated when the population in the last swarm band reaches a certain threshold density. However, the experimental data showed no connection between nutrient (glucose) depletion and the onset of different phases in swarmer cell morphogenesis: swarming was initiated well before depletion of nutrients occurred, even at low glucose levels (Rauprich *et al.*, 1996). Gaisser and Hughes (1997) characterized a locus encoding a putative non-ribosomal peptide-polyketide fatty acid synthase (*nrp*) as the site of one transposon insertion causing impaired swarmer cell differentiation. The locus encodes two tandem

ABC transporters, indicating that the end product(s) of this locus are likely to be secreted. Neither the end product, nor the role of the *nrp* operon in the developmental program of *P. mirabilis,* are currently known. However, the products of bacterial polyketide and peptide synthase enzymes have diverse functions as antibiotics, toxins and siderophores (Stachelhaus and Marahiel, 1995), and some are involved in bacterial developmental pathways (Katz and Donadio, 1993). Therefore, further investigations of the *nrp* locus function may contribute to our understanding of *P. mirabilis* swarming.

Allison *et al.* (1993) identified a single amino acid, glutamine, as sufficient to signal the initiation of swarmer cell differentiation by supplementing minimal medium unable to support swarming. The proposed dual role for glutamine, both initiating differentiation and directing the migration (Allison *et al.*, 1993), has not been widely embraced by other research groups working on *P. mirabilis*. While the data published by Allison and co-workers seem convincing, these findings are contradicted by earlier careful nutritional studies (Hoffman, 1974) and could not be reproduced by other laboratories (Stickler, personal communication). Therefore, other yet-unknown factors in addition to glutamine are likely to be involved in the initiation of swarmer cell differentiation by *P. mirabilis*.

Recently, Lai *et al.* (1998) characterized a swarming-defective *P. mirabilis* *ppa* (*Proteus* P-type ATPase) mutant generated by transposon mutagenesis. The *ppa* gene product is closely related to the ATPases transporting cations across bacterial membranes (Silver and Walderhaug, 1992). This observation

suggested that ion homeostasis has an effect on swarmer cell differentiation (Lai *et al.*, 1998).

Gene expression analysis in *P. mirabilis* and other swarming bacteria demonstrated the universal nature of sensing via monitoring of flagellar rotation to produce the differentiated swarmer cell (Alberti and Harshey, 1990; Allison *et al.*, 1993; Belas *et al.*, 1995; McCarter *et al.*, 1988). In these studies, mutations that affected flagellar filament function resulted in abnormal swarming phenotypes. Additionally, inhibition of flagellar rotation by high viscosity, and tethering of flagella by specific antisera, triggered differentiation (Belas *et al.*, 1995; Belas, 1996, 1994). These results indicated that flagella function as tactile sensors of external conditions that relay signals to the transcriptional network inside the cell (Belas, 1996a).

Hay *et al.* demonstrated an involvement of Lrp (leucine-responsive regulatory protein) in swarmer cell development (Hay *et al.*, 1997). The *lrp* mutant was severely impaired in swarming motility, differentiation and production one of the virulence factors (hemolysin), while production of other virulence factors (urease and protease) was not significantly affected. This indicated that the Lrp-mediated induction of development proceeds along at least two branches: one leading to overproduction of flagella and hemolysin, and a second activating urease and protease synthesis. Lrp is likely to activate hyperflagellation through the *flhDC* master regulon (see below). It is not known whether hemolysin induction is activated directly by Lrp. The exact role for Lrp is not defined, though Lrp is pivotal to the expression of at least 40 genes of diverse

function in *E. coli* (Newman and Lin, 1995). This abundant small basic protein bends DNA and may function as a chromosome organizer, similar to the role played by histones in a eukaryotic cells. It is possible, that Lrp acts in concert with other global regulators (such as H-NS, IHF, cyclic AMP-receptor protein) to alter DNA topology and allow multiple layers of gene regulation in response to environmental cues (cell density, osmolarity, temperature, nutrient depletion) that affect global levels of transcription (Hay *et al.*, 1997; Shin and Park, 1995; Silverman and Simon, 1974). Swarmer cells contain multiple condensed nucleoids that can be visualized by fluorescent staining to be evenly spaced along the entire length of the bacterial filament (Belas, 1992). It is reasonable to suggest that in swarmer cells certain coding regions (such as flagellum biogenesis genes) could be preferentially overexpressed by being located on the periphery of nucleoids where they would be easily accessible to the transcriptional machinery. Furthermore, the copy number of such genes would be greatly amplified due to the presence of multiple identically organized nucleoids in the swarmer cell. Under this form of gene expression regulation, protein chromosome organizers like Lrp would be critical.

Relationship of swarming to myxobacterial fruiting body formation

Swarmer cell differentiation is only one of many types of developmental behaviour exhibited by prokaryotes. Myxobacteria display one of the best studied and the most striking of types of multicellular behavior, where individual bacterial cells move, prey and sporulate as a single multicellular organism (Kim

et al., 1992). In response to starvation, thousands of myxobacteria aggregate to construct multicellular fruiting bodies. During this developmental process, many of the participating cells lyse to facilitate the sporulation and survival of the remaining bacterial cells. The resulting myxospores are dormant and capable of surviving adverse environmental conditions for long periods. Myxobacteria do not produce flagella, but rather translocate by gliding, which requires solid surfaces, cell-cell signaling and contact, and is significantly slower than swarming motility.

At first glance, swarming by *Proteus mirabilis* and fruiting body formation by *Myxococcus xanthus* appear to be very different. Unlike fruiting body formation, swarmer cell development is generally not initiated in response to nutrient depletion, but rather occurs on rich media. Upon closer examination, however, these two types of developmental behaviour share a number of similarities. Both developmental processes are only apparent on solid media. Swarming bacteria and myxobacteria produce copious extracellular "slime", which facilitates surface translocation, probably by reducing surface tension. For myxobacteria, extracellular slime plays an important role in cell-cell communication (Shimkets, 1990). Cell-cell contact and/or signaling have been suggested to play a role in the swarming motility of *P. mirabilis*, based on the reported inability of an individual swarmer cell to migrate (Allison and Hughes, 1991). A more likely explanation suggests that the number of swarmer cells in an individual raft is a function of surface tension. Thus, Williams *et al.* (1976) observed motility of individual swarmer cells when surface tension was

sufficiently decreased by the addition of surfactants. The motility of individual swarmer cells does not eliminate the possibility of cell-cell contact and/or communication at stages preceding the outward migration, but currently no supporting data are available for this hypothesis.

M. xanthus has two motility systems that become advantageous when these bacteria grow on different surfaces (Shi and Zusman, 1993). The two systems differ both genetically and morphologically, with system A having at least 21 genetic loci and moving mainly single cells, and system S having at least 10 genetic loci and moving groups (rafts) of cells (Hodgkin and Kaiser, 1979). Although *P. mirabilis* produces only one type of flagellum in both swarmer and swimmer cells (Hoeniger, 1965), this organism uses two different motility systems (swimming and swarming) to move in different environments, similar to the pattern seen for myxobacteria. Lastly, the *frz* genes (related to chemotaxis genes) play an important role in myxobacterial gliding (Ward and Zusman, 1997). Likewise, several laboratories have described a possible role for chemotaxis genes in the swarming motility of *P. mirabilis* (Allison *et al.*, 1993; Belas *et al.*, 1991). However, these reports are in conflict with earlier studies that effectively disproved an involvement of chemotaxis in *P. mirabilis* swarmer cell differentiation (Hoffman, 1974; Williams *et al.*, 1976). Interestingly, a recent publication on the role of chemotaxis machinery in the swarming of *E. coli* cells may provide a possible explanation that would reconcile these contradictory reports (Burkart *et al.*, 1998). The authors demonstrated that neither saturation of chemoreceptors (methyl-accepting proteins) with corresponding

chemoeffectors, nor mutations abolishing the ligand binding, prevented swarming by *E. coli*. However, communication of the chemoreceptors with the CheA kinase was essential, since mutations in the signaling domain of the Tsr chemoreceptor that inhibits CheA kinase abolished swarming. Based on the above observations, it was suggested that an intact chemotaxis machinery, rather than chemotaxis itself, is required for swarming by *E. coli*. It remains to be determined whether the *P. mirabilis* chemotaxis machinery is sufficiently similar to that of *E. coli* before any conclusion may be drawn for *P. mirabilis* swarming.

Virulence determinants of *P. mirabilis*

Flagella and swarming motility

A growing number of genes has been implicated in the swarming motility of *P. mirabilis*. Biochemical and transposon mutagenesis studies demonstrated that *P. mirabilis* synthesizes only a single flagellin, FlaA (filament subunit) (Bahrani *et al.*, 1991, Belas *et al.*, 1991). Mutations in other flagellum biogenesis genes result in defective swarmer cell differentiation. These genes, which also play a role in swarmer cell elongation, include the *fliL* gene, the homologue of which is required for flagellum gene expression and normal cell division in *Caulobacter crescentus* (Stephens and Shapiro, 1993). Other genes of this type are *fliG*, *flgH*, *flhA*, *fliD* and *flgN*. The *fliG* gene encodes a component of the flagellar switch; *flgH* encodes the basal-body L ring (Belas *et al.*, 1995). The *flhA* gene product is required for flagellum biogenesis (Macnab, 1992) and is a member of a putative signal-transducing receptor family involved in diverse

cellular processes (Gygi *et al.*, 1995; Carpenter and Ordal, 1993). The *fliD* gene encodes flagellum filament capping protein (Belas and Flaherty, 1994), while *flgN* encodes a facilitator (putative chaperone) of flagellum assembly (Gygi *et al.*, 1997). Generally, mutations in flagellum biogenesis genes that interfere with the normal function and expression of flagella result in abnormal swarmer cell development, thus indicating the pivotal role of flagellar filament in swarmer cell differentiation and multicellular migration (Belas, 1996).

Flagellum biogenesis is an integrated part of swarmer cell differentiation by *P. mirabilis*. Therefore, understanding of the regulation of flagellum biogenesis is required for elucidation of the regulation of the developmental program. *P. mirabilis* flagellum biogenesis genes are organized in a three-tier hierarchy, as is also true for these genes in E. *coli* and *S. typhimurium* (Furness *et al.*, 1997; Macnab, 1992). Class I genes are expressed from σ^{70}-specific promoters, while σ^{28} activates transcription of the class II and class III genes (Furness *et al.*, 1997, Belas and Flaherty, 1994; Hughes *et al.*, 1993). Flagellum biogenesis is coupled to cell division by the action of the class I *flhDC* master operon, which encodes a transcriptional regulator (Furness *et al.*, 1997). In *E. coli*, FlhD and FlhC activate transcription of class II flagellum genes, while FlhD represses cell division (Liu and Matsumura, 1994; Pruß and Matsumura, 1996). Currently, two negative feedback loops have been characterized within the hierarchy of the *P. mirabilis* flagellar genes. Flagellum assembly is coupled to flagellum gene expression by the expression of the anti-σ^{28} gene *flgM* (Brown and Hughes, 1995). In cells defective for flagellum assembly, FlgM post-

translationally inhibits σ^{28}, resulting in repression of class II and class III genes. Additionally, a second feedback loop inhibits cell elongation in class II mutants defective in flagellum assembly (Furness *et al.*, 1997).

Flagella antigenic variation

P. mirabilis contains at least three different copies of the flagellin gene (*flaA*, *flaB* and *flaC*), and is capable of synthesizing immunologically different flagella by rearranging DNA within the *flaA* locus (Belas, 1994). The *flaB* and *flaC* genes are usually silent, but may become active during the genetic rearrangement process. Because flagellin, also called H-antigen, is highly antigenic, changes in flagellar antigenicity through genetic recombination may provide the bacteria with a powerful mechanism for avoiding the host immune response. *P. mirabilis* flagellar antigenic variation has been demonstrated *in vitro* for spontaneous motile revertants of the *flaA*$^-$ mutants (Belas, 1994). This observation prompted Belas (1996a) to suggest that flagellar antigenic variation may occur *in vivo*, since it would provide an opportunity to swarmer cells to avoid immobilization by immunoglobulins. Tethering of the bacteria via their flagella would prevent their motility and impose a selective pressure for new antigenic variants (Belas, 1996a).

Urease

Urea is the main nitrogenous excretion product of mammals. Urease (urea amidohydrolase) catalyses the hydrolysis of urea to ammonia and carbon dioxide, resulting in elevation of the urine pH (Mobley *et al.*, 1995; Mobley and Hausinger, 1989). Urease activity has been detected in over 200 bacterial

species (Mobley *et al.*, 1995). Bacterial ureases are unique in their requirement for nickel as a cofactor and in the presence of a large number of cystine residues (Rando *et al.*, 1990; Sriwanthana *et al.*, 1993). Posttranslational insertion of nickel into assembled apourease is necessary for enzyme activity (Rando *et al.*, 1990). In *P. mirabilis,* this enzyme functions in the cytoplasm (Jones and Mobley, 1988).

P. mirabilis swarmer cell differentiation is co-ordinated with increased expression of a number of virulence factors, including urease (Allison, *et al.*, 1992a; Falkinham and Hoffman, 1984). The contribution of urease to *P. mirabilis* virulence was assessed in a mouse model with isogenic *ureC* mutant strains (Jones *et al.*, 1990). The studies defined urease as a critical virulence factor for colonization, urolithiasis (urinary stone formation) and development of acute pyelonephritis (kidney inflammation) (Johnson *et al.*, 1993). Urease plays a major role in kidney and bladder stone formation, which may protect uropathogens from antibiotics, antibodies, and urease inhibitors (Clapham *et al.*, 1990; McLean *et al.*, 1988; Mobley and Hausinger, 1989). Stone formation is a hallmark of infection with *P. mirabilis*. The rise in local pH due to urease activity causes precipitation of magnesium and ammonium phosphate (struvite) and calcium phosphate (apatite) crystals (Mobley and Hausinger, 1989). In addition, the *P. mirabilis* capsule was implicated in this process (Dumanski *et al.*, 1994). The growing stone protects the *P. mirabilis* cells within from the action of antibiotics and, eventually, can obstruct urine flow (Mobley, 1996).

The 6.45-kb *P. mirabilis* urease operon is structurally homologous to the urease operons of *Providencia stuartii* and *Klebsiella aerogenes* (Jones and Mobley, 1988; Mobley, 1996). The operon includes eight open reading frames: *ureR* and *ureA-G* (Mobley and Hausinger, 1989). The *ureABC* genes are flanked upstream by *ureD* and downstream by *ureEFG* (Jones and Mobley, 1989). The *ureA*, *-B* and *-C* genes encode three structural enzyme subunits α, β and γ, respectively. By analogy to *K. aerogenes* urease, the *P. mirabilis* enzyme is believed to be a trimer of trimers with three active sites (Jabri *et al.*, 1995, Mobley, 1996). UreD, UreE and UreF polypeptides are accessory factors responsible for the insertion of nickel ions into the apoenzyme (Jones and Mobley, 1989). Two nickel ions are bound into each of the active sites of the enzyme (Jarbi *et al.*, 1995). Mobley and Belas (1995) suggested that during assembly of the active enzyme UreD functions as a urease-specific chaperon. The role of UreG is not clearly defined, but this protein is required for the full enzymatic activity of urease (Sriwanthana *et al.*, 1993).

Urease activity is induced by urea and is under control of UreR. The regulatory gene *ureR* is located 400 bp upstream of *ureD* and is divergently transcribed. The UreR polypeptide contains a helix-turn-helix DNA-binding motif and signature sequences of the AraC family of transcriptional regulators (Nicholson *et al.*, 1993). Therefore, UreR is likely to regulate expression of the urease operon in the manner similar to that described for the AraC transcriptional regulator family. Urease activity increases during swarmer cell morphogenesis and diminishes during the consolidation phase when elongated cells revert to

short vegetative rods (Allison *et al.*, 1992a; Falkinham and Hoffman, 1984). Urease activity may be controlled by a developmental signal, since swarmer cells produce substantial levels of the enzyme on laboratory media containing no urea (Falkinham and Hoffman, 1984). It is postulated that binding of urea to UreR triggers urease expression (D'Orazio *et al.*, 1996; D'Orazio and Collins, 1995; Nicholson *et al.*, 1993); therefore, in the absence of urea, a developmental factor might augment the activity of UreR. Experimental data from our laboratory show that growth conditions, growth phase, and possibly posttranscriptional activities can modulate urease activity (Toptchieva *et al.*, 1998).

Hemolysin

Almost all *P. mirabilis* strains have the ability to lyse erythrocytes from a large number of animal species (Senior and Hughes, 1987). The lytic activity is associated with 166-kDa protein named HpmA (Swihart and Welch, 1990). The genetic locus encoding this hemolysin consists of two genes, *hpmB* and *hpmA*, in that transcriptional order, and contains a ferric uptake regulator (Fur) binding site in the promoter region overlapping the –35 region of the proposed *hpm* promoter. Studies of HpmA production in the absence of Fe^{2+} or in a *fur* background were inconclusive and, at this point, the extent of Fur regulation of the *hpm* genes remains unknown (Uphoff and Welch, 1990). Both *hpm* genes show approximately 50% nucleotide sequence similarity to the hemolysin determinants of *S. marcescens* (Uphoff and Welch, 1990). It is believed that *Proteus* spp. use the HpmA hemolysin to damage uroepithelium and gain entry into the kidney (Mobley, 1996). However, the role of hemolysin in virulence is not clear, and its

relative contribution to colonization and histopathology in mouse model studies is surprisingly small despite potent *in vitro* cytotoxicity (Mobley, 1996).

Metalloprotease

Most *P. mirabilis* strains secrete a protease that cleaves immunoglobulin A1 (IgA1) and IgA2 (Loomes *et al.*, 1990). The structural gene for this metalloprotease, *zapA*, encodes a 488-amino acid protein which is secreted by an ATP-dependent transport system. The ZapA protein sequence is homologous to that of a *S. marcescens* zinc-metalloprotease (Wassif *et al.*, 1995). While experimental evidence demonstrated that the protease is produced in a human host, the contribution of this enzyme to virulence remains to be elucidated (Senior *et al.*, 1991).

Invasiveness

Peerbooms *et al.* (1984, 1985) demonstrated that all *P. mirabilis* and *P. vulgaris* strains, including isolates from patients with urinary tract infections (UTI) and from healthy individuals, were able to invade cultured Vero (African green monkey kidney) cells. The data obtained by Allison *et al.* (1992a) indicated that *P. mirabilis* swarmer cells were the invasive forms of this bacterium, suggesting a major role for this morphological form in colonization of the host during UTI. Thus far, however, other researchers have not confirmed these results. Moreover, Chippendale *et al.* (1994) and Rózalski *et al.* (1997) found that upon addition to monolayers of human renal epithelial cells and mouse fibroblasts, respectively, swarmer cells rapidly de-differentiated into short swimmer cells and then were internalized in this form without subsequent replication. Therefore,

whether swimmer or swarmer cells are able to invade mammalian cells remains an unresolved issue.

Iron acquisition

Virtually all bacteria, pathogenic or otherwise, have developed mechanisms for scavenging iron, which may include secretion of high-affinity iron chelators called siderophores. Unlike many other genera of the *Enterobacteriaceae*, none of the *Proteus-Morganella-Providencia* (PMP) group produce common types of siderophores (Rózalski *et al.*, 1997). Drechsel *et al.* (1993) suggested that bacteria of the PMP group instead produce α-keto acids as novel siderophores by the action of amino acid deaminases. α-Keto acids with aromatic or heteroaromatic side chains (phenylpyruvic acid from phenylalanine, or indolylpyruvic acid from tryptophan) have the most significant siderophore activity in growth promotion and/or iron transport assays (Drechsel *et al.*, 1993). However, in uropathogenic *P. mirabilis*, the structural gene for amino acid deaminase is not regulated by iron availability (Massad *et al.*, 1995). The possibility still remains that amino acid deaminase expression is regulated by iron availability *in vivo*, despite the fact that this regulation was not observed under the laboratory conditions. Alternatively, *P. mirabilis* may utilize different mechanisms for iron acquisition.

Currently, iron-dependent regulation has been demonstrated only for a single locus associated with swarmer cell differentiation: the *nrp* operon encoding non-ribosomal peptide/polyketide synthase functions (Gaisser and Hughes, 1997). Immediately distal to the *nrp* operon is the *irpP* gene, encoding a

homologue of the *Bacillus subtilis* ComX pheromone precursor associated with *Bacillus* development (Cosmina *et al.*, 1993; Magnuson *et al.*, 1994). Transcription of both the *nrp* operon and the *irpP* gene is up-regulated under iron-limiting conditions, consistent with a possible Fur-binding site 5' of the *irpP* gene (Gaisser and Hughes, 1997); the 5' end of the *nrp* operon has not been characterized. Disruption of the *nrp* locus by transposon mutagenesis resulted in abnormal swarmer cell development, but the role of the *nrp* locus and the *irpP* gene in *Proteus* physiology remains to be elucidated (see above).

Genome organization of *Proteus* spp.: extrachromosomal DNA and phages

Plasmids

Beginning in 1970, a large number of often-contradictory reports were published describing detection and characterization of large conjugative or cryptic plasmids in *Proteus* strains. The majority of the detected plasmids were reported to be associated with drug and/or heavy-metal resistance. Similar to what has been found for other bacterial pathogens, the emergence of drug resistance in *Proteus* is most likely associated with the widespread use of antimicrobial agents. Heavy-metal resistance determinants could potentially provide bacteria with a selective advantage in their natural environment (outside an animal host).

In *Enterobacteriaceae,* plasmids representing a wide range of incompatibility (Inc) groups often contain genes required for the assembly of conjugative pili which serve as an attachment sites for filamentous and tailed

phages (Bradley, 1980). Co-evolution of plasmids and phages has been suggested as an explanation for the specificity that restricts phage adsorption to pili encoded by only one or a few closely related Inc group(s) (Dennison and Hedges, 1972; Bradley *et al.*, 1982).

Incompatibility group J plasmids, encoding resistance to kanamycin and mercuric ions, were detected in South African *Proteus* strains (Coetzee *et al.*, 1972; Hedges, 1975). Similarly, a plasmid carrying ampicillin-, streptomycin- and sulphonamide-resistance determinants was described for an Indian strain of *P. mirabilis* (Matthew *et al.*, 1979). However, numerous attempts to isolate plasmid DNA from these strains have failed, leading to the suggestion that the incJ plasmids may exist in a chromosomally integrated form (Nugent, 1981; Hedges, 1975). In the last decade, several groups reported detection of plasmid-borne resistance to β-lactam (pAP1), carbenicillin (pCS229), gentamycin and β-lactam combined (self-transferrable 54-kb plasmid) in clinical isolates of *P. mirabilis* (Araque *et al.*, 1997; Ito and Hirano, 1997; Mariotte *et al.*, 1994; Watanabe *et al.*, 1991). In 1988, Konkoly Thege and Nikolnikov published another report of multiresistant *P. mirabilis*. All 60 strains described in their study were resistant to 11-16 antibiotics and harbored 2-4 plasmids, whereas sensitive strains were devoid of plasmids (Konkoly Thege and Nikolnikov, 1988).

Genetic determinants of plasmid-borne heavy-metal resistance in *Proteus* spp. remain largely uncharacterized. The heavy metals to which plasmid-borne resistance has been reported are mercury, lead, zinc, cobalt, copper and chromium (Olukoya *et al.*, 1997; Trevors, 1987; Dobritsa *et al.*, 1985). Similarly,

genetic determinants of the intrinsic tellurite resistance of *Proteus* bacilli (Fleming, 1932) have not been characterized.

Bacteriophages

A number of *P. mirabilis* phages have been described over the last three decades. Generalized transducing phages 5006M, 13M and 34 are responsible for lysogenic conversion, which results in immunity to super-infection (Pretorius and Coetzee, 1979; Krizsanovich, 1973). The host strains were cryptically lysogenic, and cryptic non-inducible prophage were occasionally liberated (Krizsanovich, 1973). Bradley and colleagues isolated two phages, C-2 and J, from sewage (Bradley *et al.*, 1982). Both of these phages were able to propagate, but failed to form plaques on *P. mirabilis* strains carrying IncC and IncJ plasmids, respectively. Therefore, plasmid-borne determinants may provide protection from phage predation to *Proteus* bacilli.

Tellurite resistance, phage inhibition and colicin resistance

In 1932 and 1940 Alexander Fleming reported that some *E. coli* and *Proteus* isolates were tellurite resistant (Fleming, 1932; Fleming and Young, 1940). Until now the genetic basis of the tellurite resistance of *P. mirabilis* has not been investigated. My work describes the characterization of the *P. mirabilis* chromosomal gene cluster that mediates tellurite resistance, as well as colicin resistance and phage inhibition. The following sections describe our current limited knowledge of the molecular mechanisms underlying each of the three

phenotypes associated with this locus, and the potential selective advantage provided by these phenotypes to the host bacterium.

Tellurite resistance

Tellurium compounds are used in the manufacture of batteries, in the production of rubber, and in other industrial processes (Browning, 1969). The oxyanions of tellurium, tellurite and tellurate, are highly toxic for most microorganisms, especially Gram-negative bacteria (Summers and Jacoby, 1977; Turner *et al.*, 1995), even though some Gram-positive bacteria, such as *Corynebacterium diphtheriae*, *Enterococcus faecalis* and most *Staphylococcus aureus*, are naturally resistant (Summers and Jacoby 1977; Hoeprich *et al.*, 1960; Skadhauge, 1950). Ninety five percent of bacteria isolated from film-processing sludge were multiply resistant to tellurite, mercury, and silver; 10% and 22% of bacteria isolated from Boston city sewage and hospital waste, respectively, were tellurium resistant (Taylor and Summers, 1979). Resistant Gram-negative bacteria produce jet-black colonies on solid medium supplemented with potassium tellurite, as the result of internal deposition of elemental tellurium in the periplasm (Bradley *et al.*, 1988; Taylor *et al.*, 1988). Several research groups have speculated that reduction to non-toxic metallic tellurium is the resistance mechanism (Taylor *et al.*, 1988, Bradley *et al.*, 1988, Lloyd-Jones *et al.*, 1991). However, this hypothesis is likely to be incorrect, since tellurite-sensitive *E. coli* cells also deposit metallic tellurium in the periplasm, but die in the process; furthermore, attempts to identify specific reductase activity in strains carrying plasmid-borne tellurite determinants have failed (Lloyd-Jones *et*

al., 1991). Studies with radiolabelled tellurite have failed to detect any substantial differences in the uptake or efflux levels between *E. coli* strains with and without tellurite-resistance determinants, indicating that active efflux is not a resistance mechanism (Lloyd-Jones *et al.*, 1991). Previously, the toxicity of tellurite was attributed to its oxidizing ability. However, the standard reduction potential of tellurite reduction is too negative for the reaction $TeO_3^{2-} + 3H_2O + 4e^- \rightarrow Te^0 + 6OH^-$ to proceed spontaneously (Turner *et al.*, 1995). Therefore, the specific mechanism of toxicity remains unknown. Generally, heavy-metal stress is attributed to a combination of factors, including the inhibition of protection and repair systems (Stohs and Bagehi, 1995).

Tellurite-resistant strains that can withstand concentrations up to 500-fold greater than those that kill susceptible bacteria have been isolated in variety of bacterial species from hospital water and soil environments (Summers and Jacoby, 1977; Bradley, 1985; Jobling and Ritchie, 1987). Tellurite can enter the *E. coli* cells through the phosphate uptake pathway; therefore, mutation of chromosomal genes of the phosphate uptake pathway can result in resistance (Tomas and Kay, 1986). However, tellurite resistance is usually mediated by plasmid-borne genetic determinants.

Summers and Jacoby (1977) reported that tellurite resistance was encoded by the transferable IncH plasmids of Gram-negative bacteria. Over a decade later, the *ter* gene clusters were cloned from the IncH12 plasmids pMER610 and pR478 isolated from *Alcaligenes* spp. and *Serratia marcescens*, respectively (Jobling and Ritchie, 1987; 1988; Whelan *et al.*, 1995). Sequence

analysis of these two *ter* gene clusters revealed the presence of seven loci, *terZ*, *terA*, *terB-terF*, which collectively encode three phenotypes: phage-inhibition (Phi), colicin resistance (PacB) and tellurite resistance (Te^r) (Whelan *et al.*, 1995). Transposon mutagenesis of the plasmid R478 from *S. marcescens* demonstrated that genes governing the three phenotypes associated with the *ter* gene cluster are genetically linked. Thus, these phenotypes require some common component of the R478-encoded *ter* gene cluster, but disruption of other parts of the system has variable effects on expression of these phenotypes (Whelan *et al.*, 1995). However, the underlying biochemical mechanisms remain elusive despite considerable efforts by many researchers (Lloyd-Jones *et al.*, 1994, 1991; Taylor *et al.*, 1988; Turner *et al.*, 1995; Walter and Taylor, 1992). The *terC* gene encodes a highly hydrophobic product that is predicted to be an integral membrane protein (Jobling and Ritchie, 1988). The *terE* and *terD* gene products are highly similar with 66.5% identity at the amino acid level, and are functionally interchangeable, strongly suggesting that these genes resulted from ancestral gene duplication (Jobling and Ritchie, 1988). The amino acid sequences of TerZ, TerD and TerE, as well as of TerF and TerA, are related (Whelan *et al.*, 1997). In addition, various subsets of the five putative gene products contain domains with highly similar amino acid sequences (Whelan *et al.*, 1995). Transposon mutagenesis indicated that *terD* is essential for all three phenotypes, *terZ* is necessary for PacB (colicin resistance) and Te^r (tellurite resistance), and *terC* is required for Te^r, while no mutations were isolated in *terB*,

which suggests that *terB* is important for all three phenotypes and for the viability of the host in general (Whelan *et al.*, 1995).

Phage inhibition associated with IncH plasmid-borne *ter* determinants

Phage inhibition (Phi) was found in association with the tellurite resistance conferred by IncH plasmids (Taylor and Summers, 1979). The Phi determinants interfere with the propagation of specific phages (I, T1, T5, T7 and φ80), causing reductions in plaque numbers or in the efficiency of plating (Taylor and Grant, 1977). Watanabe *et al.* (1966) and Revel and Georgopoulos (1969) showed that phage DNA is restricted in cells carrying IncH plasmids. This type of phage inhibition differs from other types of plasmid-encoded phage-inhibition determinants, because no modification of phage DNA occurs. That is, the progeny of a bacteriophage which manages to replicate in a bacterial host expressing Phi factors are faced with the same resistance levels in the next bacterial cell containing the determinant (Revel and Georgopoulos, 1969; Taylor and Summers, 1979; Watanabe *et al.*, 1966). An ability to defend itself against phage predation might provide a bacterium with a selective advantage. Based on a continuous culture model, Feldgarden *et al.* (1995) suggested that many large plasmids (including cryptic ones) could be maintained in natural populations simply by selection for their role in phage defence. According to this model, possession of both types of phage resistance, plasmid-borne and chromosomal, is often the most prevalent outcome, despite apparent redundancy of the two systems (Feldgarden *et al.*, 1995).

Colicin resistance

The term 'bacteriological warfare' is commonly associated with the use of bacteria by humans as agents of toxicity against humans. However, bacteria themselves make extensive use of this concept in their constant competition for survival. They routinely synthesise a large and varied family of protein toxins (bacteriocins). Antibiotic proteins known as colicins were first reported in 1925 by the Belgian microbiologist André Gracia, who described the property of certain coliform bacteria to produce cell-free substances (colicins) that killed other coliform strains. Colicin K (Jesaitis, 1970) and colicin E1 (Goebel and Kreft, 1974) production have been described for *Proteus* bacilli. The colicinogenic bacteria are immune to the colicin they produce, but not to other colicins (Luria and Suit, 1987). Some colicin receptors have been identified as specific outer membrane proteins that might also serve as bacteriophage receptors or in the transport of external substrates (e.g., iron, vitamin B_{12}) (Di Masi *et al.*, 1973; Kadner and Bassford, 1978). A common origin was suggested for all colicin determinants, based on remarkable similarities of genetic organization and segment sequence similarity of the genes encoding diverse colicins. Luria and Suit (1987) hypothesised that the outer membrane serves as an organelle from which colicins have evolved. Protein-motif comparison studies are required to test this hypothesis.

IncH plasmids mediate resistance to all pore-forming colicins by a yet-unknown mechanism. Pore- or channel-forming colicins are believed to kill susceptible *E. coli* and related species by forming holes in the cytoplasmic

membrane, and thus reducing the electrochemical membrane potential (Braun *et al.*, 1994; Konisky, 1982; Pugsley 1984a, 1984b). Colicin structural genes are always closely linked to other genes encoding immunity protein(s) and, possibly, a lysis gene, which facilitates colicin release from the cell (Brickman and Armstrong, 1996). Colicins are highly effective bactericidal weapons, since an individual colicin molecule is capable of killing a single cell (Luria and Suit, 1987). The reactive regions of colicin 5 and the corresponding immunity protein were localized near the inner side of the cytoplasmic membrane, suggesting that colicin 5 (and perhaps other pore-forming colicins) is inactivated by the immunity protein immediately prior to the lethal colicin-pore formation in the cytoplasmic membrane (Pilsl and Braun, 1995). The TonB-ExbB-ExbD uptake system has been shown to be involved in uptake of colicins B and D, phages φ80 and T1, and many other ligands (Braun and Herman, 1993; Postle, 1990). Based on the observations that these specific phages and colicins were associated with both IncH Te^r determinants and the TonB uptake system, Whelan and co-workers (1995) suggested the existence of a link between the TonB system and the IncH Te^r determinants. This group suggested that resistance mechanism(s) affect a common element in the transport of ligands (including colicins and phages) to the cytoplasmic membrane.

Colicins, more generally termed bacteriocins (see p. 31), and phages are often used by bacteria to gain an upper hand in interstrain antagonism. One of the most peculiar forms of such antagonism was described by Dienes in *Proteus* bacilli (Dienes, 1946). When incompatible strains of *Proteus* species are allowed

to swarm towards each other, a line of partial or complete growth inhibition demarcates the border along which swarmer cells of the two strains meet. The phenomenon forms the basis of the Dienes test, which has been extensively used to determine the relationship between *Proteus* strains in cross-infection studies and has served as an epidemiological tool (Story, 1954; Skirrow, 1969; Herruzo-Cabrera *et al.*, 1988). Proticine (bacteriocins) production is an important determinant of Dienes compatibility, because the formation of Dienes lines is prevented at the temperature inhibitory to proticine production (42°C) (Senior, 1977b). However, proticine production alone fails to explain formation of Dienes lines between two non-producing strains, or between two proticine-resistant strains producing different proticines (Senior, 1977b). Clearly, further genetic experiments are needed to define the determinants of proticine production and sensitivity, as well as compatibility determinants in the Dienes test. Interest in the Dienes phenomenon has waned due to the development of other diagnostic procedures.

Significance of the Te^r gene cluster

The plasmid-borne *ter* gene cluster confers the three phenotypes of colicin resistance (PacB), phage inhibition (Phi), and tellurite resistance (Te^r) (Whelan *et al.*, 1995). Among these three phenotypes, colicin resistance (PacB) is likely to be the most advantageous to the host bacterium. Fifty percent of human *E. coli* isolates produce colicins, and 80% of the colicin-producing strains in the *E. coli* Reference Centre are human isolates (Pilsl and Braun, 1995; Riley and Gordon, 1992), suggesting that colicins are important weapons in competitions among

bacterial populations in the human host environment (Braun *et al.*, 1994). Since a majority of IncH plasmids that contain the *ter* gene cluster come from clinical isolates of *Serratia* and *Salmonella*, the phenotypes associated with these plasmids, i.e., Phi, PacB and heavy-metal resistance, can supply powerful protection for the host in a variety of environments (Whelan *et al.*, 1995).

Tellurite resistance is neither unique to the IncH plasmids, nor exclusively plasmid-borne. Currently, at least three additional and distinct Te^r determinants have been described in *Enterobacteriaceae*: the *telAB* genes from the *E. coli* chromosome; *arsABC* mediated tellurite resistance; the *kilAtelAB* locus of IncPα plasmids (Bradley, 1985; Hou and Taylor, 1994; Taylor *et al.*, 1994; Turner *et al.*, 1992; Walter *et al.*, 1991).

The *kilAtelAB* locus on the broad-host-range IncPα plasmid RK2 mediates tellurite resistance (Bradley, 1985; Walter *et al.*, 1991). Although normally silent, expression of this determinant can be induced by growth of the host *E. coli* cells containing RK2 on low concentrations (5 μg/ml) of potassium tellurite. Sequence analysis of this tellurite resistance determinant revealed a mutation in the *telB* gene, resulting in the replacement of a serine residue by cysteine, and associated with expression of high level tellurite resistance (MIC 512 μg/ml) compared to that mediated by the *arsABC* genes (Goncharoff *et al.*, 1991; Walter *et al.*, 1991).

Moderate levels of tellurite resistance are specified by the *arsABC* genes (arsenicals-resistance determinants) from the IncFI plasmid R773 (Turner *et al.*, 1992). Ter mediated by *arsABC* depends on efflux of TeO_2^{3-} out of the bacterial

cell.

Tellurite resistance determinants also include the *tehAB* genes in the *E. coli* chromosome (Taylor *et al.*, 1994). Although phenotypically silent in most *E. coli* strains, the *tehAB* operon overexpressed from a multicopy plasmid vector resulted in moderate levels of tellurite resistance (MIC 128 μg/ml) (Turner *et al.*, 1994). However, these different determinants confer variable levels of resistance to the host cell and show no sequence similarity to each other, do not share common genetic organization, and the number of genes within these Te^r operons varies greatly (Hou and Taylor, 1994). In addition, these systems are believed to mediate tellurite resistance by completely different but yet unknown biochemical mechanisms (Turner *et al.*, 1995). The presence of several different Te^r determinants with no sequence similarities indicates that the evolution of Te^r determinants may occur quite readily; therefore, this phenotype may be important to bacteria in the natural environment, or may be associated with colonization of the human body (Hill *et al.*, 1993: Walter *et al.*, 1991).

Although direct evidence is still lacking, a link might exist between swarmer-cell differentiation and *ter* gene function. Cell elongation, coordinated with an inhibition of septation, is a hallmark of swarmer cell development. Recent work suggests that an additional R478-borne *ter* locus (comprising *terX*, -*Y* and -*W*), when expressed in *E. coli*, leads to inhibition of cell division and lethal filamentation that is associated with the expression of the *terZ* locus from recombinant plasmids (Whelan *et al.*, 1997). Complementation analysis defined a 1.95-kb R478 fragment containing the *terW* gene as a minimal protective

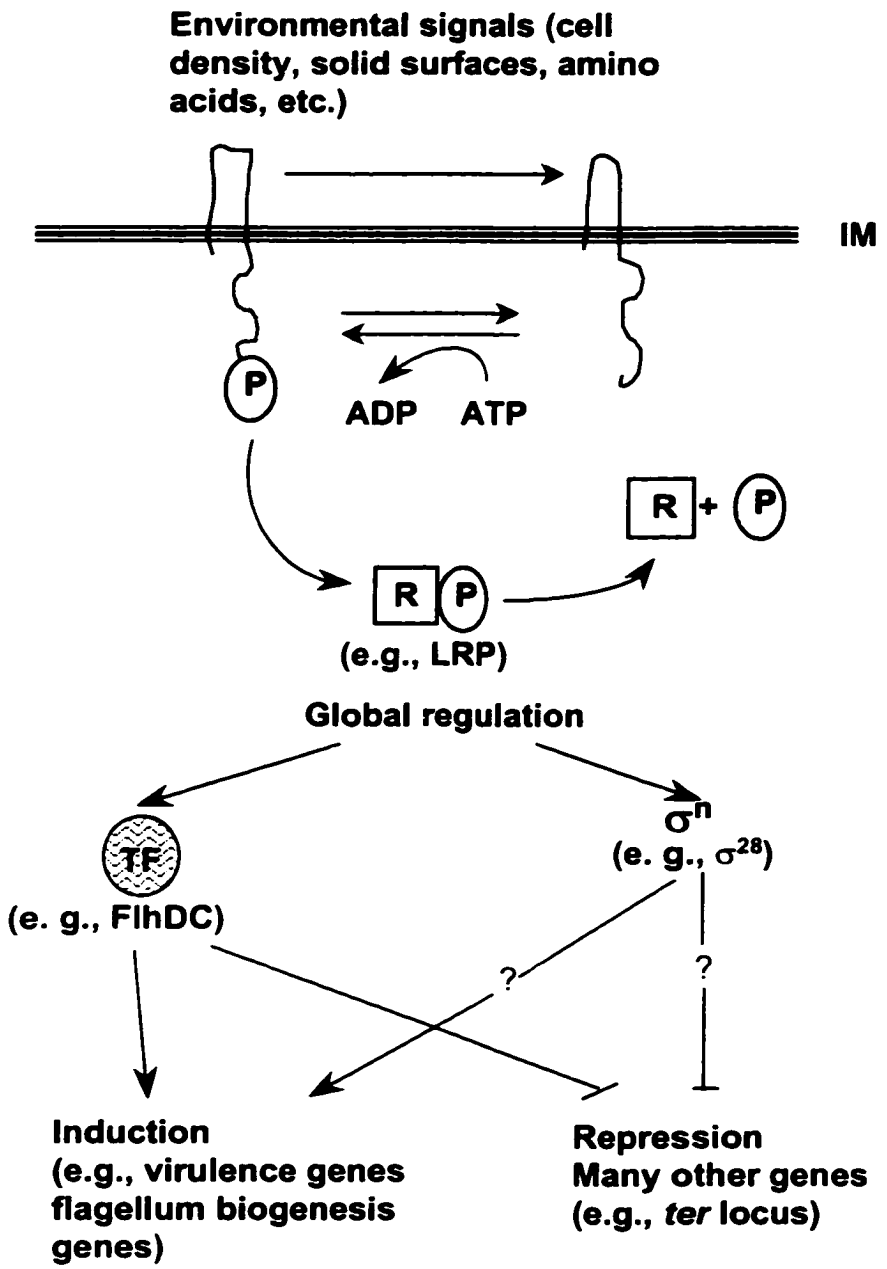
region required to abolish filamentation associated with the *terZ* locus. Additionally, expression of various subclones of the R478 *terZ* locus in *E. coli* suggested, but did not prove, that increased potassium tellurite resistance was associated with filamentation. The biochemical roles of the putative TerX, TerY and TerW proteins have not been determined, and these genes show no similarity to the *E.coli* cell-division genes or other known bacterial genomic sequences. Whelan *et al.* (1997) hypothesised that TerW may interact with the *terZ* promoter region and function as a transcriptional regulator. However, no DNA-binding motifs were detected within the TerW amino acid sequence. Elucidation of a possible relationship between the *terWYX* genes and the inhibition of cell division presents an exciting challenge and merits further investigation.

Research objectives

Figure 1 schematically depicts a hypothetical model of regulatory cascade control that might be associated with swarmer cell differentiation. According to this diagram, the developmental process is triggered when several appropriate environmental signals (e.g., cell density, nutrient availability) are received by a membrane-spanning sensor/transducer protein (two-component regulatory system). Recently, Belas *et al.* (1998) identified *rsbA* (regulator of swarming behaviour), a genetic locus that may function as a sensor of environmental conditions required to initiate swarming migration. The transducer, through phosphorylation, activates a global response regulator, such as the leucine

Figure 1. Model for a regulatory cascade controlling swarmer cell differentiation.

The top line represents the bacterial inner membrane (IM) harbouring the membrane-associated sensor/transducer protein (wavy line). R – a global response regulator, which is activated through phosphorylation (P – phosphate residue) by the sensor/transducer protein. The global response regulator protein can activate the transcription of other transcriptional factors (TF), or trigger a cascade of alternative sigma factors (σ^n). Alternative sigma factors and transcription factors can induce expression of the swarming associated genes and repression of other genes (see text). Alternative sigma factors can modulate the transcription of the target genes indirectly through several intermediate steps (represented by the question marks), leading to induction (up-regulation) of swarmer- specific genes (e. g., urease, flagella biogenesis) and repression (down-regulation) of swimmer-specific or non-essential genes (apparently including *ter* genes).


Environmental signals (cell density, solid surfaces, amino acids, etc.)
IM
P
ADP
ATP
R
+
P
R
P
(e.g., LRP)
Global regulation
TF
(e. g., FlhDC)
σn
(e. g., σ28)
?
?
Induction
(e.g., virulence genes
flagellum biogenesis
genes)
Repression
Many other genes
(e.g., ter locus)

responsive protein (LRP). This response regulator in turn activates the transcription of genes encoding other transcription factors, cascades of alternative sigma factors and regulons. This regulatory cascade results in the induction of swarming-associated genes (flagellum-biogenesis and virulence genes) and repression of others, including the *ter* genes (Figure 1).

The purpose of this investigation was to elucidate signal-transduction pathways and developmentally regulated genes of *Proteus mirabilis*. I used transposon mutagenesis to generate chromosomal promoter-*lacZ* fusions and applied a screen to identify mutant strains harbouring a promotorless mini-Tn5*trp'-'lacZ* transposon in developmentally regulated genes. Most of the research in *P. mirabilis* has been focused on virulence genes (e. g., hemolysin, protease, and urease), which are up-regulated in swarmer cells. In this study, I have characterised a chromosomal cluster of genes and provide the first molecular analysis of a down-regulated locus in swarmer cells. This cluster includes six genes that have sequences similar to those of members of the *ter* gene cluster found on large conjugative IncH12 plasmids in Gram-negative bacteria. I describe the use of Tn5-transposon mutagenesis to generate a promoter-reporter-gene fusion that disrupts the *terC* coding region in the chromosome of *P. mirabilis*. The insertion resulted in decreased levels of potassium tellurite resistance in the mutant strain. Using northern-blot and primer extension analysis, I uncovered evidence that the expression of the *ter* locus is positively regulated in response to potassium tellurite. These studies further demonstrate that resistance to tellurite is chromosomally encoded and an

integral feature of *Proteus* bacilli. Based on these observations, I hypothesise that *P. mirabilis* may be the evolutionary origin for dissemination of the plasmid-borne *ter* loci among enteric bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids and media.

The *P. mirabilis* and *E. coli* strains, plasmids and cosmids used in this study are listed in Table 1. Bacterial stock cultures were maintained at -70°C in nutrient broth containing 10% DMSO or in LB broth (Miller, 1972) containing 15% (v/v) glycerol. *P. mirabilis* strain S2 (WT) was obtained from the Victoria General Hospital, Halifax, and the isogenic rifampicin-resistant mutant strain S2R was isolated by plating the WT strain on LB agar supplemented with rifampicin (30 μg/ml). Plasmid constructions were carried out with *E. coli* strain JF626 and vectors pBR322 (Bolivar *et al.*, 1977) and Bluescript M13 (Short *et al.*, 1988). The Bluescript M13 vector allows the X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) system to be used for detection of cloned inserts. Antibacterial agents were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; potassium tellurite (Sigma Chemical Co., St. Louis, MO), 20 to 300 μg/ml; rifampicin, 50 μg/ml. LB medium (BDH Inc., Toronto) was used for all experiments, unless otherwise indicated. LB lacking sodium chloride (5 g yeast extract plus 10 g tryptone [Difco Laboratories, Detroit, MI] per L) solidified with 2% agar (Life Technologies, Paisley, Scotland) was used as the non-swarming medium.

Table 1. Bacterial strains, plasmids and oligonucleotides.

Bacterial strain	Description	Reference or source
Escherichia coli		
CC118 (λpir)	Δ(*ara-leu*), *araD*, Δ*lacX74*, *galE*, *galK*, *pho*A20, *thi-1*, *rpsE*, *rpoB*, *argE*(Am), *recA1*, λ*pir*	Herrero *et al.* (1990)
JF626	Δ(*lac pro*), *thi*, *rpsL*, *supE*, *endA*, *sbcB15*, *hsdR4*, (F' *traD36*, *proAB*, *lacI*^{q}, *lacZm15*)	Ron Taylor
LE392	*supF*, *supE*, *hsdR*, *galK*, *trpR*, *metB*, *lac*, *tonA*	Silhavy *et al.* (1984)
SM10 (λpir)	*thi-1*, *thr*, *leu*, *tonA*, *lacY*, *supE*, *recA*::RP4-2Tc::Mu, Km^r, λ*pir*	Miller and Mekalanos (1988)
S17-1 (λpir)	Tp^r Sm^r *recA*, *thi*, *pro*, *hsdR*$^-$M$^+$ RP4:2-Tc:Mu:Km Tn7, λ*pir*	Kenneth Timmis
DT2378	pKFW4a and pDT2790 host, Rif^r, Nal^r	Diane Taylor
Proteus mirabilis		
S2	Wild-type clinical isolate	VGH
S2R	Spontaneous Rif^r derivative of S2	This study
S2R-6/39		This study

Rif^r Km^r pUT/miniTn5 lacZ1 x S2R

Vectors	Description	Reference
Plasmids		
pUT mini Tn5-*lacZ*1	Ap^r, Km^r; delivery plasmid for miniTn5 *lacZ*1	De Lorenzo *et al.* (1990)
pBR322	Cloning vector; Ap^r Tc^r	Bolivar *et al.* (1977), Sutcliffe (1979)
pBluescript	M13 phagemid T_7 T_3 *lacI, lacZ*; Ap^r	Short *et al.* (1988)
pHC79	Cosmid vector; Ap^r Tc^r	Hohn and Collins (1980)
pKFW4a	5.8-kb *Bam*HI - *Eco*RI subclone of theR478 *terZ* locus, Cb^r (subtoxic clone)	Whelan *et al.*(1995)
pDT2790	1.95-kb *Bam*HI subclone of *terW*, Km^r	Whelan *et al.* (1997)
pBOC20	delivery vector, *sacB*, Chr^r	Nicholas Cianciotto
Bacteriophages		
M13 tg131	single-stranded DNA sequencing	Kieny *et al.* (1983)
M13 tg130	vectors	

Oligonucleotides	(Canadian Life Technologies, Burlington, ON)
TEREF-F	CGCTAATAAAAGCGCCAAGATG
TEREF-R	TTGTTGIAGWGGRATRTTTTG
TERZR	CCAAAGAGTCCACCGAGAAATCCC

Antibiotic resistances: Ap^r, ampicillin; Km^r, kanamycin; Tc^r, tetracycline; Rif^r, rifampin, Nal^r, nalidixic acid, Chr^r, chloramphenicol, Cb^r , carbenicillin

DNA manipulations

The recombinant DNA protocols used were those of Sambrook *et al.* (1989). Restriction endonucleases, T4 DNA ligase, and RNAse were purchased from GIBCO BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA) and used according to the manufacturer's directions.

Extraction of chromosomal DNA

Bacterial cultures were grown overnight at 37°C in liquid medium. Cells were collected by centrifuging 15 ml of culture at 7000 X g for 10 min. The cell pellets were suspended in 0.8 ml of TE buffer, pH 8 (10 mM Tris.HCl pH 8.0, 1 mM EDTA pH 8.0) and transferred to 1.5-ml microfuge tubes (Sarstedt, Germany). Lysozyme (100 µl of 20 mg/ml) (Sigma Chemical Co.) was added to cell suspensions and incubated at room temperature for 10 min. Following this incubation, 10 µl of 20% sodium dodecyl sulfate (SDS) (Sigma Chemical Co.)

and 100 µl of Proteinase K (10 mg/ml; Sigma Chemical Co.) were added and the suspensions were further incubated on a rotating platform for 1 h at 37°C. Then, buffered phenol (made by extracting distilled phenol once with 100 mM Tris.HCl pH 8.0 and then with 10 mM Tris.HCl pH 8.0, or until the pH of the phenol was close to neutral) was added to the microfuge tubes and mixed well by rotating the tubes. The tubes were incubated for 1 h at 37°C on a rotating platform. Following this incubation, the tubes were centrifuged for 10 min at 7000 X *g* and the aqueous (top) phase was transferred to a fresh microfuge tube. Then, a mixture of 24 parts chloroform and 1 part isoamyl alcohol was added to the top of the tubes, the contents were mixed thoroughly by hand for 2 min, and the tubes were centrifuged for 5 min at 7000 X *g*. The aqueous phase was transferred to a fresh microfuge tube, and 0.1 volume of 3 M sodium acetate pH 4.8 was added to the tube. The contents of the tubes were mixed well by hand. Then, 2.5 volumes of 95% ethanol were added to the tubes and mixed thoroughly by hand. The DNA was allowed to precipitate at room temperature for 5 min. After the 5-min incubation, the precipitated DNA was spooled on a glass rod and suspended in 0.5 ml of TE pH 8.0. The contaminating RNA in the sample was removed by adding 3 µl of RNAse I (10 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) and incubating for 1 h at 37°C. Then, the DNA was precipitated again by adding 0.1 volume sodium acetate and 2.5 volumes of 95% ethanol and incubating the tubes for 5 min at room temperature. The precipitated DNA was spooled using a glass rod and immediately washed

with a gentle squirt of 95% ethanol. The DNA was resuspended in 0.25 ml of TE and stored at 4°C.

Southern blot hybridization assays

These assays were performed as originally described by Southern (1975) and modified by Sambrook *et al.* (1989). Briefly, DNA fragments were generated using appropriate restriction endonucleases according to the manufacturer's suggestions, and then separated by electrophoresis through 0.85% or 1.5% agarose gels. The agarose gels were cast by dissolving 0.85 g or 1.5 g of agarose (Boehringer Mannheim Biochemicals) in 100 ml of 1 X TAE buffer (50 X TAE per 1 liter of distilled water: 242 g Tris base, 57.2 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0). The DNA samples were mixed with 2 μl of 10 X TAE loading buffer (30% glycerol and 0.1% bromophenol blue in 10 X TAE buffer), loaded onto the gel and resolved by electrophoresis in 10-cm trays at 80 V. The gel was viewed using an UV transilluminator and photographed on Polaroid 667 instant film with a Polaroid MP-4 Camera and Wratten 80A red filter (Fotodyne, Inc.). The DNA was transferred to nylon membranes (Hybond-N+, Amersham Life Science Inc., Arlington Heights, IL) using a gel-transfer apparatus (VacuGene XL, Pharmacia) following the manufacturer's protocol. First, depurination solution (0.2 N hydrochloric acid) was passed through the gel for 15 min, followed by denaturation with 1 M sodium hydroxide for 30 min. The membrane was then washed with 2 X SSC (per 1 liter of 20 X SSC solution in distilled water: 175.3 g of sodium chloride, 88.2 g of sodium citrate, pH adjusted

to 7.0 with 10 N sodium hydroxide) and air dried for at least 30 min. The dried membrane was sandwiched between two sheets of Whatman 3M paper and baked under vacuum for 2 h at 80°C. After baking, the membrane was soaked in 2 X SSC and placed in a capped glass tube containing 20 ml of pre-hybridization solution (12 ml formamide, 7.2 ml of 20 X SSC, 480 μl of 50 X Denhardts solution [1% bovine serum albumin, 1% polyvinylpyrrolidone, 1% Ficoll], 48 μl of 0.5 M EDTA, 240 μl 10% SDS, 160 μl single-stranded fish sperm DNA [10 mg/ml]) and incubated overnight in a Hybridization Incubator (Lab-Line Instruments, Inc., Melrose Park, IL) at 37°C. The radiolabelled DNA probes were made by polymerase chain reaction (PCR) or the random-priming method developed by Feinberg and Vogelstein (1983). Oligonucleotide primers were used to amplify the appropriate DNA sequences by PCR. [α-^{32}P]dCTP (65 μCi in a 50-μl reaction mixture) was incorporated into the PCR fragments by decreasing the dCTP molar concentration in the deoxynucleotide triphosphate reaction mixture from 125 μM to 30 μM. Amplified fragments were extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1). The random-priming protocol was carried out using Random Primed DNA Labeling Kit (Boehringer Mannheim Biochemicals) as suggested by the manufacturer. Mixed of random hexanucleotide primers was hybridized to a DNA restriction fragment and complementary DNA strands were synthesized using Klenow enzyme. [α-^{32}P]dCTP (50 μCi in a 25-μl reaction) was present in the reaction and incorporated into the newly synthesized complementary DNA probe. All

radiolabelled DNA probes were purified by using Ultrafree-MC 30,000 NMWL filter units (Millipore Corporation, Bedford, MA). Briefly, 300 μl of TE buffer was added to a tube containing radiolabelled products, transferred in a Ultrafree-MC filter unit and centrifuged at 3000 X *g* until almost all the liquid had passed through the filter. The filter was then washed well with 150 μl of TE buffer and the liquid remaining in the filter was transferred to a fresh microfuge tube. The radiolabelled DNA probes were stored at -20°C inside a plexiglass container. Approximately 8 $X10^6$ cpm of denatured radiolabelled DNA were added to the pre-hybridization mixture and incubated overnight at 37°C. Then the nylon membranes were washed using different concentrations (see below) of SSC containing 0.1% SDS. At the end of each wash, the centre and the edges of the membrane were monitored for radioactivity using a survey meter (Model 3 Survey Meter, Ludlum Measurements, Inc., Sweetwater, TX) to determine the effectiveness of washes by decrease in background counts at the edges of the membrane. Typically, the washing protocol included the following steps. The filter was washed with 5 X SSC/ 0.1 % SDS for 20 min at room temperature, followed by a 10-min wash at 55°C, followed by a 10-min wash with 2 X SSC 0.1% SDS at 55°C, followed by a 5-min wash with 1 X SSC 0.1% SDS at 55°C, and finally for 2 min with 0.2% SSC 0.1% SDS at room temperature. If background radioactivity was still high in comparison to the radioactivity at the center of the membrane, the last wash was repeated at 55°C. At this point, if the background radioactivity was sufficiently low, the filter membrane was wrapped in

plastic wrap, placed in a cassette containing intensifying screens and exposed to Kodak XOmat AR X-ray film for 12-24 h at -70°C. In some instances, temperatures as high as 65°C were used for washing to increase stringency.

Colony blot hybridization

This procedure was performed as described by Sambrook *et al.* (1989). Isolated colonies (in pure culture) were grown overnight on Nitrocellulose (NC) disks placed on solid culture media. Four pieces of Whatman 3M paper were cut to fit on the bottoms of four large Pyrex glass dishes. Each piece of filter paper was then saturated with one of the following: Solution 1 - 10% SDS; Solution 2 (denaturing) - 0.5 M sodium hydroxide, 1.5 M sodium chloride; Solution 3 (neutralizing) - 1.5 M sodium chloride, 0.5 M Tris.HCl pH 7.4; and Solution 4 - 2 X SSC. The excess liquid was poured off so that no liquid accumulated on the top of the filter papers. Then, the NC filters were picked up with blunt-end forceps and placed colony side up on the Whatman paper saturated with 10% SDS. The filters were exposed in this way to SDS for 3 min and transferred in similar fashion onto the other sheets of filter paper saturated with the various solutions for 5 minutes each. Finally, the NC disks were placed colony side up on a fresh sheet of Whatman 3M paper and allowed to air dry at room temperature for at least 30 min. The dried disks were then sandwiched between two pieces of Whatman 3M paper and baked at 80°C under vacuum for 1-2 h. The baked disks were floated on 2 X SSC until they become thoroughly wet from

underneath and then were submerged in the same solution for an additional 5 min. The disks were transferred to a large glass beaker containing 200 ml of Prewash solution (5 X SSC, 0.5 % SDS, 1mM EDTA) and incubated with gentle agitation (to prevent filters from sticking to one another) for 30 min at 50°C. Following this incubation, the colony debris was gently scraped off the surface of the disks using Kimwipes soaked in Prewash solution and the disks were transferred to prehybridization solution. Probing with radiolabelled DNA and subsequent washing of the disks were performed as outlined for the Southern blot hybridization assays.

RNA extraction

Bacterial cultures were grown overnight with aeration in liquid medium at 37°C. Bacterial culture (5 ml) was centrifuged at 7000 X *g* for 10 min and the cell pellets were placed on ice. The RNA was extracted by the hot SDS/acid phenol method (Hoffman *et al.*, 1992). All solutions used in the RNA extraction procedure were made using sterile water treated with 0.1% diethyl pyrocarbonate (DEPC, Sigma Chemical Co.). Briefly, the bacterial cells were lysed by adding 3 ml of boiling TES buffer (50 mM Tris.HCl pH8.0, 1 mM EDTA, 50 mM NaCl) containing 1% SDS, vortexed for 5 sec, and placed in boiling water for 30 sec. Then 3 ml of 65°C acid phenol (pH 5.0) was added to the suspension, vortexed briefly, and placed in 65°C water bath for 5 min (the acid phenol was made by equilibrating double-distilled phenol with an equal volume of 500 mM sodium

acetate followed by 2 to 3 changes of 50 mM sodium acetate pH 4.8). The tubes were then centrifuged at 7000 X *g* for 10 min and the aqueous layer was carefully removed to a fresh tube, leaving behind the contaminating protein and DNA in the phenolic phase. The extraction with acid phenol was repeated in the same manner, and then the aqueous layer was extracted once with an equal volume of chloroform:isoamyl alcohol (24:1) to remove the traces of phenol. The RNA was precipitated by adding 10% (vol/vol) of 3 M sodium acetate and 2.5 X volumes of ice-cold 95% ethanol. After 30 min of incubation at -70°C to maximize the RNA precipitation, the sample was centrifuged at 13,000 X *g* for 10 min. The supernatant was discarded and the pellet was washed with 70% ethanol. The RNA pellet was dried under vacuum and resuspended in 50 μl of sterile DEPC-treated water. To estimate the concentration of RNA, a portion of the RNA sample was diluted appropriately and the absorbance at 260 nm was determined. The RNA solution was dispensed in 20-μg portions and dried under vacuum in sterile microfuge tubes. The vacuum-dried samples were stored at -20°C.

Northern blot hybridization assays

These assays were performed as described by Kroczek and Siebert (1990). The RNA was separated by formaldehyde-agarose gel electrophoresis. To prepare the gel, 0.5 g of agarose (Boehringer Mannheim Biochemicals) was dissolved in 36.7 ml of distilled water and 5 ml of 10 X 4-morpholinepropane-

sulfonic acid (MOPS)/EDTA buffer (per 300 ml: 31.4 g MOPS, 6 ml 0.5 EDTA pH 8.0, pH adjusted to 7.0 with 10 N sodium hydroxide). Before the gel was cast, 8.3 ml of 37% formaldehyde were added to the melted agarose. The solidified gel was "pre-electrophoresed" in 1 X MOPS/EDTA buffer for 30 min in a 14-cm gel tray at 60 V before loading the RNA samples. The RNA samples were resuspended in 11 μl of gel loading buffer consisting of 2.2 μl of Buffer A (294 μl 10 X MOPS/EDTA, 706 μl DEPC-treated sterile distilled water), 4.8 μl formaldehyde/formamide solution (89 μl formaldehyde, 250 μl of formamide), 2 μl gel loading buffer (50 mg ficoll, 5 mg bromocresol green, 322 μl of buffer A, 178 μl of 37% formaldehyde, 500 μl formamide) and 2 μl of ethidium bromide (0.5 mg/ml). The samples were then heated to 65°C for 15 min, quickly cooled on ice and then loaded onto a gel. At the same time, 3 μl of 0.24 - 9.5 kilobases RNA ladder (Gibco BRL Products, Burlington, ON) were similarly treated and loaded onto the gel. The RNA samples were then resolved by electrophoresis for 5-6 h in a 14-cm gel tray at 60 V. The gel was viewed using a UV transilluminator, photographed and then the RNA was transferred to a nylon membrane (Hybond-N+, Amersham Life Science Inc.) using a gel-transfer apparatus (VacuGene XL, Pharmacia) according to the manufacturer's instructions. First, the formaldehyde was removed by passing through the gel DEPC-treated water for 5 min. Next, an alkaline solution (50 mM sodium hydroxide, 10 mM sodium chloride) was passed through the gel for 5 min. The alkaline solution was neutralized by passing through a solution of 0.1 M Tris.HCl for 5 min. Finally, the RNA was transferred

using 20 X SSC for 30 min. Following the transfer, the membrane was wrapped in plastic wrap, placed on a transilluminator and exposed to UV light at the "high" setting for 5 min. Then, the membrane was placed between two sheets of Whatman 3M paper and baked under vacuum for 1-2 h at 80°C. The baked membrane was placed in a capped glass tube containing 20 ml of Northern blot pre-hybridization solution (per 20 ml: 5 ml 20 X SSC, 0.2 ml 10% SDS, 0.4 ml single-stranded fish sperm DNA [10 mg/ml], 12.6 ml of sterile distilled water) and incubated overnight in a Hybridization Incubator (Lab-Line Instruments, Inc., Melrose Park, IL) at 42°C. The probing and washing of the membrane were carried out essentially as described for the Southern blot hybridization essays.

Primer extension reaction

Primer extension was performed by hybridizing the end-labelled oligonucleotide primer TERZR (5'-CCAAAGAGTCCACCGAGAAATCCC) to *P. mirabilis* S2 RNA. The end-labeling of TERZR was done using T4 polynucleotide kinase (New England Biolabs) and the end-labelled oligonucleotide was purified using a Sep-Pack C_{18} cartridge (Millipore Corp., Milford, NH). For the end-labeling reaction the following was added to a sterile microfuge tube: 3 μl of 10 X kinase buffer (0.5 M Tris.HCl pH 7.6, 0.1 M $MgCl_2$, 1 mM EDTA), 1 μl of dithiothreitol (DTT), 1 μl of 30 mM spermidine, 12.5 μl of [γ-^{32}P]ATP (125 μCi), and 200 ng of the oligonucleotide primer, and the reaction volume was adjusted

to 28 μl with sterile distilled water. Then, 20 units of T4 polynucleotide kinase (2 μl) were added, and the contents of the tube were mixed well and incubated at 37°C for 1 h. Following incubation, the kinase reaction was resolved by 20% PAGE. The gel was prepared by mixing 25 g of urea, 25 ml of 40% acrylamide (per 100 ml: 38 g acrylamide, 2 g bis-acrylamide [Pharmacia Biothech]), 5 ml of 1 M Tris borate buffer [TBE] (1 L of 10 X TBE: 108 g Tris base, 55 g boric acid, 40 ml of 0.5 M EDTA pH 8.0) and 35 mg of ammonium persulphate. TEMED (15 μl) was added to the mixture immediately prior to casting of the gel in a 18 cm X 16 cm gel apparatus (Vertical Slab Gel Unit, Model SE 400, Hoefer Scientific Instruments, San Francisco, CA). The gel was allowed to polymerize and was then electrophoresed for 1 h at 300 V prior to loading of the samples. Then, the kinase reaction was mixed with 4-5 μl of gel loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and the entire sample was loaded onto the gel. The samples were electrophoresed for 3-4 h at 300 V. After electrophoresis, the gel was wrapped in plastic wrap and exposed to Kodak film for 1 min. The resulting autoradiograph was superimposed onto the gel and the radioactive band, corresponding to the end-labelled primer, was excised and placed in a sterile tube containing 3 ml of Sep-Pack elution buffer (100 mM Tris.HCl pH 8.0, 5 mM EDTA, 0.5 M NaCl). The gel slice was crushed and incubated in elution buffer overnight at 60°C to elute the end-labelled primer from the gel into the buffer. Following incubation, a Sep-Pack cartridge was prepared for the oligonucleotide purification by attaching it to a 10-ml syringe

(without the plunger) and passing 10 ml of methanol through the cartridge in a drop-wise manner. Then, 10-20 ml of sterile distilled water was passed through the cartridge in the same manner to wash out the methanol. Next, the DNA solution in elution buffer was transferred to the syringe and slowly passed through the cartridge. The cartridge was rinsed by 3 X passage of 1 ml of Sep-Pack elution buffer, followed by one passage of sterile distilled water (20 ml). The end-labelled primer was eluted by passing 3 ml of methanol-TEAAc solution (100 mM triethylamine-acetate buffer pH 7.3, 50% methanol) through the cartilage. The stock 1 M triethylamine-acetate (TEA) buffer was prepared as follows: in a flask on ice with constant stirring, 0.2 M of triethylamine were added dropwise to 100 ml of 0.2 M acetic acid, the pH adjusted to 7.3 with TEA or acetic acid, and the final volume adjusted to 200 ml. The eluate was collected in 3 microfuge tubes (1 ml per tube). The oligonucleotide was recovered by drying the liquid in a Speed Vac concentrator (Savant Instruments Inc., Hicksville, NY). The DNA pellet from each tube was dissolved in 10 μl of DEPC-treated water, pooled together, and the radioactivity incorporation was measured using a Wallac 1410 liquid scintillation counter (Pharmacia).

For each primer-extension reaction, 10-60 μl of RNA was added to 1.2 X 10^7 cpm of end-labelled oligonucleotide primer and the final mixture volume was adjusted to 27 μl with DEPC-treated distilled water. Three μl of annealing buffer (100 mM Tris.HCl, 2.5 M potassium chloride) was added to each tube and the tubes were placed in MiniCycler™ thermocycler (MJ Research Inc., Watertown,

MA) programmed as follows: 80°C for 5 min, 65°C for 5 min, 42°C for 10 min, and 37°C for 20 min. After completion of the reaction, the annealed material was precipitated by addition of 70 μl of DEPC-treated water, 10 μl of 3 M sodium acetate, and 260 μl of ice-cold 95% ethanol, followed by 30-min incubation at -20°C. The pellet was washed with 70% ethanol and dried under vacuum. The dried pellet was resuspended in 7 μl of DEPC-treated water. Then, 13 μl of reverse transcriptase buffer (1 M Tris.HCl pH 8.3, 1 M $MgCl_2$, 1M DTT, 50 mM each of dATP, dCTP, dGTP, and TTP, and actimomycin D [2 mg/ml]) and 200 U of reverse transcriptase (Gibco/BRL) were added to the reaction. The mixture was incubated for 1 h at 42°C. Following the incubation, the free RNA was digested by adding 1 μl of RNAse (10 mg/ml) [bovine pancreas; Boehringer Mannheim] and incubating for 30 min at 37°C. The reaction volume was adjusted to 100 μl with TE. The cDNA was purified by phenol:chloroform:isoamyl alcohol (24:24:1) extraction and ethanol precipitation. The cDNA was pelleted by centrifugation, dried under vacuum, and resuspended in 10 μl of gel loading buffer. The cDNA samples were resolved on a polyacrylamide sequencing gel beside a sequencing ladder corresponding to the *terZ* promoter region. The sequencing ladder was generated using the TERZR oligonucleotide primer. The sequencing gel electrophoresis is described below.

Extraction of plasmid DNA

Plasmid DNA was isolated by the method of Birnboim and Doly (1979). Broth medium cultures were inoculated with a single bacterial colony and incubated overnight with aeration at 37°C. For small-scale plasmid DNA preparations, 5 ml of bacterial culture were pelleted by centrifugation for 10 min at 5000 X *g* and the pellet was suspended in 200 µl of Solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris.HCl pH 8.0). The tube was vortexed and placed on ice for 5 min. To each tube, 400 µl of Solution 2 (0.2 N sodium hydroxide, 1% SDS in sterile water) was added, gently vortexed and placed on ice for 10 min. 300 µl of 5 M potassium acetate, pH 4.8, were then added, gently mixed and placed for 5 min at -70°C. The tubes were centrifuged at 7000 X *g* for 15 min and 750 µl of the supernatant were transferred to a microfuge tube. The plasmid DNA was precipitated by addition of 450 µl of ice-cold isopropanol and placing for 5 min at -70°C. The tubes were then centrifuged at 7000 X *g* for 15 min, the supernatant was discarded and the pellet was washed with 70% ethanol, dried under vacuum and resuspended in 100 µl of TE buffer. The contaminating RNA in samples was digested by adding 1 µl of RNAse I (10 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) and incubation for 1 h at 37°C. Plasmid DNA was further purified by serial extractions with equal volumes of buffered phenol, buffered phenol:chloroform:isoamyl alcohol (24:24:1) mixture and chloroform:isoamyl alcohol (24:1) mixture. After each extraction the top (aqueous) layer was transferred in a fresh microfuge tube. Then the plasmid

DNA was precipitated by adding 10% (vol/vol) of 3 M sodium acetate, pH 4.8, and 2.5 volumes of ice-cold 95% ethanol. The tubes were placed at -70°C for 30 min to facilitate precipitation. The DNA was then precipitated by centrifugation at 7000 X *g* for 10 min, and the pellet was washed twice with 70% ethanol, dried under vacuum, resuspended in 50 μl of TE buffer and stored at 4°C.

Transformation of plasmid DNA into bacterial cells

The method used was described by Sambrook *et al.* (1989) and based on observations of Mandel and Higa (1970) and experimental work of Cohen *et al.* (1972).

Preparation of competent cells: Appropriate *E. coli* strains were grown overnight in LB broth at 37°C with aeration. The next day, 50 ml of fresh LB broth were inoculated with 1 ml of the overnight culture and incubated under the same conditions until the culture had reached an OD_{660} of 0.3. The culture (40 ml) was centrifuged at 5000 X *g* for 2 min, the cells were resuspended in half of the original volume (20 ml) of sterile 0.1 M calcium chloride and placed on ice for 20 min. The cells were then pelleted by another centrifugation at 5000 X *g* for 2 min and re-suspended in one-tenth of the original volume (4 ml) of sterile 0.1 M calcium chloride. The cells were held at 4°C for several hours to increase the efficiency of transformation. Competent cells prepared in this way were discarded if not used within 48 hours.

Transformation: For transformation, 300 μl of competent cells suspended in 0.1 M calcium chloride were added to a microfuge tube containing 10-20 μg of plasmid DNA (or an entire ligation mixture) and the tube was gently swirled to mix the contents. The tube was cooled on ice for several hours. The tube was then placed in a 42°C water bath for 3 min, and then immediately cooled on ice. Then, the bacterial suspension was transferred to a fresh microfuge tube and 500 μl of LB broth was added. The cells were allowed to grow without antibiotic selection for 45 min at 37°C with aeration. Then, the bacterial cells were pelleted by brief centrifugation, resuspended in 200 μl of LB broth supplemented with appropriate antibiotics and spread on solid medium containing appropriate antibiotics. The plates were incubated overnight at 37°C.

DNA sequencing and analysis

The procedure is based on the dideoxy chain-termination method (Sanger *et al.*, 1977). Both double-stranded DNA fragments cloned in pBluescript and single-stranded DNA fragments cloned in M13 phagemids were used. Single-stranded DNA was isolated from *E. coli* strain JF626 infected with M13 cloning vectors as described in The M13 Cloning and Sequencing Handbook (T7 Sequenase™ version 2.0 dGTP Reagent Kit, Amersham, IL). Double-stranded and single-stranded DNA sequencing was performed with Sequenase (version 2.0) kit from United States Biochemical (Oakville, ON), and [^{35}S]dATP from NEN/Life Science Products (Boston, MA). The synthetic oligonucleotide primer

5'-GTTTTCCCAGTCACGAC supplied in the Sequenase Sequencing Kit was used, unless specialized sequencing primers were designed and used.

Double-stranded DNA sequencing. The plasmid templates to be sequenced were created by subcloning desired DNA fragments (generated by digestion with appropriate restriction endonucleases) in the corresponding restriction sites in pBluescript KS or SK. When a single endonuclease was used, the pBluescript DNA was treated with 1 U of calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim) for 30 min at 37°C to remove 5' phosphates from the DNA fragment and to prevent subsequent self-ligation. The insert and the vector were purified using a Geneclean II Kit (Bio 101 Inc., Mississauga, ON) and ligated together using T4 ligase. The ligation mix was then transformed into competent *E. coli* JF626 cells, which were spread on LB agar plates supplemented with ampicillin (150 μg/ml), 100 mM isopropylthio-β-D-galactosidase (IPTG) solution in distilled water and of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (20mg/ml) in dimethylformamide. The plates were incubated overnight at 37°C and a single white colony resulting from this incubation was used for subsequent recombinant plasmid DNA extraction as described above.

Double-stranded templates were denatured immediately prior to sequencing reactions by the alkaline-denaturation method as suggested by the manufacturer. Briefly, 3-5 μg of plasmid DNA were combined with 0.1 volume of 2 M NaOH, 2 mM EDTA and the mixture was incubated for 30 min at 37°C. The

solution was neutralized by addition of 0.1 volume of 3 M sodium acetate (pH 5.1) and the DNA was precipitated with 3 volumes of ice-cold 95% ethanol (-70°C, 15 min). The DNA was pelleted by centrifugation and washed twice with ice-cold 70% ethanol. The pellet was dried under vacuum and resuspended in 7 μl of sterile distilled water.

For the annealing step, up to 7 μl of the template DNA, 1 μl of primer oligonucleotide and 2 μl of the Reaction Buffer (Sequenase Kit) were combined in a sterile microfuge tube (if necessary the volume was adjusted to 10 μl with sterile distilled water). The primer was annealed to the template by heating for 2 min at 65°C and then allowing the mixture to cool slowly to < 35°C over 30 minutes. The tubes were centrifuged briefly and placed on ice. While the tubes with the annealing mixture were cooling, 2.5 μl of each termination mixture (G, T, C and A) were added to four separate labeled sterile microcentrifuge tubes; the tubes were pre-warmed for 1 min at 37°C prior to termination step. For the labeling reaction, ice-cold annealed DNA mixture (10 μl) was combined with 1 μl of 0.1 M dithiothretiol, 2 μl of the labeling mix (5-fold dilution in distilled water), 1 μl of [^{35}S]dATP and 2 μl of diluted Sequenase polymerase (8-fold dilution in ice-cold Sequenase dilution buffer). The contents of the tubes were mixed and incubated at room temperature for 2-5 min. Then, 3.5 μl of this labeling reaction were added to each pre-warmed termination tube. Solutions were mixed and termination reactions were incubated at 37°C for an additional 5 min. The

reactions were stopped by adding 4 μl of the Stop Solution to each tube. The tubes were stored at -20°C.

Single-stranded DNA sequencing. The DNA fragments to be sequenced were first cloned into M13 phage (Tg 130 and Tg 131) (Kieny *et al.*, 1983). The M13 Cloning and Sequencing Handbook (Amersham) was used as a guide for the generation of single-stranded templates for sequencing.

First, replicative forms (RF) of the M13 phage were prepared. For this, single-stranded M13 DNA was transformed into competent *E. coli* JF626 cells as described previously. After transformation, the *E. coli* cells were added to a 13-ml test tube containing 40 μl of 100 mM IPTG, 40 μl of X-gal (20 mg/ml), and 3 ml of molten H top agar (per 1 liter of distilled water: 10 g of Bacto-tryptone, 8 g of sodium chloride and 8 g of Bacto-agar) kept at ≈40°C. The contents of the tubes were quickly and thoroughly mixed by rolling the tubes between the palms of the hands and poured immediately on fresh pre-warmed LB agar (plates were swirled to make sure that the entire surface was evenly covered with the molten agar mixture), left at room temperature to solidify, and incubated inverted overnight at 37°C. Following this incubation, a single blue-coloured plaque was used to inoculate a 1-ml culture of fresh *E. coli* JF626 cells (obtained by adding 0.1 ml of an overnight bacterial culture to 10 ml of LB broth). The inoculated cultures were incubated at 37°C for 4-5 h with aeration (approx. 100 rpm). The cells were pelleted by centrifugation in microfuge tubes for 5 min at a high setting, and 1 ml of the supernatant was transferred to a flask containing 100 ml

of fresh LB broth and 1 ml of the overnight culture of *E. coli* JF626. This culture was incubated at 37°C for 4-5 h with aeration (approx 100 rpm), the cells were pelleted by centrifugation and the RF DNA was prepared by the standard plasmid DNA extraction protocol described above.

To generate single-stranded templates for sequencing, both the DNA fragments to be sequenced (insert) and the RF of the phage (vector) were digested with suitable restriction endonucleases. When a DNA fragments were generated using single endonuclease, the RF DNA was treated with 1 U of calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim) for 30 min at 37°C to prevent self-ligation. The insert and the vector were purified using a Geneclean II Kit (Bio 101 Inc., Mississauga, ON) and ligated together using T4 ligase. The ligation mix was then transformed into competent *E. coli* JF626 cells, which were then mixed with H top agar, X-gal and IPTG and spread on LB agar as described in the previous section. After overnight incubation at 37°C, a single white plaque (made by a phage containing an inserted DNA fragment) was used to inoculate 1.5 ml of a fresh *E. coli* JF626 culture (1 ml of overnight culture in 100 ml of fresh LB broth) in 13-ml test tubes. A single blue plaque was also selected randomly to serve as a negative control. The tubes were incubated 5 h at 37°C with aeration. The contents were then transferred to sterile microfuge tubes and centrifuged for 5 min at maximum setting. The supernatants were transferred to fresh microfuge tubes (care was taken not to transfer any bacterial cells). Polyethylene glycol 6000 (PEG)/sodium chloride (20% PEG, 2.5 M

sodium chloride, stored at 4°C) (200 μl) was added to each tube, mixed well and incubated 15 min at room temperature. The tubes were centrifuged for 2 min, the supernatants were discarded and 100 μl of TE buffer was added to the phage pellets. Then 50 μl of buffered phenol was added to the tubes, and the contents were vortexed 15-20 sec and incubated at room temperature for 15 min. The tubes were then centrifuged for 3 min and the aqueous layers were transferred to fresh microfuge tubes. The single-stranded DNA was precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5.1, and 2.5 volumes of ice-cold 95% ethanol. The DNA pellet was washed with 70% ethanol, dried under vacuum and re-suspended in 20 μl of TE buffer. The phage DNA (2 μl) was loaded on a 0.85% agarose gel in 1 X TAE and subjected to electrophoresis. The relative migration of DNA from the white plaques was compared to that of DNA from the blue plaque (the recombinant phage would contain additional DNA and migrate more slowly) to confirm the presence of the insert DNA.

Annealing, labeling and termination reactions were performed as outlined for double-stranded sequencing.

Preparation of sequencing gel. A 20 cm X 40 cm BRL sequencing gel apparatus (model S2, Life Technologies Inc., Gaithersburg, MD) with a 20-well shark's tooth comb was used for sequencing. The glass plates were thoroughly cleaned with detergent and rinsed with 95% ethanol. The surface of the smaller plate was covered evenly with Rain Away (Wynn's Canada Ltd., Mississauga, ON) using Kimwipes. Then, 2 spacers were glued with rubber cement to the

edges of the larger plate. Sequencing gels were poured using The Otter™ sequencing gel caster (Owl Scientific Inc., Cambridge, MA).

To prepare the sequencing gel, 36 g of urea, 11.25 ml of 40% acrylamide (38% acrylamide, 2% bisacrylamide in distilled water, sterilized by filtration), 7.5 ml of 5 X TBE (10 X TBE, per 1 liter of distilled water: 108 g Tris base, 55 g boric acid, 49 ml 0.5 M EDTA pH 8.0) and 10 ml of distilled water were placed in a 100-ml glass beaker and stirred with low heat on a magnetic stirrer/hot plate to dissolve the urea completely. The volume of the solution was adjusted to 75 ml with distilled water with continuous stirring. Then, 380 μl of 10% ammonium persulfate (APS) in distilled water and 20 μl of N,N,N',N'-tetramethylethylenediamine (TEMED, described earlier) were added and the gel was cast immediately using a 60-ml syringe. Before the gel solidified, the combs were placed upside down to make a trough for the wells and the glass plates were clamped together with spring clamps. The gel was allowed to polymerize. Then, the clamps and the combs were removed, the plates were rinsed with water, and dried with a paper towels, and the gel was mounted on the electrophoresis apparatus. The top and bottom chambers were filled with 0.5 X TBE buffer and the combs were inserted to form wells. The gel was pre-electrophoresed for approximately 1 h at 50 W before loading the samples. The samples were heated at 80°C for 2 min, loaded on the gel and electrophoresed until the bromophenol blue dye had run off the gel. Then, another set of samples was heated and loaded into a new set of wells and electrophoresis was

continued. Depending on the length of the sequence to be determined, up to 3 sets of samples were loaded per electrophoretic run. After electrophoresis was completed, the 0.5 X TBE buffer was drained from the upper chamber and the glass plates with the gel were removed and placed on a large metal tray. The glass plates were separated using a plastic wedge (Hoefer Scientific Instruments, San Francisco, CA), leaving the gel on the larger plate. Fixing solution (10% glacial acetic acid, 10% methanol in distilled water) was sprayed on the gel. Then, the gel was carefully lifted onto a sheet of 3M Whatman paper and dried on a Drygel Sr. slab gel drier (Hoefer Scientific Instruments) for 1 h at 80°C. The dried gel was then exposed to Kodak X-ray film and the sequence was determined from the autoradiographic pattern.

Automated DNA sequencing. Some of the sequencing reported here was performed on a LiCor 4000L automated sequencer by the Dalhousie University - NRC Institute for Marine Biosciences Joint Laboratory. The reactions were carried out using standard primers.

DNA sequence analysis. Computer programs for arrangement and analysis of sequence were from the GCG software suite (Genetics Computer Group Inc., University of Wisconsin). Relatedness between predicted polypeptides was detected with the BLAST (Altschul *et al.*, 1990) and FASTA (Pearson, 1990) programs of the National Center for Biological Information (NCBI), Bethesda, MD. CLUSTAL W (Thompson *et al.*, 1994) was used for multiple alignments of putative amino acid sequences.

Transposon mutagenesis

The miniTn*5-lacZ*1 promoter-probe mini-transposon carried on the suicide vector pUT was used to mutagenize *P. mirabilis* strain S2R (de Lorenzo *et al.*, 1990). The recipient strain *P mirabilis* S2R and the donor strain, *E. coli* SM10 (*λpir*) containing the delivery vector with the mini-transposon, were grown overnight in LB medium at 37°C with aeration in the presence of required antibiotics. Samples of 100 μl of each culture were mixed in 5 ml of sterile 10 mM $MgSO_4$ and then filtered through a 1.3-mm-diameter Millipore type HA 0.45 μ filter. The filter was then placed on LB agar and incubated for over 8 h. The cells from the surface of the filter were then suspended in 5 ml of sterile 10 mM $MgSO_4$, and 0.1 ml of this suspension was spread on LB 2% agar without NaCl (non-swarming medium) supplemented with rifampicin to counterselect the donor strain and with kanamycin to select transconjugant recipient cells carrying the transposon marker. The plates were incubated at 37°C overnight (until transconjugant colonies became visible). Individual colonies of Km^r Rif^r transconjugant colonies were transferred using sterile wooden toothpicks into 96-well tissue-culture plates with covers (Linbro Flow Laboratories Inc., McLean, VA); each well contained 150 μl of freezing medium (per 1 liter of distilled water: 8 g Nutrient broth [BDH, Darmstadt, Germany], 10% v/v dimethyl sulfoxide). Then, the covered plates were incubated on a rocking platform at 37°C for several hours to allow the growth of bacteria. The plates were then stored at -70°C.

Construction of a *P. mirabilis* genomic library

Genomic libraries from *P. mirabilis* strain S2 and S2R-6/39 were constructed with large chromosomal DNA fragments (≥20 kb), generated by the partial digestion with *Sau*3AI restriction endonuclease, and ligated into cosmid vector pHC79 that had been digested with *Bam*HI restriction endonuclease and treated with CIP (Boehringer Mannheim) as described above. The ligation mixtures were packaged in recombinant lambda phage using *in vitro* Gigapack® II Plus packaging extracts (Stratagene Ltd., La Jolla, CA) as suggested be the manufacturer.

*Sau*3AI partial digestion of chromosomal DNA; ligation into pHC79. *P. mirabilis* chromosomal DNA was extracted as described above. The concentration of the chromosomal DNA preparation was determined using the following formula (Sambrook *et al.*, 1989):

DNA concentration (μg/ml) = OD_{260} X 50 X dilution factor.

The following solutions were added to a sterile microfuge tube kept on ice: 41 μl of 10 X *Sau*3A I buffer, 4.1 μl of 100 X (10 mg/ml) bovine serum albumin (BSA), chromosomal DNA preparation to a final concentration of 0.055 μg/μl, and the final volume was adjusted to 410 μl with sterile distilled water. Eight fresh microfuge tubes were labeled 1 through 8 and placed on ice. Then, 100 μl of the DNA-buffer mixture were transferred to tube 1, and 50 μl to tubes 2-7; tube 8 remained empty. Next, 5.5 U of *Sau*3AI were added to tube 1, and 2-fold serial dilutions of the restriction endonuclease in chromosomal DNA mixture were

prepared. Starting with tube 1, 50 μl of the DNA/endonuclease solution were removed and transferred to tube 2 and mixed well by pipetting. Then, 50 μl of the solution from tube 2 was transferred to tube 3 and the contents were mixed. This process was repeated with the remaining tubes, until 50 μl of the DNA solution was transferred from tube 7 to tube 8. The tubes were incubated at 37°C for 30 min, followed by a 10-min incubation at 65°C to inactivate the restriction endonuclease. Following incubation, 10 μl of the digest from each tube were loaded on a 0.6% agarose gel in 1 X TAE and electrophoresed with molecular weight standards to determine the size range of the resulting DNA fragments. The gels were photographed and the tubes containing DNA fragments of the desired range were stored at -70°C. The restriction digests were repeated several times (if necessary, incubations at 37°C were shortened to prevent excessive digestion of the DNA). The tubes were pooled together, and large DNA fragments were purified by phenol:chloroform extraction and ethanol precipitation at -70°C. DNA fragments (insert) were pelleted by centrifugation, washed with 70% ethanol and dried under vacuum. The dried pellet was resuspended in 20 μl of TE buffer and stored at -70°C.

To obtain an optimal insert-to-vector (pHC79) ratio, several ligation reactions, containing different concentrations of insert DNA, were set up in sterile 100-μl microfuge tubes. The tubes were incubated overnight at 14°C, and then ligation mixtures were stored on ice. Then, 4 μl of each ligation reaction were loaded on a 0.6% agarose gel in 1 X TAE and subjected to electrophoresis. The

gel was photographed, and tubes containing optimal ligation reactions were determined (by scoring for disappearance of the band corresponding to the linearized vector). Selected ligation reactions were stored at -70°C.

Preparation of host cells. Cells of *E. coli* strain LE392 from the frozen stock were spread on LB agar and incubated overnight at 37°C. A single colony was used to inoculate 50 ml of LB broth supplemented with 0.2% (v/v) maltose (made by dilution of 20% maltose stock solution) and 10 mM $MgSO_4$. This broth culture was incubated for 4-6 h at 37°C with aeration (cultures were not grown to greater than OD_{600} = 1, to minimize the number of non-viable cells). Bacteria were pelleted by a 10-min centrifugation at 2000 rpm. The pellet was gently resuspended in half of the original volume with sterile 10 mM $MgSO_4$. The OD_{600} of the bacterial suspension was determined, and the cells were diluted to OD_{600} = 0.5 with sterile 10 mM $MgSO_4$ just before use. If bacterial cells were not used immediately, they were stored undiluted at 4°C for no longer than 48 h.

Packaging, titration and plating of the library. A set of *in vitro* Gigapack® II Plus packaging extracts was removed from the -70°C storage and placed on ice. At the same time the tube containing the Sonic Extract (Gigapack® II Plus) was placed at room temperature to thaw. Freeze-thaw Extract (Gigapack® II Plus) was quickly thawed between the fingers until the extract just began to thaw, and 1-4 μl of the ligation mixture containing 0.1-5 μg of DNA were added immediately, and the tube was placed on ice. Quickly, 15 μl of Sonic Extract was added to the tube containing DNA. The contents were gently mixed by pipetting,

taking care to avoid air bubbles. The tube was centrifuged briefly to collect the contents at the bottom and incubated at room temperature (22°C) for 100 min to allow packaging. Then, the reaction was stopped by the addition of 500 μl of SM buffer (per 1 liter: 5.8 g NaCl, 2.0 g $MgSO_4 \cdot H_2O$, 50 ml of 1 M Tris.HCl [pH 7.5], 5 ml of 2% [w/v] gelatin). Chloroform (20 μl) was added to the tube and the contents were mixed gently. The tube was centrifuged in a microcentrifuge briefly to sediment the debris. The supernatant was removed and stored at 4°C.

To titer the packaged cosmid library, 1:10 and 1:50 dilutions of the final packaged reaction in SM buffer were made in a separate sterile microfuge tubes. Then, 25 μl of the final packaged reaction, of each dilution, were mixed with 25 μl of the OD_{600} = 0.5 *E. coli* LE392 host cells. The phage (packaged reaction) and bacteria were incubated for 30 min at room temperature to allow phage adsorption to the cells. Then, 200 μl of LB broth was added to each sample. To allow time for expression of the ampicillin resistance encoded by the cosmid vector, the tubes were incubated for 1 h at 37°C and gently shaken every 15 min. Then, the cells were pelleted by centrifugation, resuspended in 50 μl of fresh LB broth, and spread on pre-warmed LB-ampicillin plates. The plates were inverted and incubated at 37°C overnight. Once individual colonies became visible, the optimal dilution of the final packaged reaction was selected based on the number of colonies per plate. The remaining packaged reaction was incubated with host cells and plated on LB-ampicillin agar as described above to generate 1500-2000

colonies. Individual bacterial colonies were transferred into 96-well plates as described for transposon mutagenesis.

Construction of a ~3-kb *Eco*RI - *Pst*I *P. mirabilis* chromosomal DNA fragment library in pBluescript

The size range of desired DNA fragments was identified by screening *P. mirabilis* chromosomal DNA digested with various restriction endonucleases by Southern hybridization (as described above). Then, a sample of genomic DNA preparation and both pBluescript KS and SK DNA were digested with relevant restriction enzymes (*Eco*RI and *Pst*I), and the resulting fragments were separated by gel electrophoresis. Chromosomal DNA fragments of the desired size range of 2.3 - 3.6 kb (insert) and bands corresponding to linearized plasmid DNA (vector) were excised from the agarose gel. The insert and the vector were purified using a Geneclean II Kit (Bio 101) and ligated together using T4 ligase. The ligation mix was then transformed into competent *E. coli* JF626 cells as described above. Transformants were spread on LB-ampicillin agar and transferred to 96-well plates as described for the cosmid library construction. Between 500 and 600 colonies were collected per each insert/vector ligation reaction. Both pBluescript KS and SK were used as cloning vectors to facilitate subsequent sequencing analysis, and to allow the subcloning of DNA fragments that might be toxic to the host cells.

Molecular cloning of PCR products by the T-A overhang method

The cloning vector was digested with *Eco*RV in a 20-μl final reaction volume. Then, the volume of the digestion reaction was brought up to 200 μl with sterile water, and the plasmid DNA was purified by phenol/chloroform/ isoamyl alcohol extraction with ethanol precipitation as described above. After precipitation, plasmid DNA was resuspended in 10 μl of sterile water.

To carry out the T-addition reaction the following were added to a fresh microfuge tube: 10 μl (5 μg) blunt-ended vector DNA, 20 μl 10 X *Taq* DNA polymerase buffer (Pharmacia Biotech), 20 μl of 5 mM TTP (Gibco BRL), 1 μl (5 U) *Taq* DNA polymerase (Pharmacia Biotech), 49 μl of sterile H_2O. The tube was incubated for 2 h at 75°C. After incubation, plasmid DNA was purified by phenol:chloroform:isoamyl alcohol extraction with ethanol precipitation as described above.

PCR amplicons were separated by gel electrophoresis and eluted from agarose using a Geneclean kit. A-addition reactions were not required, since PCR utilized *Taq* polymerase, which added adenosine residues to the 3' end of each strand of the resulting amplicon. T4 ligation reactions with purified vector and PCR amplicons were set up and incubated overnight at 14°C. After incubation, ligation reactions were transformed into competent cells of *E. coli* JF626 strain as described above.

Cloning of the *ter* gene cluster

P. mirabilis strain S2R-6/39 chromosomal DNA fragments encoding the *ter* gene cluster were identified by Southern hybridization. Relevant DNA fragments were subcloned from the 2 cosmid constructs and by shotgun cloning of 3-kb *Eco*RI – *Pst*I fragments.

To subclone chromosomal DNA sequences flanking the site of the mini-transposon insertion, chromosomal DNA from *P. mirabilis* S2R-6/39 was partially digested with *Sau*3AI and resulting fragments were ligated into the *Bam*HI site of pBR322 and transformed into competent *E. coli* JF626 cells. Transformants were spread on LB agar supplemented with ampicillin and kanamycin (a Km^r cassette is encoded by the mini-transposon). Orientation and size of the sub-cloned sequences were determined by restriction mapping. The cloned fragments were sequenced, labeled with [α-^{32}P]dCTP by random priming and used as probes to screen the cosmid library to identify DNA fragments containing additional upstream and downstream regions. Cosmid DNA yielding strong hybridization signals was extracted (as described for the isolation of plasmid DNA) and digested with a panel of restriction endonucleases, and the resulting fragments were screened by the second round of Southern blot hybridization. The relevant fragments were identified by comparison of the restriction maps of the positive clone and the probe.

When no positive hybridization signal was detected by screening of the genomic library with a particular probe, *P. mirabilis* S2 chromosomal DNA was digested with various restriction endonucleases and hybridized with the same

probe. This hybridization provided information about the size range of the DNA fragments encoding the *ter* genes. Thus, chromosomal DNA was digested with the relevant restriction enzymes, the resulting fragments were separated by gel electrophoresis, and DNA fragments of the desired size range were excised and ligated into pBluescript vector. Following transformation, the presence of the relevant sequences in the recovered recombinant constructs was confirmed by Southern blot hybridization.

β-galactosidase assay

β-galactosidase assays were conducted as described by Miller (Miller, 1972). Swarmer-cells and short-cell populations were isolated as previously described (Hoffman, 1974). Briefly, Petri plates (9 cm diameter) containing LB agar were centrally inoculated with a drop of overnight broth culture of wild-type or mutant strains of *P. mirabilis*. After 3-4 hours of incubation at 37°C a single swarmer band was apparent. The central "mother" colony was cut out from the plate and swarm cells were harvested from the remaining agar section by washing the cells from the agar surface with 5 ml of PBS (per 1 liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 pH 7.4) and collecting the cell suspension into a 13-ml centrifuge tube. Cell suspensions were pelleted by centrifugation in a Varifuge 20 RS centrifuge (Heraeus Sepatech) for 5 min at 6,000 rpm, resuspended in PBS and used directly for the appropriate experiments. Swarming motility was monitored by phase-contrast microscopy and the purity of a swarmer-cell population was determined by counting the

mean percentage of elongated cells using a Gram-stained sample (Branson, 1972).

When bacteria were grown in broth cultures, 1 ml of log phase cells were pelleted by centrifugation for 5 min at 6,000 rpm. The pellet was resuspended in 1 ml of Z buffer (per liter, 16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.25 g $MgSO_4$, 2.7 ml 2-mercaptoethanol, pH adjusted to 7.0). In each assay, 100-200 μl of the cell suspension was used. The following solutions were added to sterile glass test tubes: 100-200 μl of the cell suspension, 1 ml of Z buffer, 2 drops of chloroform, 1 drop of 0.1% SDS. The contents were vortexed at the high setting for 10 sec and the tubes were placed in a 28°C water bath. To each tube, 0.2 ml of *o*-nitrophenyl β-D-galactoside (ONPG, 4 mg/ml; Sigma Chemical Co.) in 0.1 M phosphate buffer (per 1 liter of distilled water: 16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, pH adjusted to 7.0) were added. The tubes were mixed by vortexing for 5 sec and placed back in a 28°C water bath until the liquid in the tubes turned a yellow colour. At that point the reaction was stopped by addition of 1 ml of 1 M $NaCO_3$. The time interval between the addition of ONPG and the termination of the reaction was monitored using a laboratory timer. Each reaction was performed in triplicate. The absorbances at wave lengths of 420 nm and 550 nm were determined for each tube. The cell density immediately prior to the reaction was determined from the absorbance of the cell suspension at 600 nm. The specific activity of β-galactosidase was

determined, in units/mg of protein (nmol of *o*-nitrophenol/min/mg of protein), using the following equation:

$$\text{Units} = 1000 \times (OD_{420}-1.75 \times OD_{550})/t \times v \times OD_{600}$$

t = time of the reaction in minutes (time between addition of ONPG and 1 M Na_2CO_3).

v = volume of cell suspension used in the assay, in ml.

RESULTS

To begin a study of genes associated with swarmer cell development, I adapted a system of transposon mutagenesis to randomly generate insertion mutations. Differential β-galactosidase (reporter gene) activity under development-permissive conditions served as the screen to identify developmentally regulated genes among the bank of generated mutants. The approach resulted in the identification of a chromosomal gene cluster encoding resistance to tellurite ions (tested during this study), pore-forming colicins, and inhibition of phage attachment (implied by sequence similarity to the characterized *ter* gene clusters). This study appears to be the first analysis of a developmentally repressed gene cluster in *P. mirabilis*. My work describes the genetic characterization of this *ter* gene cluster and the molecular basis of inducibility of one of the phenotypes (tellurite resistance) encoded by this locus. Extensive amino acid sequence analysis was used to develop a possible model for action of the putative Ter proteins and to identify evolutionary implications for dissemination of plasmid-borne *ter* loci.

Transposon mutagenesis and characterization of the *P. mirabilis* mutants

Transposon mutagenesis with the mini-transposon Tn5*trp'-'lacZ* (see appendix), that lacks a promoter but contains a Shine-Dalgarno and ATG translation start codon, was used to introduce random mutations into the

chromosome of *P. mirabilis* (De Lorenzo *et al.*, 1990). The *lacZ* reporter gene of mini Tn*5trp'-'lacZ* permitted identification of transcriptionally active and developmentally regulated promoters by monitoring levels of ß-galactosidase during swarmer-cell differentiation.

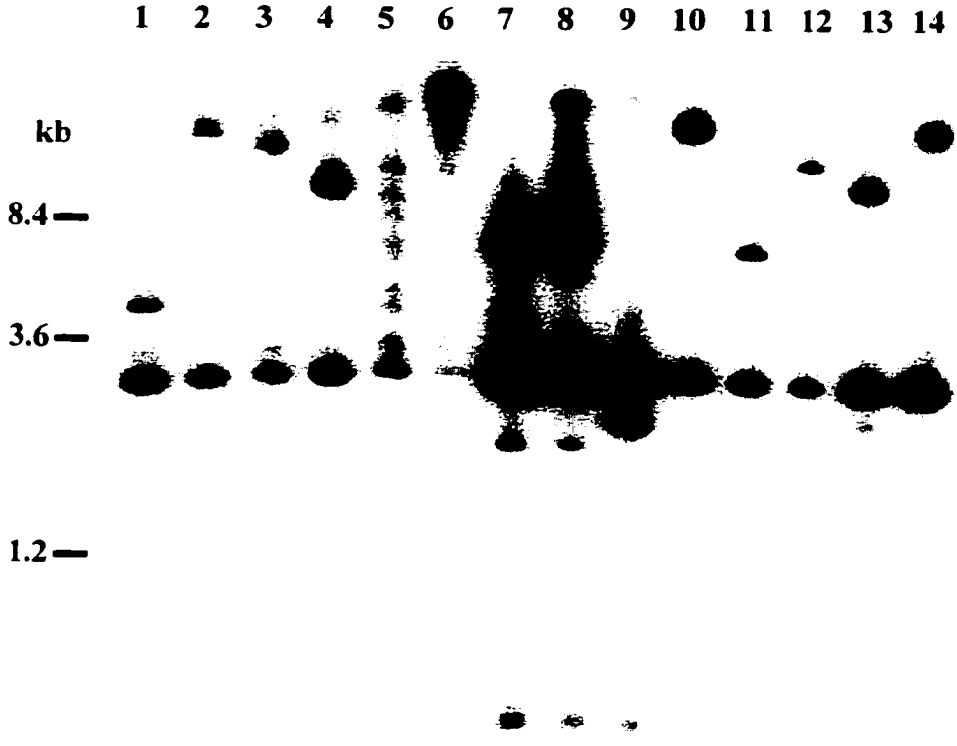
Preliminary mutagenesis attempts indicated that all of the *P. mirabilis* stock strains in our laboratory collection were poor recipients for the conjugative transfer of plasmids from the donor *E. coli* strain. Therefore, I obtained twenty clinical isolates of *P. mirabilis*. Following several attempted conjugations using spontaneous rifampicin-resistant variants of these clinical isolates, a single clinical isolate (*P. mirabilis* strain S2R) was identified as a suitable recipient. Filter matings between *E. coli* strain S17-1 *λpir* (pUT::miniTn5*trp'-'lacZ*) and *P. mirabilis* S2R were performed essentially as described (De Lorenzo and Timmis, 1992). The mini-transposon construct was delivered on a suicide vector, pUT (see appendix), that requires the λ *pir* gene for segregation of the plasmid DNA following replication (De Lorenzo *et al.*, 1990), and thus was not maintained in the recipient strains of *P. mirabilis*. Transconjugants were plated onto non-swarming medium supplemented with rifampicin to counter-select the *E. coli* donor cells, kanamycin to select for transposon-containing strains and X-gal to monitor the reporter gene activity. Both blue and white colonies (β-galactosidase-positive and -negative, respectively) of *P. mirabilis* were transferred to the swarming medium supplemented with X-gal to screen for non-swarming mutants, for abnormal swarming and for a differential expression of β-galactosidase (color change) associated with swarmer-cell development. All

kanamycin-resistant colonies were also screened for ampicillin resistance as an indicator of integration of the delivery plasmid into the chromosome of *P. mirabilis*. In total, 168 transconjugants were recovered and characterized. The recovered transconjugants can be divided in 5 groups based on swarming motility pattern and reporter-gene activity. The first group included the majority of transconjugants (68%) that exhibited normal swarming and constitutive expression of the reporter gene (*lacZ*) activity (blue or white throughout the swarmer cell developmental cycle). The second group included 5% of transconjugants that exhibited normal swarming plus differential expression of β-galactosidase encoded by the reporter gene. The third group included less than 1% of transconjugants that displayed aberrant pattern of swarming with differential expression of β-galactosidase. The fourth group included 17% of the recovered colonies that were constitutively blue or white and exhibited an aberrant pattern of swarming. The fifth group included 9% of transconjugants that were non-swarming and were constitutively blue or white. This distribution of transconjugant' phenotypes was in agreement with previous studies of Tn*5* mutagenesis in *P. mirabilis* (Belas *et al.*, 1991).

Figure 2 displays the results of a genomic Southern blot of mutant chromosomal DNA probed with a DNA fragment internal to the pUT::Tn*5trp'-'lacZ1* construct. The hybridization signals noted at 3 kbp and at 0.6 kbp were derived from a two internal *Pvu*II recognition sites within the pUT delivery vector (see appendix). These sites were not indicated on the physical map of this construct provided by De Lorenzo and Timmis (1992). The third major signal

Figure 2. Southern hybridization of mutant chromosomal DNA with reporter-gene (*lacZ*) sequences

Lanes 1-14 contain *Pvu*II digested chromosomal DNA isolated from 14 randomly selected *P. mirabilis* transconjugant strains. A 6-kbp *Bam*HI fragment containing the *lacZ*-Km and R6K regions of pUT::Tn5*trp*'-'*lacZ* was used as the hybridization probe. Lambda DNA *Bst*EII digest molecular-weight standards were used during electrophoresis and corresponding molecular weights are shown on the left.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
kb
8.4
3.6
1.2

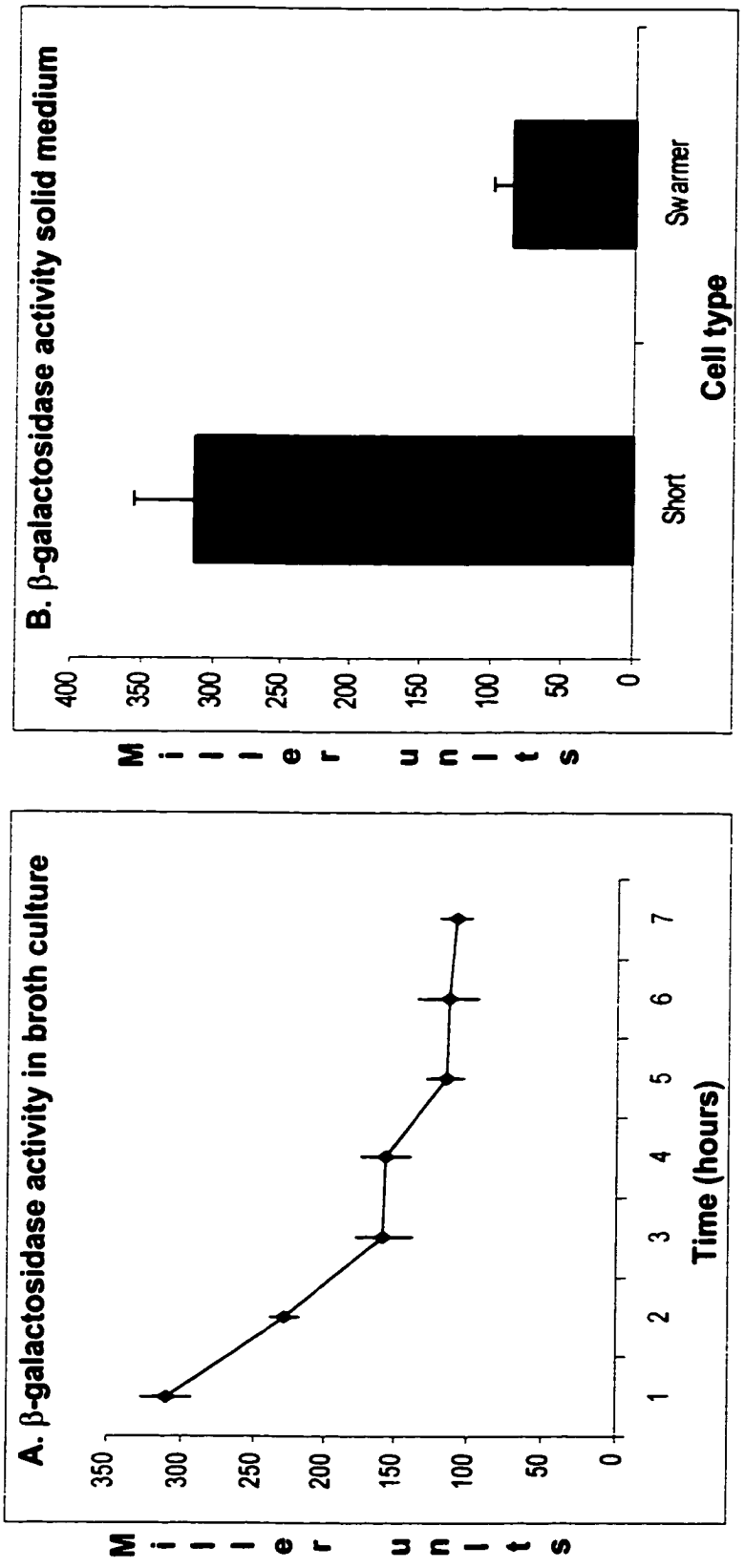
demonstrates the randomness of the site of transposition insertion in selected mutants. Of note, additional weak hybridization bands present in some of the lanes (*e. g.*, lane 5-8) are likely to be due to incomplete digestion of the chromosomal DNA samples used for hybridization screening.

Characterization of *P. mirabilis* S2R-6/39

One of the transconjugants (*P. mirabilis* S2R-6/39) drew attention by its differential pattern of β-galactosidase expression (Figure 3B): short cells of this mutant produced on average 312 Miller units of enzyme, while long swarmer cells produced 85 Miller units. The difference in β-galactosidase levels between swarmer cells and short cells was evaluated by the t-test to be statistically significant (p value = 0.001). The cells in broth culture also showed a gradual decline of β-galactosidase levels as a function of increase in population density (Figure 3A). These results indicated that the promoter driving the expression of the gene(s) at the site of the mini-transposon integration is regulated, with maximum levels of expression in short cells followed by a four-fold repression in swarmer cells. Thus, the expression of the reporter-gene activity in the mutant strain showed both growth phase dependent regulation in broth culture, as well as developmental regulation on solid medium. The mutant strain was not impaired in its ability to undergo swarmer cell differentiation. This mutant strain was chosen for further characterization.

Figure 3. Control of the *lacZ* reporter-gene expression in *P. mirabilis* S2R-6/39 by developmental cycle and cell density

β-galactosidase activity (Miller units) of the *P. mirabilis* mutant strain grown on either solid medium (B) or in broth culture (A).


A. β-galactosidase activity in broth culture
Miller units
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
Time (hours)
B. β-galactosidase activity solid medium
Miller units
400
350
300
250
200
150
100
50
0
Short
Swarmer
Cell type

Cloning and sequence analysis of the DNA fragments flanking the site of mini-transposon insertion

To characterize the site of mini-transposon insertion within the chromosome of *P. mirabilis* S2R-6/39 (mutant), attempts were made to clone mini-Tn*5* and flanking chromosomal DNA sequences into pBR322, pBluescript and pHC79 vectors using a "shotgun" method. Only ligation mixtures with pBR322 cloning vectors yielded Km^r transformants. A single recombinant plasmid was recovered and named pAT9501. The physical map of the plasmid was constructed, and is depicted in Figure 4. Restriction analysis indicated that this construct contains a 5.9-kbp *Sau*3A insert comprising a transposon-derived truncated *lacZ* coding region, followed by the kanamycin-resistance cassette, and approximately 2.8 kbp of *P. mirabilis* chromosomal DNA. Unfortunately, this construct did not contain sequences 5' to the *lacZ* coding region as well as upstream promoter and regulatory sequences. However, sequence analysis of the 3' chromosomal DNA fragment could identify the locus disrupted by the mini-transposon insertion, and the fragment subsequently served as a hybridization probe for the identification of the upstream sequences.

Southern blot hybridization analysis confirmed that a 2.5-kbp *Cla*I - *Not*I fragment was indeed derived from the genome of *P. mirabilis* (Figure 5). When mini-transposon sequences from pAT9501 were used as a hybridization probe to screen chromosomal DNA from the wild-type (WT) and mutant strains of *P. mirabilis*, the hybridization signals were only detectable in the lane with the mutant DNA (Fig 5A, lanes 2 and 3). However, both mutant and WT strains

Figure 4. Physical map of the pAT9501

pBR322-derived sequences are indicated by the open line including *amp* (ampicillin-resistance determinant, indicated by the chequered arrow). The 5.93-kbp chromosomal DNA *Sau*3A fragment of the mutant was cloned in the *Bam*HI site of pBR322. MiniTn*5*-derived sequences: *trp'-'lacZ*, truncated *lacZ* coding region (solid line); Km, kanamycin-resistance cassette (chequered line). *P. mirabilis* chromosomal DNA is shown as a thin line, with open arrows indicating predicted direction of transcription and location of the *terC* and *terD* partial open reading frames. The numbers indicate the positions of relevant restriction endonuclease recognition sites mapped by restriction digestion. The underlined *Bam*HI recognition sites were disrupted when a *Sau*3A DNA fragment containing the mini-transposon-derived and chromosomal sequences from the mutant *P. mirabilis* strain was subcloned.

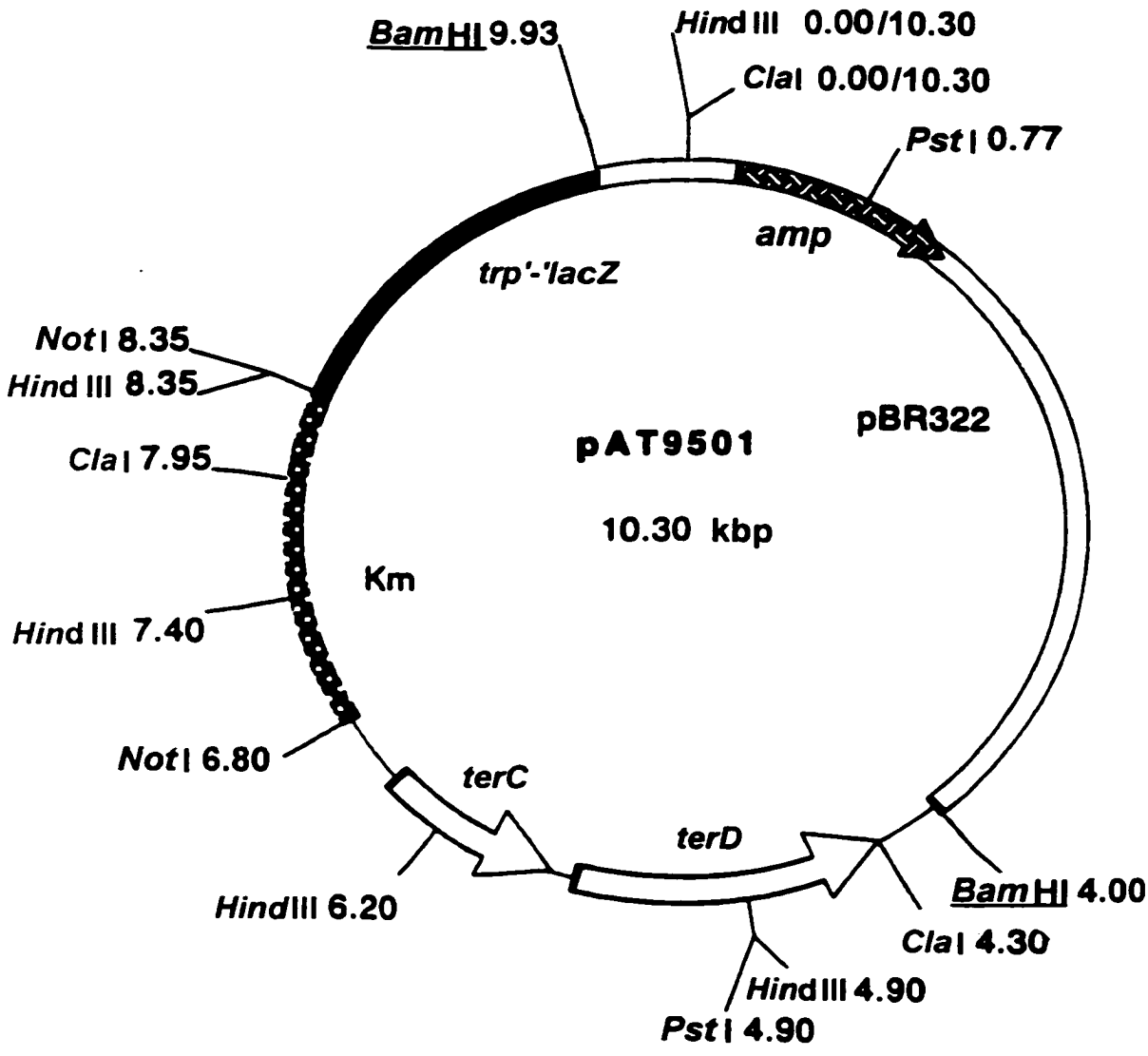
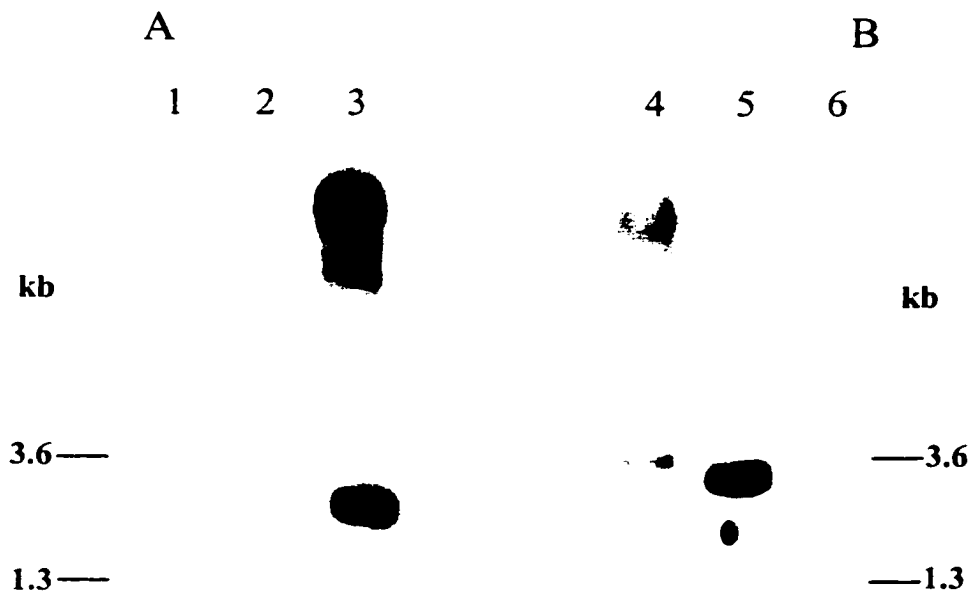

BamHI 9.93
HindIII 0.00/10.30
ClaI 0.00/10.30
PstI 0.77
amp
trp'-'lacZ
NotI 8.35
HindIII 8.35
ClaI 7.95
pBR322
pAT9501
10.30 kbp
Km
HindIII 7.40
NotI 6.80
terC
terD
HindIII 6.20
BamHI 4.00
ClaI 4.30
HindIII 4.90
PstI 4.90

Figure 5. Southern blot analysis of DNA fragments derived from pAT9501

Lanes 1 and 6, molecular weight ladder; lanes 2 and 5, *P. mirabilis* S2R chromosomal DNA digested with *Cla*I; lanes 3 and 4, *P. mirabilis* S2R-6/39 chromosomal DNA digested with *Cla*I. Molecular weight standards are shown on the left of panel A and on the right of panel B. (**A**) A 1.95-kbp *Cla*I - *Not*I fragment (see Figure4: from *Not*I site at 8.58 kbp position to *Cla*I site at 10.30 kbp position) containing minitransposon sequences was used as a hybridization probe; (**B**) A 2.5-kbp *Cla*I - *Not*I fragment (see Figure 4: from *Cla*I site at 4.30 kbp position to *Not*I site at 6.80 kbp position) containing *P. mirabilis* S2R-6/39 chromosomal DNA was used as a hybridization probe.


A
B
1
2
3
4
5
6
kb
kb
3.6
3.6
1.3
1.3

gave strong hybridization signals when screened with sequences flanking the Km^r cassette (Fig. 5B, lanes 5 and 6).

The nucleotide sequence of the subcloned chromosomal DNA fragment was determined to identify the putative open reading frame disrupted by the mini-transposon insertion. For this, the 2.5-kbp *Not*I - *Cla*I fragment from pAT9501 was subcloned into pBluescript KS, and a partial nucleotide sequence of the fragment was determined. Two partial putative open reading frames were identified and the genes were named *terC* and *terD* (Figure 4), based on high degrees of similarity of these ORFs with the previously described *ter* genes carried by the large conjugative plasmids pMER610 and R478 in Gram-negative bacteria (Whelan *et al.*, 1997; Jobling and Ritchie, 1988).

Comparative levels of potassium tellurite resistance in the WT and mutant strains of *P. mirabilis*

Well-characterized plasmid-borne *ter* loci contain 7 genes that mediate three phenotypes: phage inhibition, resistance to pore-forming colicins, and resistance to potassium tellurite (Whelan *et al.*, 1995). Restriction analysis of pAT9501 showed that mini-Tn5 transposon integrated into the *terC* coding region. Therefore, levels of tellurite resistance of the WT and the mutant strains of *P. mirabilis* were compared to assess the effect of *terC* disruption on this phenotype. Portions (100 μl) of broth-grown bacterial cells were spread on non-swarming agar supplemented with various concentrations of potassium tellurite (50 - 300 μg/ml), followed by incubation at 37°C. Both WT and the mutant

strains were able to grow at equal rates in the presence of low concentrations of potassium tellurite (<150 μg/ml). However, when the growth medium contained potassium tellurite at greater than 150 μg/ml, the mutant strain formed much smaller colonies (Figure 6). These results indicated that the mutant strain has reduced levels of potassium tellurite resistance (MIC 150 μg/ml), likely due to the transposon insertion in the *terC* coding region. The high levels of potassium tellurite resistance associated with the WT strain of *P. mirabilis* are comparable to the levels of potassium tellurite resistance previously reported for plasmid-borne *ter* loci (Whelan *et al.*, 1995).

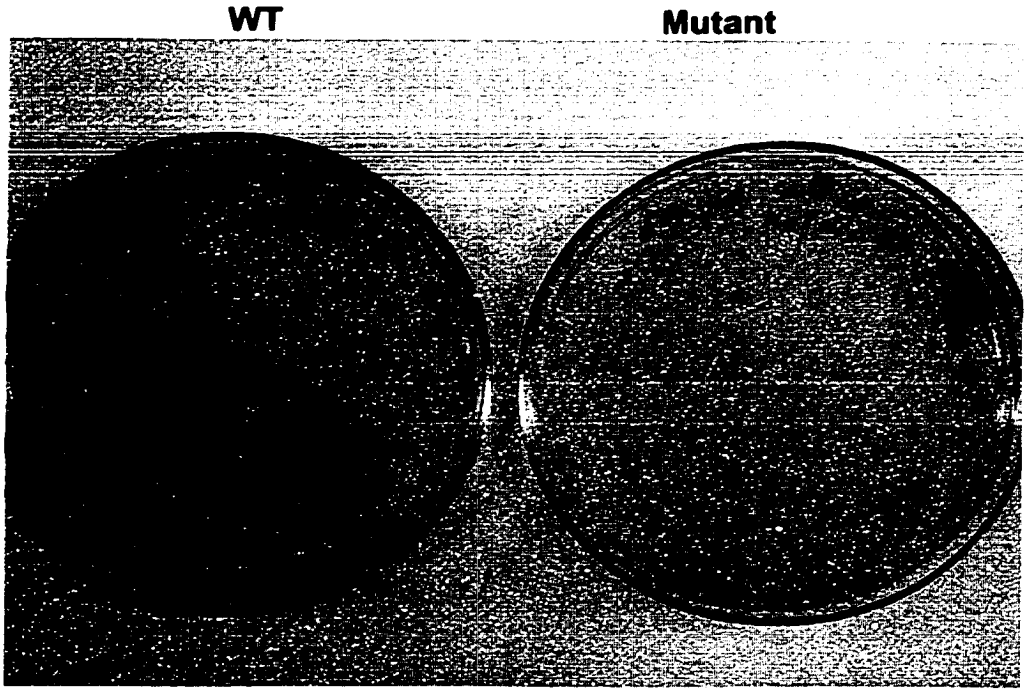
Next, β-galactosidase assays were conducted to assess the effect of potassium tellurite on the reporter-gene activity. However, only background levels of β-galactosidase production (≈ 6 Miller units) were detected in mutant cells grown in the presence of potassium tellurite in either broth culture or on solid medium. This result suggested that either the chromosomal locus disrupted by mini-transposon insertion was not transcriptionally active in the presence of potassium tellurite, or this chemical had a detrimental effect on the synthesis and/or stability of β-galactosidase. Alternatively, decreased levels of potassium tellurite resistance could reflect a polar effect of the mini-transposon on the expression of downstream genes.

Complementation analysis

Among the three phenotypes associated with the gene clusters in Gram-negative bacteria, tellurite resistance was the easiest to distinguish, by scoring

Figure 6. Comparative tellurite resistance levels in the WT and mutant *P. mirabilis*

Levels of tellurite resistance were determined by plating both WT (S2R) and mutant (S2R-6/39) strains on solid medium supplemented with potassium tellurite. Bacterial cell suspensions (100 μl) in PBS were plated on non-swarming medium containing potassium tellurite at 150 μg/ml. Cell suspensions were diluted in PBS to equal optical density immediately before plating. Plates were incubated for 36 h at 37°C.


WT
Mutant

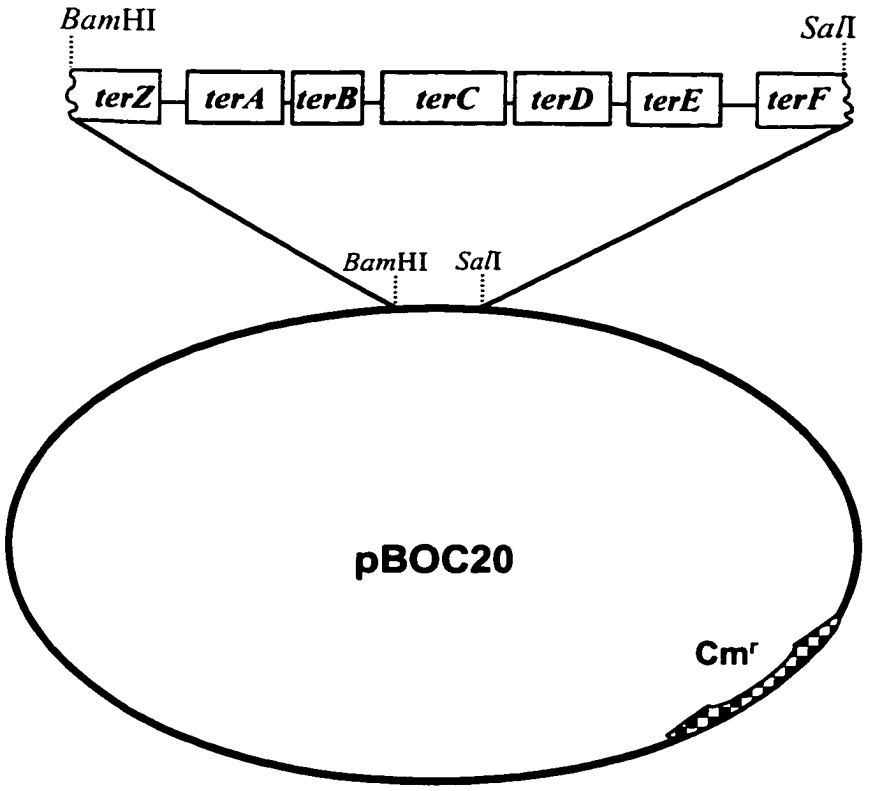
jet-black colonies on solid medium supplemented with potassium tellurite. Based on the transposon mutagenesis studies, Whelan *et al.* (1995) concluded that the *terC* gene from the large-conjugative plasmid R478 is required for the tellurite-resistance phenotype. Therefore, this phenotype was used to determine whether the putative ORF disrupted by the mini-transposon in the mutant strain was functionally equivalent to the previously described plasmid-borne *terC* gene.

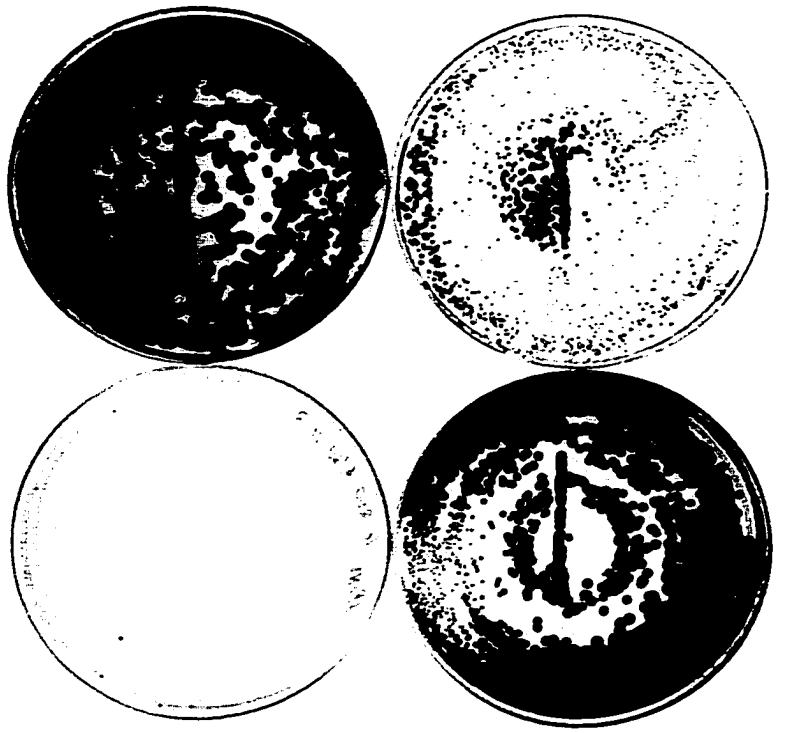
To restore wild-type levels of tellurite resistance in the mutant strain, R478-derived *ter* genes were subcloned from plasmid pKFW4a (Whelan *et al.*, 1995) into pBOC20 (obtained from Dr. Cianciotto), as the 5.8-kbp *Bam*HI -*Sal*I fragment containing an incomplete R478-derived *ter* locus. Figure 7A diagrams the resulting construct, named pAT9804. This construct contains the complete *terA-terE* genes plus partial coding regions for the *terZ* and the *terF* genes from R478 (Whelan *et al.*, 1995). *E. coli* strain JF626 normally was highly susceptible to potassium tellurite and was unable to grow in the presence of this chemical at the concentrations tested in this study (50-250 μg/ml). However, this study established that transformation with pAT9804 enabled host *E. coli* strain JF626 to grow in the presence of potassium tellurite, indicating that a Te^r phenotype is encoded within the 5.8-kbp *Bam*HI - *Sal*I region of R478. The Te^r phenotype was inducible in the *E. coli* JF626 genetic background by prior growth on medium supplemented with low concentrations of potassium tellurite. Furthermore, when pAT9804 was introduced into the mutant *P. mirabilis* strain by conjugation, this construct restored WT levels of tellurite resistance in this strain (Figure 7, panels B, D and E). The presence of

Figure 7. Complementation analysis of *P. mirabilis* S2R-6/39

(**A**). Construction of the pAT9804. A partial R478 *ter* locus was subcloned on a 5.8-kbp *Bam*HI - *Sal*I fragment from pKFW4a into corresponding restriction sites of pBOC20. Open boxes indicate complete coding regions; wavy lines indicate partial coding regions, truncated by digestion with restriction endonucleases during subcloning; chequered box indicates pBOC20-derived chloramphenicol-resistance determinant. (**B-E**) The resulting construct (pAT9804) was introduced by conjugation into the WT and mutant strains of *P. mirabilis*. Levels of tellurite resistance in the transconjugant and parental strains were determined by plating bacteria on solid non-swarming medium supplemented with potassium tellurite at 200 μg/ml. Cell suspensions in PBS were diluted to equal optical density immediately prior to plating. Plates were incubated for 4 days at 37°C.

A.


BamHI
SalI
terZ
terA
terB
terC
terD
terE
terF
BamHI
SalI
pBOC20
Cmr


B. WT
C. WT + pAT9804
D. Mutant
E. Mutant + pAT9804

pAT9804-derived sequences within transconjugant strains was confirmed by selection for chloramphenicol resistance (encoded by the pBOC20 vector) and by Southern blot hybridization with a 3.0-kbp fragment containing the Cm^r determinant. These results suggested that the putative *terC* gene, disrupted by transposon mutagenesis in this study, is required for the tellurite resistance phenotype, similar to the plasmid-borne *terC* genes.

Interestingly, overexpression of the plasmid-borne Te^r proteins from the high-copy plasmid pAT9804 in the WT *P. mirabilis* strain had an inhibitory effect on the growth in the presence of tellurite (Figure 7, panels B and C). This effect was likely one of gene dosage. Alternatively, these proteins may require a tight stoichiometric ratio for proper function. Selective overexpression of some of the Ter proteins would disrupt such ratio and result in decreased resistance to potassium tellurite. In summary, complementation restored the tellurite-resistance phenotype in the *terC* mutant strain. These complementation results, and the high degree of sequence similarity between putative chromosomal and plasmid-borne *ter* genes (see below), strongly suggested the presence within the *P. mirabilis* genome of additional *ter* genes, homologous to the plasmid-borne *terZ*, *terA*, and *terB* genes.

Screening of *P. mirabilis* and *P. vulgaris* strains for resistance to potassium tellurite

The genetic basis of potassium tellurite resistance in *Proteus* spp. has not been previously investigated, with the exception of an early report by Fleming

(1932). To investigate how widespread tellurite resistance is among *Proteeae*, I screened 30 clinical isolates of *P. mirabilis* and four strains of *P. vulgaris* for ability to grow in the presence of potassium tellurite (50-250 μg/ml), by spreading bacterial cells on solid medium supplemented with potassium tellurite. Following 4-day incubation period, all of the strains were able to form jet-black colonies on solid medium at the concentrations tested, reinforcing the conclusion that resistance to potassium tellurite is an integral feature of this genus.

Construction and screening of *P. mirabilis* genomic DNA libraries in the cosmid vector pHC79

Based on the above results, the *P. mirabilis* chromosomal DNA fragment from pAT9501 was used as a hybridization probe to screen *P. mirabilis* S2R genomic DNA, and to identify and clone regulatory sequences upstream of the site of the mini-transposon insertion. To facilitate cloning and sequencing analysis of the chromosomal DNA at the site of mini-transposon insertion, cosmid libraries of genomic DNA from both the WT and mutant *P. mirabilis* strains were constructed in cosmid vector pHC79.

First, the genomic library of the mutant DNA was screened for mini-transposon sequences by scoring for kanamycin resistance. Of the 1400 transformants recovered by selection for vector-encoded ampicillin resistance, none were resistant to kanamycin. This result was likely due to the toxic effect

specified by the plasmid-borne *ter* genes on the host *E. coli* cells, as previously reported (Whelan *et al.*, 1997).

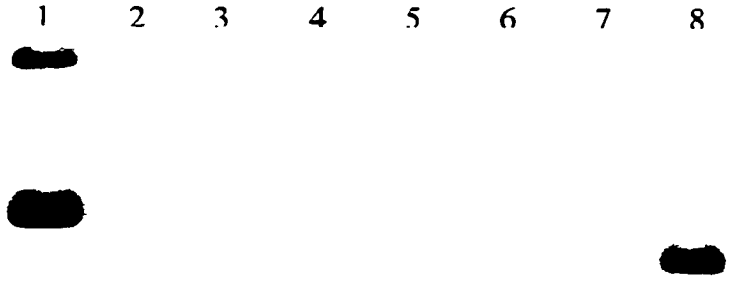
Using a subcloned *P. mirabilis* chromosomal DNA fragment from pAT9501 as a hybridization probe, the cosmid bank of wild-type chromosomal DNA was screened and a single colony, containing the cosmid construct PM2-P1-C8, was identified. Cosmid DNA from the positive clone and from four randomly chosen negative colonies was digested with *Sal*I and *Cla*I restriction endonucleases to separate cosmid vector (backbone) from cloned genomic fragments (insert), and the resulting DNA fragments were screened by a second Southern blot hybridization using the same probe (Figure 8). The probe hybridized specifically to *P. mirabilis* genomic sequences and confirmed the presence of the desired DNA sequences in cosmid PM2-P1-C8, but not in cosmids used as negative controls (Figure 8A, lanes 1-6). No hybridization signals were detectable in lanes containing the pUT suicide delivery vector or the pHC79 cosmid vector used in this study (Figure 8A, lanes 7 and 8).

To identify a fragment of appropriate size for sequence analysis, cosmid DNA from PM2-P1-C8 was digested with several restriction endonucleases and the fragments were screened by additional rounds of Southern hybridization (Figure 8B). Based on the restriction map of pAT9501 (Figure 4), a 2.8-kbp *Sal*I-*Pst*I fragment (Figure 8B, lane 12, indicated by the arrowhead) appeared to contain sequences upstream of the site of mini-transposon insertion. This

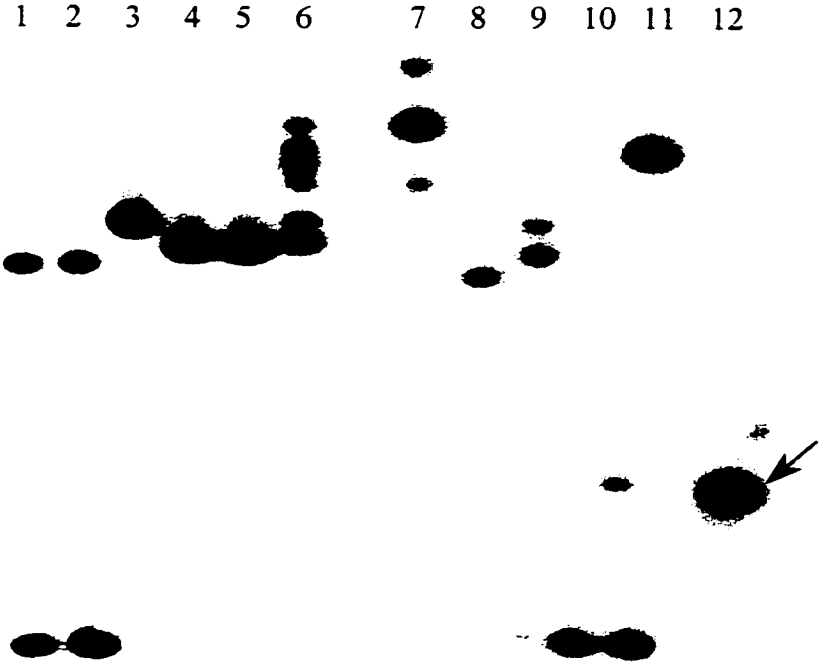
Figure 8. Southern blot analysis of *P. mirabilis* S2R genomic DNA library cosmids with a chromosomal DNA fragment flanking the site of mini-transposon insertion in *P. mirabilis* S2R-6/39

A 1.8-kbp *Not*I - *Pst*I DNA fragment from pAT9501 (flanking 3' of the Km^r cassette) was used as a hybridization probe. (**A**) Cosmid DNA from PM2-P1-C8 (lane 1), PM2-P6-H3 (lane 2), PM2-P10-B7 (lane 3), PM2-P10-C7 (lane 4) and PM2-P11-B1 (lane 5) digested with *Sal*I and *Cla*I; *Bam*HI digest of pHC79 (lane 6); pUT digested with *Bam*HI and *Eco*RI (lane 7); pAT9501 digested with *Not*I and *Cla*I (lane 8). (**B**). PM2-P1-C8 cosmid DNA digested with *Hin*dIII (lane 1), *Hin*dIII and *Cla*I (lane 2), *Cla*I (lane 3), *Cla*I and *Pst*I (lane 4), *Pst*I (lane 5), *Pst*I and *Bam*HI (lane 6), *Bam*HI (lane 7), *Pst*I and *Hin*dIII (lane 8), *Hin*dIII and *Bam*HI (lane 9), *Sal*I and *Hin*dIII (lane 10), *Sal*I (lane 11), *Sal*I and *Pst*I (lane 12). The arrowhead indicates the cosmid DNA fragment chosen for further characterization.

A.



B.



fragment was subcloned into pBluescript KS for further analysis and named pBKS-SP.

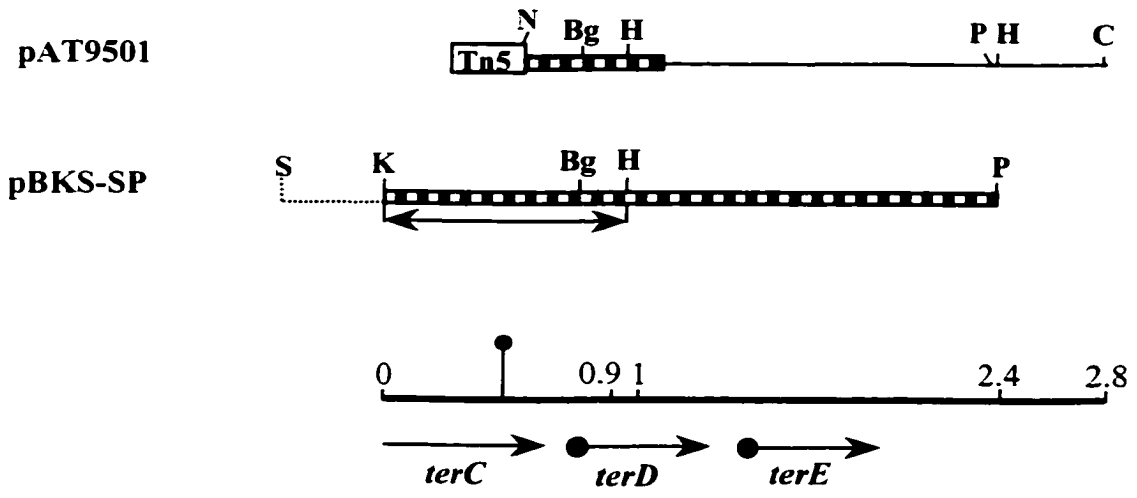
Restriction analysis confirmed that pBKS-SP and pAT9501 contained overlapping fragments of *P. mirabilis* chromosomal DNA (Figure 9). Sequencing of the *Sal*I-*Pst*I fragment revealed an additional open reading frame named *terE* located immediately downstream of the *terD* open reading frame. Sequence analysis of DNA immediately downstream of the Km^r cassette in pAT9501 placed the transposon insertion within the 3' region of the *terC*. A BLAST search (Altschul *et al.*, 1990) of the DNA sequences subcloned into pBKS-SP as query sequences showed that this plasmid contained a 2.4-kbp *Kpn*I-*Pst*I fragment of *P. mirabilis* chromosomal DNA, while the remaining 0.4-kbp *Sal*I- *Kpn*I fragment was derived from cosmid vector pHC79. These results indicated that cosmid construct PM2-P1-C8 did not contain sequences 5' of the *terC* coding region.

Shotgun cloning of the partial *terB* and 5' of the *terC* coding regions

Cosmid libraries of WT and mutant genomic DNAs were screened with the 1.07-kbp *Kpn*I - *Hin*dIII fragment (Figure 9, double-headed arrow) to identify cosmid constructs containing overlapping and additional upstream DNA sequences, as compared to the previously characterized *ter* coding sequences from cosmid PM2-P1-C8. However, no additional positive colonies were identified. This result, together with the previously published observations of the toxic effect of the *ter* genes on *E. coli* host cells (Whelan *et al.*, 1997), suggested that large chromosomal DNA fragments encoding an entire *P. mirabilis ter* locus

Figure 9. Physical map of the *P. mirabilis* S2R and S2R-6/39 chromosomal DNA regions at the site of the mini-transposon insertion

The top two lines show restriction maps of overlapping DNA fragments subcloned from pAT9501 and pBKS-SP. The open box indicates mini-Tn5 derived sequences, dotted lines indicate sequences derived from the cosmid vector pHC79, and chequered boxes represent completely sequenced fragments. The thin line in the pAT9501 map represents DNA fragment that was not sequenced. The double-headed arrow indicates the 1.07-kbp *Kpn*I-*Hin*dIII fragment used as a hybridization probe in the subsequent experiments. The third line shows the physical organization of 3 putative ORFs: *terC*, *-D* and *-E*. Complete coding regions are represented by arrows with solid circles at the 5' end, whereas the incomplete ORF (*terC*) is represented by the arrow. Arrows point in the predicted direction of transcription. The insertion site of the mini-Tn*5-lacZ* transposon within the *terC* coding region is indicated by the "lollipop". The third line also shows the positions of restriction sites and sizes of the DNA fragments kbp. Restriction endonuclease recognition sites: Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; P, *Pst*I; S, *Sal*I. The double-headed arrow indicates the 1.07-kbp *Kpn*I-*Hin*dIII fragment used as a hybridization probe in the subsequent experiments.

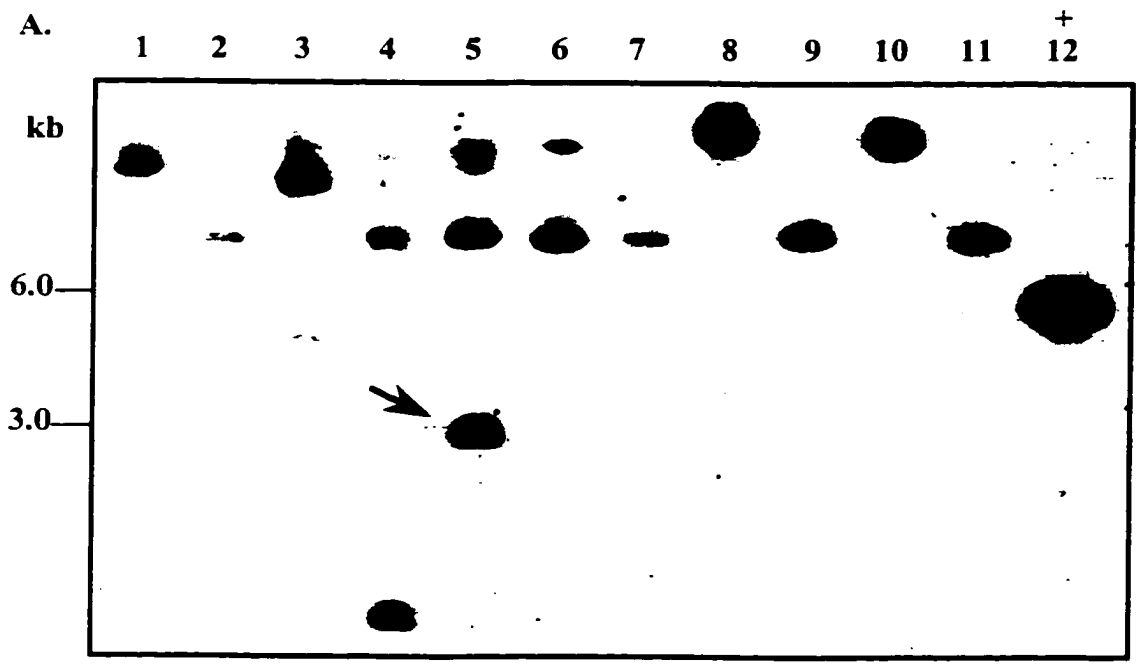

pAT9501
N
Bg
H
P
H
C
Tn5
pBKS-SP
S
K
Bg
H
P
0
0.9
1
2.4
2.8
terC
terD
terE

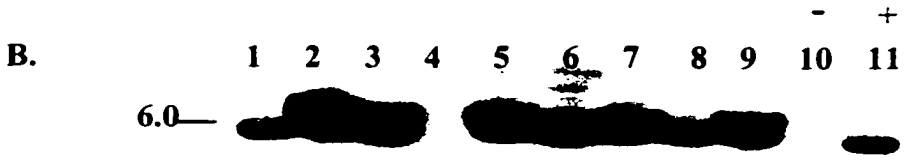
would be toxic to the host *E. coli* strain LE392 cells. As a consequence, the *ter*-positive cosmid constructs would be under-represented in the genomic libraries. Therefore, a shotgun-cloning approach was used to isolate smaller (1-4 kbp) fragments containing the overlapping sequences from the chromosomal DNA of *P. mirabilis* S2R. Portions of *P. mirabilis* S2R chromosomal DNA were digested with 22 endonucleases (single- or double-digestion restriction reactions), and the resulting DNA fragments were separated by gel electrophoresis and hybridized with the 1.07-kbp *Kpn*I - *Hin*dIII probe. Based on the restriction map of pBKS-SP, a 3.0-kbp *Eco*RI - *Pst*I fragment (Figure 10A) was estimated to contain over 0.5 kbp of the upstream sequences, and to contain the 5' part of the *terC* ORF. Since no other overlapping DNA fragments of manageable size were identified, this fragment was chosen for further characterization.

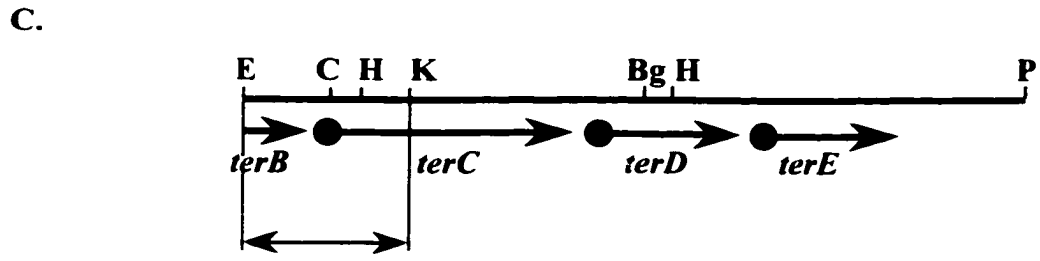
A library of *Eco*RI - *Pst*I chromosomal DNA fragments in the 3-kbp size range was generated using pBluescript KS and SK cloning vectors. The resulting 1200 bacterial colonies were screened by Southern blot hybridization with the 1.07-kbp *Kpn*I - *Hin*dIII DNA fragment as the probe. Plasmid DNA was isolated from the colonies displaying strong hybridization signals, and the presence of the desired inserts was confirmed by Southern hybridization with restricted plasmid sequences using the same 1.07-kbp probe. The sizes of the inserts were estimated by gel electrophoresis of the recombinant constructs linearized by restriction digestion (Figure 10B). One of the recombinant plasmids was termed pBSK-EP and used for further analysis.

Figure 10. Southern blot analysis of *P. mirabilis* S2R chromosomal DNA to identify the *terB* coding region

The 1.07-kbp *Kpn*I-*Hin*dIII fragment from pBKS-SP was used as a hybridization probe. The minus sign indicates a negative control pBluescript SK, and the plus sign indicates the positive control, pBKS-SP. Molecular weight sizes are shown on the left of each panel. (**A**) *P. mirabilis* S2R chromosomal DNA digested with *Bam*HI (lane 1), *Eco*RI and *Bam*HI (lane 2), *Bgl*II (lane 3), *Eco*RI and *Bgl*II (lane 4), *Eco*RI and *Pst*I (lane 5), *Eco*RI and *Pvu*II (lane 6), *Eco*RI and *Sal*I (lane 7), *Xba*I (lane 8), *Eco*RI and *Xba*I (lane 9), *Xho*I (lane 10), *Eco*RI and *Xho*I (lane 11). The arrowhead indicates the 3.0-kbp *Eco*RI – *Pst*I fragment subcloned into pBluescript SK for further characterization. (**B**) Lanes 1-9 (except lane 4) contained recombinant pBluescript constructs with 3.0-kb *Eco*RI - *Pst*I inserts (digested with *Pst*I). (**C**) Physical organization of putative ORFs encoded within the 3.0-kbp *Eco*RI - *Pst*I fragment. The double-headed arrow indicates the 0.64-kbp *Eco*RI - *Kpn*I fragment used as a hybridization probe in subsequent experiments. Restriction enzyme symbols are the same as in Figures 8 and 9.


A.
1 2 3 4 5 6 7 8 9 10 11 +12
kb
6.0
3.0


B.
- +
1 2 3 4 5 6 7 8 9 10 11
6.0


C.
E C H K Bg H P
terB
terC
terD
terE

1 kb

The restriction map of pBSK-EP confirmed the presence of an additional 0.64-kbp of upstream sequence. Sequence analysis of the insert revealed a partial *terB* coding region, followed by the complete *terC*, *-D* and *-E* genes (Figure 10C).

Cloning and sequence analysis of the *terZ* and *terA* coding regions from the cosmid library of the *P. mirabilis* WT genomic DNA

Initially, an attempt was made to identify and clone chromosomal DNA fragments encoding the remaining genes from the *ter* locus by repeated the shotgun cloning. The nylon membranes containing *P. mirabilis* S2R chromosomal DNA fragments (described in the previous section) were stripped to remove the radioactive probe and then hybridized with the 0.64-kbp *Eco*RI - *Kpn*I DNA fragment from pBSK-EP (Figure 10C, double-headed arrow). A strong hybridization signal corresponding to a 4.8-kbp *Hin*dIII fragment was detected using this probe (Figure 11), but not when the same nylon membrane was re-probed with the 1.07-kbp *Kpn*I - *Hin*dIII probe containing sequences downstream of the mini-transposon. Based on the restriction map of the pBSK-EP construct, the 4.8-kbp *Hin*dIII fragment was estimated to contain 4.3 kbp of upstream DNA sequences in addition to a 0.47-kbp *Eco*RI - *Hin*dIII fragment overlapping the insert from PBSK-EP. The fragment was sufficiently large to include the remaining members of the *P. mirabilis ter* gene cluster and to contain putative regulatory sequences located farther upstream.

Next, a plasmid library of *P. mirabilis* S2R *Hin*dIII chromosomal DNA fragments was constructed using pBluescript SK and KS vectors. Recombinant plasmid constructs were transformed into *E. coli* JF626 cells and six transformants were randomly chosen, following which recombinant plasmid DNA was isolated and shown by gel electrophoresis to contain inserts of the desired sizes. Overall, 1300 transformants were recovered and screened by hybridization with the 0.64-kbp *Eco*RI - *Kpn*I fragment containing partial *terB-terC* coding regions (Figure 10C, double-headed arrow). No positive colonies were obtained using this approach. As in the previous experiments, this result was likely due to toxicity specified by the fragment that was selected for subcloning.

When the same probe was used to screen cosmid libraries of the *P. mirabilis* WT and the mutant genomic DNA, two cosmids, PM2-P8-B4 and PM2-P12-E2, gave strong hybridization signals (Figure 12A). The PM2-P8-B4 cosmid construct was chosen for further analysis (the second cosmid, PM2-P12-E2, failed to grow when an attempt was made to subculture the frozen stock culture on LB agar). To identify a fragment containing the upstream sequences, PM2-P8-B4 cosmid DNA was isolated and digested with several restriction endonucleases. The resulting fragments were separated by electrophoresis and screened by Southern blot hybridization with the radiolabelled 0.64-kbp *Eco*RI-*Kpn*I fragment (Figure 10C, double-headed arrow). Based on the restriction map of pBSK-EP, two of the fragments that gave a strong hybridization signal appeared to contain the desired sequences: a 3.6-kbp *Hin*dIII fragment and a 1.38-kbp *Cla*I fragment (Figure 12B, indicated by the arrowheads). An attempt

Figure 11. Southern blot analysis of *P. mirabilis* S2R genomic DNA to identify the *terZ* and *terA* coding regions

A 0.64-kbp *Eco*RI - *Kpn*I fragment (Figure 10C, double-headed arrow) was used as a hybridization probe. The plus sign indicates pBSK-EP (positive control, lane 12). Positions of molecular-weight standards are shown on the left. *P. mirabilis* S2R chromosomal DNA was digested with *Cla*I (lane 1), *Cla*I and *Hin*dIII (lane 2), *Eco*RI (lane 3), *Eco*RI and *Hin*dIII (lane 4), *Hin*dIII (lane 5), *Pst*I (lane 6), *Hin*dIII and *Pst*I (lane 7), *Pvu*II (lane 8), *Hin*dIII and *Pvu*II (lane 9), *Sal*I (lane 10), *Sal*I and *Hin*dIII (lane 11). The arrowhead indicates the 4.8-kbp *Hin*dIII fragment selected for further characterization.

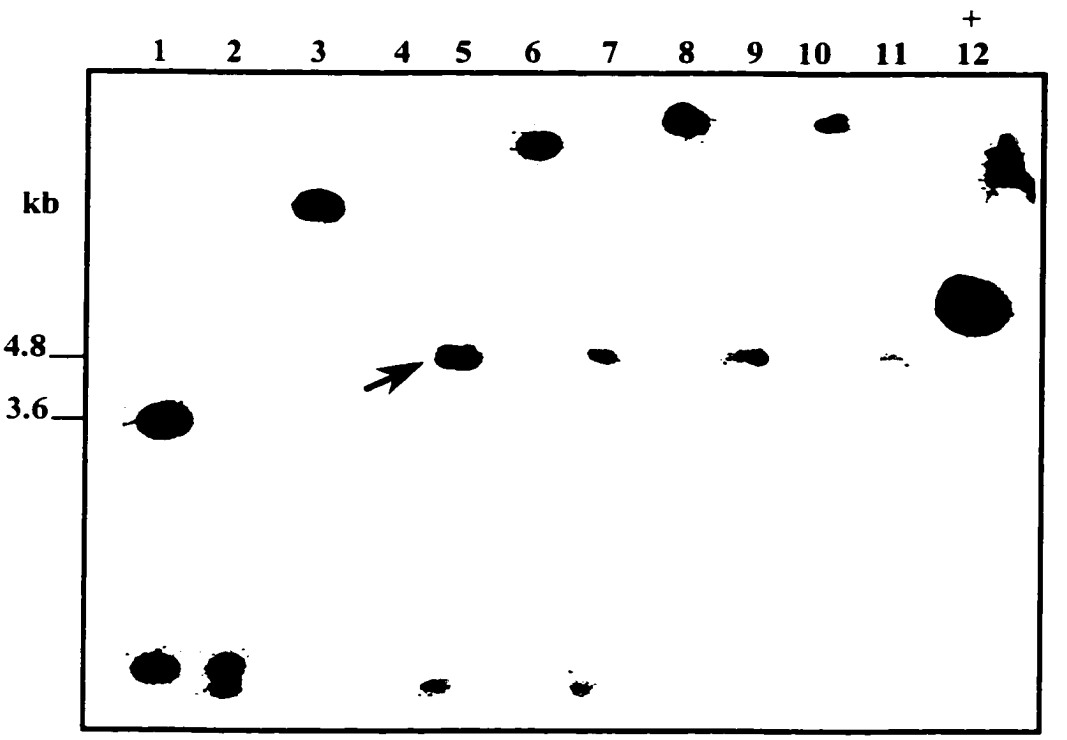
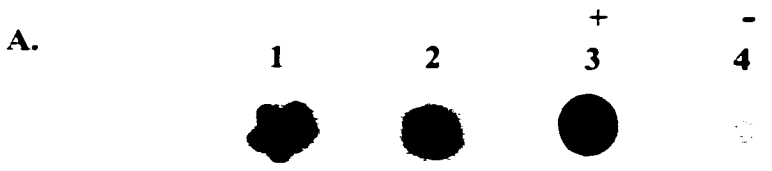
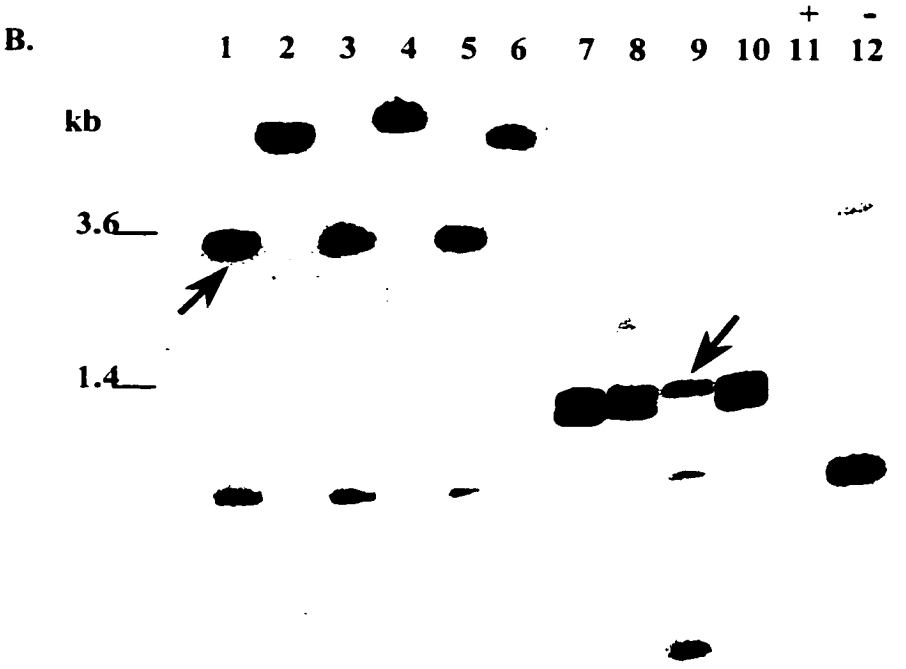
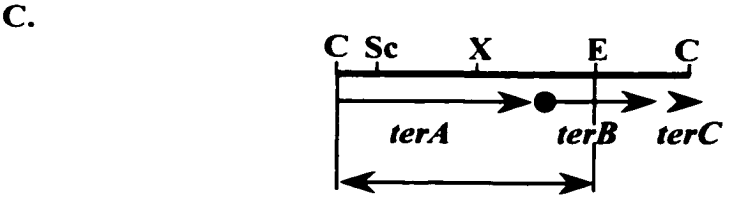

1
2
3
4
5
6
7
8
9
10
11
12
+
kb
4.8
3.6

Figure 12. Southern blot analysis of the cosmid bank of *P. mirabilis* S2R genomic DNA to identify and clone *terZ* and *terA* coding regions

A 0.64-kbp *Eco*RI - *Kpn*I fragment from pBSK-EP (Figure 10C, double-headed arrow) was used as the hybridization probe. (**A**) Colony-blot of cosmids PM2-P8-B4 (lane 1) and PM2-P12-E2 (lane 2). The plus sign indicates the positive control (pBSK-EP [A: lane 3; B: lane 11]) and the minus sign indicates the negative control (pHC79 [A: lane 4; B: lane 12]). (**B**) DNA from cosmid construct PM2-P8-B4 digested with *Hin*dIII (lane 1), *Sal*I (lane 2), *Hin*dIII and *Sal*I (lane 3), *Kpn*I (lane 4), *Kpn*I and *Hin*dIII (lane 5), *Kpn*I and *Sal*I (lane 6), *Cla*I (lane 7), *Cla*I and *Kpn*I (lane 8), *Cla*I and *Hin*dIII (lane 9), *Cla*I and *Sal*I (lane 10). Arrowheads indicate the3.6-kbp *Hin*dIII and 1.38-kbp *Cla*I fragments chosen for further characterization. Positions of molecular-weight standards are shown on the left. (**C**) Physical organization of the putative ORFs encoded by the 1.38-kbp *Cla*I fragment. Restriction endonuclease single-letter codes and other symbols are the same as in Figure 9. Sc, *Sac*I; X, *Xmn*I. The double-headed arrow indicates the 1.0-kbp *Cla*I - *Eco*RI fragment used as a hybridization probe in subsequent experiments.


A.
+
-
1
2
3
4


B.
+
-
1 2 3 4 5 6 7 8 9 10 11 12
kb
3.6
1.4


C.
C Sc X E C
terA
terB
terC

was made to subclone both fragments into pBluescript KS and SK to facilitate sequence analysis. The presence of the desired insert in the recovered constructs was subsequently verified by colony blot hybridization using the same 0.64-kbp *Eco*RI- *Kpn*I fragment as a probe. It was not possible to subclone the 3.6-kbp *Hin*dIII fragment. This result was not surprising considering the difficulties associated with the earlier attempt to subclone the 4.8-kbp *Hin*dIII chromosomal DNA fragment, which was estimated to contain the overlapping upstream sequences (Figure 11, and see above).

At the same time, the 1.38-kbp *Cla*I fragment was successfully subcloned in pBluescript SK, resulting in the pBSK-C2 construct. Restriction mapping and DNA sequence analysis of pBSK-C2 confirmed the presence of the upstream sequences encoding the putative *terA*, *-B* and *-C* genes (Figure 12C). The *terA* coding region extends 5', while the *terC* coding region extends 3' of this DNA fragment.

A high degree of sequence and gene-organization similarity of *the P. mirabilis ter* genes identified in this study with the plasmid-borne Te^r gene clusters indicated that the *P. mirabilis ter* gene cluster would likely contain an additional gene similar to the plasmid-borne *terZ* ORFs (Whelan *et al.*, 1995). The 3.6-kbp *Hin*dIII fragment from cosmid PM2-P8-B4 appeared likely to contain upstream sequences encoding the remaining *ter* genes (Fig. 12B). To identify and subclone this DNA fragment, cosmid PM2-P8-B4 DNA was digested with a number of restriction endonucleases and the resulting fragments were screened by hybridization with the radiolabelled 1.0-kbp *Cla*I - *Eco*RI fragment from pBSK-

C2, containing a fragment of the *terA-terB* coding region (Fig. 12C, double-headed arrow). Figure 13A shows the results of this Southern blot hybridization. Based on the restriction map derived from the previous experiments, two of the fragments corresponding to strong hybridization signals apparently contained the desired DNA sequences: the 3.6-kbp *EcoR*I and the 1.89-kbp *Bam*HI - *Eco*RI fragments (Fig. 13A, indicated by the arrowheads). Both fragments were subcloned into pBluescript vector cut with appropriate endonucleases. Several white *E. coli* JF626 transformants were selected, recombinant plasmid DNA was isolated and sizes of the subcloned fragments were estimated by gel electrophoresis. All of the recovered recombinant plasmids contained the desired inserts. However, subsequent endonuclease mapping indicated that in all recombinant plasmids containing the 3.6-kbp *Eco*RI fragment, the inserts had undergone physical rearrangements with the downstream *terC* coding region, and such constructs contained no additional upstream sequences. Of note, these rearrangements did not reflect the organization of the cosmid PM2-P8-B4 DNA, as was confirmed by subsequent experimental data (see below).

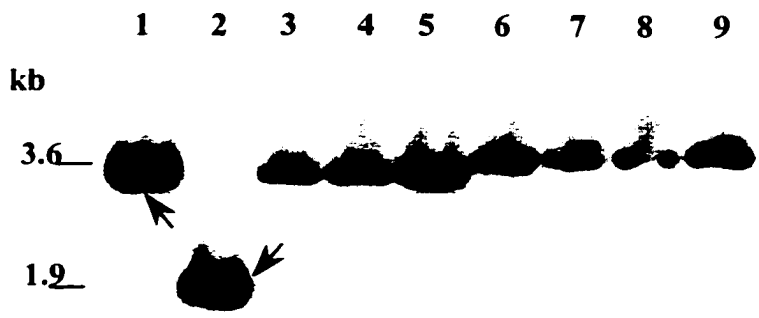
At the same time, recombinant plasmids containing the 1.89-kbp *Bam*HI - *Eco*RI insert (pBKS-BE) were analysed further. To facilitate sequence analysis, the 1.03-kbp *Bam*HI - *Sac*I fragment from pBKS-BE (Figure 13B, double-headed arrow) was subcloned into pBluescript KS, resulting in pBKS-BS. Sequencing revealed three putative ORFs: *terZ*, *terA* and *terB* (Figure 13B). The 5' region of the *terZ* ORF was not present in the DNA fragment. However, previous experiments had shown that additional upstream sequences, including the 5'

portion of the *terZ* coding region, were present in cosmid PM2-P8-B4. Therefore, a final round of Southern blot hybridization analysis and subcloning was carried out to identify these sequences. PM2-P8-B4 cosmid DNA was digested with several restriction endonucleases, and the 0.76-kbp *Cla*I DNA fragment (Figure 13B, double-headed arrow) was used as a hybridization probe to screen the resulting DNA fragments. A 2.3-kbp *Eco*RV fragment was subcloned into pBluescript SK (pBSK-Ev2) for further characterization. Based on the restriction map of the *ter* gene cluster compiled from the previous experimental data (Figure 11), this fragment was estimated to contain over 1 kbp of uncharacterized upstream sequences that would include the 5' *terZ* coding region. Additionally, restriction analysis of pBSK-Ev2 revealed the presence of a 0.7-kbp fragment derived from the pHC79 cosmid vector. To separate cosmid sequences from the *terZ* locus coding region, plasmid pBSK-Ev2 DNA was digested with a set of restriction endonucleases and the resulting DNA fragments were hybridized with the 1.03-kbp *Bam*HI - *Sac*I fragment from pBKS-BS (Figure 13, double-headed arrow). A 1.5-kbp *Cla*I fragment gave a strong hybridization signal and was chosen for further characterisation (Figure 14A). This fragment and the 0.93-kbp *Bam*HI fragment (Figure 14B, double-headed arrow) were subcloned into pBluescript SK to facilitate sequence analysis, resulting in recombinant plasmids called pBSK-C1.5 and pBSK-B2, respectively. DNA sequence analysis of these recombinant plasmids revealed an additional divergently transcribed ORF termed *orf3* located 390 bp upstream of the *terZ* ORF. The *orf3* coding region extended 5' of the *P. mirabilis* chromosomal DNA

Figure 13. Southern blot analysis of cosmid PM2-P8-B4 DNA to identify the *terA* coding region

(**A**) Southern blot hybridization. The 1.0-kbp *Cla*I- *Eco*RI fragment (Figure 12C, double-headed arrow) was used as a probe. Cosmid PM2-P8-B4 DNA was digested with *Eco*RI (lane 1), *Eco*RI and *Bam*HI (lane 2), *Eco*RI and *Hin*dIII (lane 3), *Eco*RI and *Kpn*I (lane 4), *Eco*RI and *Pst*I (lane 5), *Hin*dIII (lane 6), *Kpn*I and *Hin*dIII (lane 7), *Hin*dIII and *Pst*I (lane 8), *Hin*dIII and *Sal*I (lane 9). The arrowheads indicate the 3.6-kbp *Eco*RI and the 1.89-kbp *Bam*HI - *Eco*RI fragments chosen for further characterization. Positions of molecular-weight standards are shown on the left. (**B**) Physical organization of the putative ORFs encoded within the 1.89-kbp *Bam*HI - *Eco*RI fragment from pBKS-BE. Symbols are the same as in Figure 9. B, *Bam*HI; Ev, *Eco*RV; X, *Xmn*I; Sc, *Sac*I. The double-headed arrows indicate the 0.76-kbp *Bam*HI - *Sac*I fragment and the 1.03-kbp *Cla*I fragment used as a hybridization probes in subsequent experiments.

A.



B.

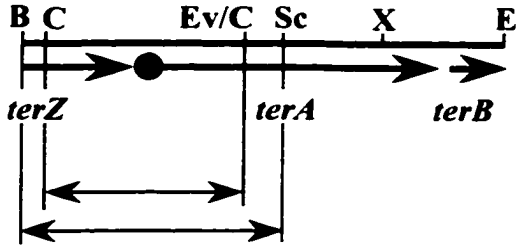
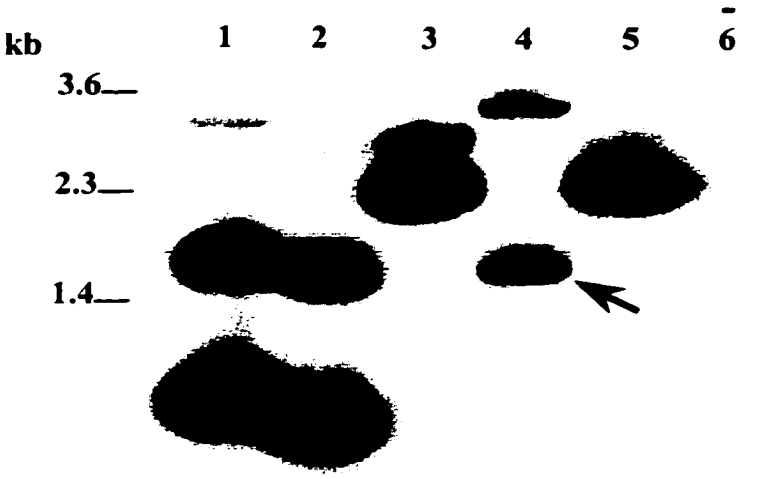


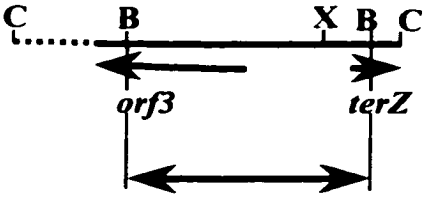
Figure 14. Southern blot analysis of plasmid pBSK-Ev2 DNA to identify the 5' *terZ* coding region

(**A**) A 1.03-kbp *Bam*HI - *Sac*I DNA fragment from pBKS-BS (Fig 13B, double-headed arrow) was used as a hybridization probe. pBSK-Ev2 plasmid DNA digested with *Bam*HI (lane 1), *Bam*HI and *Cla*I (lane 2), *Bam*HI and *Eco*RV (lane 3), *Cla*I (lane 4), *Cla*I and *Eco*RV (lane 5). The minus sign indicates the negative control pBluescript SK (lane 6). The arrowhead indicates the 1.5-kbp *Cla*I fragment selected for further characterization. (**B**) Physical organization of the putative ORFs encoded within the 1.5-kbp *Cla*I fragment from pBSK-Ev2. Symbols and restriction endonuclease single-letter codes are the same as in previous Figures. The double-headed arrow indicates the 0.93-kbp *Bam*HI fragment subcloned into pBluescript SK to facilitate sequence analysis. The dotted line indicates sequences derived from the pHC79 cosmid vector.

A.



B.



fragment subcloned into cosmid PM2-P8-B4. Interestingly, this ORF was highly similar to the putative *orf3* gene found upstream of the R478-encoded *terZ* gene cluster (Whelan *et al.*, 1996). The function of the putative *orf3* gene product is unknown, and these two *orf3* genes show no similarity to any sequences in the GenBank. Clearly, further research is required to elucidate the function of the ORFs encoded by the region upstream of the *terZ* locus.

Attempted isolation of plasmid DNA from *P. mirabilis* S2

In general, resistance to heavy metals is a common feature of bacteria occupying diverse environmental niches (Trevors *et al.*, 1985). Heavy-metal resistance has been described in human pathogens as well as in species isolated from soil, water, sewage and industrial by-products. In these bacterial populations the frequency of heavy-metal resistance is estimated to exceed 50%, and the resistance determinants are often plasmid-borne (Foster, 1983). Experimental data described in the previous section reveals significant similarity in the physical organization of the *P. mirabilis ter* gene cluster and the two previously described plasmid-borne Te^r systems (Whelan *et al.*, 1995; Jobling and Ritchie, 1987, 1988). All known *ter* determinants are carried on large conjugative plasmids, and spread easily among populations of Gram-negative bacteria. Therefore, there was a possibility that in *Proteus* sp. the tellurite-resistance determinants were also carried on a large conjugative plasmid. To investigate this possibility, an attempt was made to isolate plasmid DNA from *P. mirabilis* S2R (WT) strain. No plasmid DNA was isolated from this strain by the

method of Birnboim and Doly (1979). This result was confirmed by centrifugation of total DNA in cesium chloride – ethidium bromide gradients (Sambrook *et al.*, 1989) carried out in the collaborating laboratory of Dr. Diane Taylor. Since no plasmid DNA was isolated from this strain by either method, it is likely that the *ter* gene cluster described in this study is located on the chromosome of *P. mirabilis* S2R.

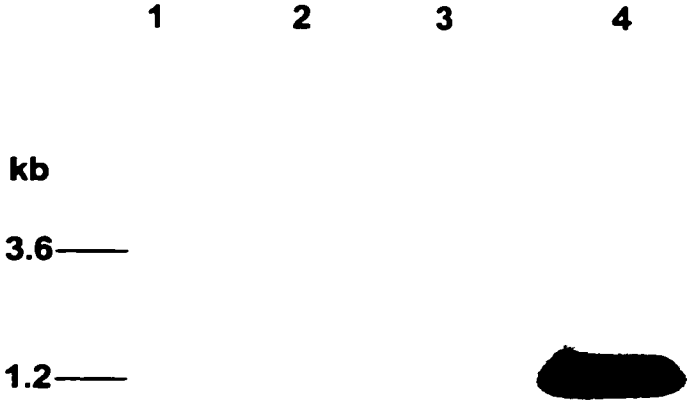
Identification of additional *ter* genes within the *P. mirabilis* S2R genome

Whelan *et al.* (1997) recently described an additional *ter* gene cluster located on the IncH12 plasmid R478. This gene cluster includes three ORFs, termed *terW, -Y* and *-X,* of unknown function. The *terW* gene protects *E. coli* host cells from toxic effects specified by the *terZ* cluster in this heterologous system. Complementation studies in the laboratory of Dr. Taylor defined a 1.95-kbp *Bam*HI fragment (pDT2790), encoding an entire *terW* gene, as a minimal protective clone.

To determine whether *P. mirabilis* also encodes a *terW* homologue, the 1.95-kbp *Bam*HI pDT2790 subclone (the *terW* coding region) was used as a hybridization probe to screen *P. mirabilis* S2R chromosomal DNA by Southern blot hybridization (Figure 15). No hybridization signal was detected despite the low stringency of washes that were used (Figure 15, lane 1 and 2), indicating that the *P. mirabilis* genome does not contain sequences homologous to the *terW* gene.

Figure 15. A search for DNA sequences homologous to the *terW* gene within *P. mirabilis* S2R chromosomal DNA by Southern blot hybridization

P. mirabilis S2R chromosomal DNA was digested with *Bam*HI (lane 1) and *Bam*HI plus *Eco*RI (lane 2). Plasmid pKFW4a DNA digested with *Bam*HI was used as a negative control (lane 3). Plasmid pDT2790 DNA digested with *Bam*HI was used as a positive control (lane 4). The *terW* coding region was excised from pDT2790 as a 1.95-kbp *Bam*HI fragment and used as a hybridization probe. Positions of molecular weight standards are shown on the left.


1
2
3
4
kb
3.6
1.2

Two plasmid-borne Te^r loci from Gram-negative bacteria contain seven *ter* genes (*terZ*, *-A* through *-F*). However, only six ORFs, termed *terZ*, *-A* through *-E*, were detected within the *P. mirabilis* S2R chromosome in this study. Over 1 kbp of chromosomal DNA extending 3' of the *terE* coding region was sequenced from the PM2-P1-C8 cosmid construct, but no ORFs were detected within this region. In fact, this region contained novel sequences with no similarity to known bacterial genes. Interestingly, when the R478-encoded *terF* coding region (the 0.9-kbp *Eco*RI – *Hin*dIII subclone of pKFW4a) was used as a hybridization probe, a strong signal was detected using *P. mirabilis* chromosomal DNA, but not with pDT2790 DNA (Figure 16A). The strong hybridization signal was likely due to the high degree of sequences similarity between *P. mirabilis* *terZ* and *terA* genes and the *terF*-coding sequence used as a hybridization probe in this experiment (see below). The weak hybridization signal in Figure 16A reflects the low degree of sequence similarity between the *terF* gene encoded by the hybridization probe and pDT2790-derived *terX* coding region (Whelan *et al.*, 1996).

To eliminate the possibility that a *terF* homologue was lost by deletion from the PM2-P1-C8 cosmid construct used to clone this region, I attempted to amplify from *P. mirabilis* chromosomal DNA putative *terE* - *terF* intergenic region by polymerase chain reaction using degenerate primers. In plasmid-borne Te^r loci, an intergenic region of less than 450 bp separates the *terE* and *terF* genes. The physical organisations of the known *ter* loci (including the one identified in this study) are similar. Therefore, it was reasonable to hypothesize that, if the *P.*

mirabilis chromosomal *ter* locus contained a *terF* homologue, the intergenic region between the *terE* and the *terF* coding regions would be small enough to be amplifiable by PCR.

Two primers, TERTF-F and TEREF-R, were designed based on the amino acid sequences of the known TerE and TerF proteins and *P. mirabilis* codon usage (Table 1). Two amplicons of 0.8 kbp and 1.0 kbp were obtained when *P. mirabilis* S2R and S2R-6/39 chromosomal DNAs were used as templates for amplification with these primers (Figure 16B). To eliminate non-specific annealing due to the degeneracy of one of the primers (TEREF-R) and the AT-rich template (*P. mirabilis* chromosomal) DNA, PCR reactions were repeated at higher annealing temperatures (50°C instead of 48°C). Products of the same size were obtained at both annealing temperatures, while no amplicons were obtained when PM2-P1-C8 cosmid DNA was used as a template. Both amplicons were TA-cloned into pBluescript SK for sequencing, resulting in pBSK-TerEFampl1 and pBSK-TerEFampl2. Sequencing data revealed that these constructs contained the *terZA* and *terBC* partial coding regions, respectively. There are known specific regions of conserved residues within the groups of ORFs in the Te^r loci (Whelan *et al.*, 1997; Jobling and Ritchie, 1988; and see below). These stretches of conserved residues most likely allowed non-specific annealing of the primers and generation of the PCR products. In addition to these blocks of similar sequences, degeneracy of one of the primers and the relatively low annealing temperatures that were used probably contributed to binding of the primers at several places within the chromosomal

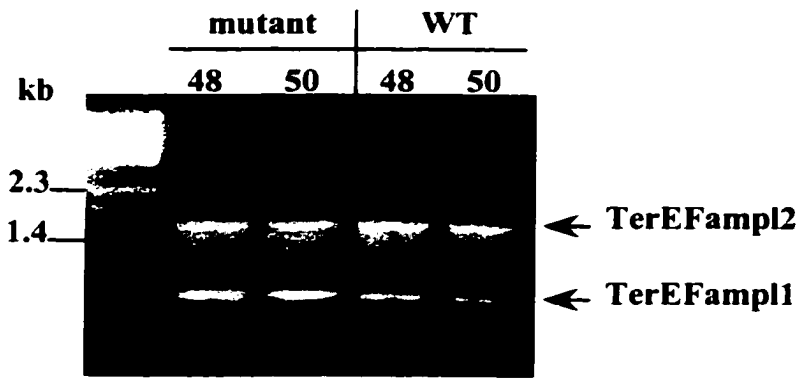
Figure 16. A search for a *terF* homologue within the *P. mirabilis* genome

(**A**) Southern blot hybridization analysis. A 0.9-kbp *Eco*RI - *Hin*dIII subclone of pKFW4a (the *terF* coding region) was used as a hybridization probe. (**B**) PCR products were obtained with TerEF-R and TerEF-F primers. *P. mirabilis* chromosomal DNA from WT and mutant strains were used as a template. Reactions were repeated at different annealing temperatures (48°C and 50°C). The arrowheads indicate the resulting amplicons. Positions of molecular-weight standards are shown on the left.

A.


PM2
pKFW4a
pDT2790

B.


mutant
WT
48
50
48
50
kb
2.3
1.4
TerEFampl2
TerEFampl1

ter region of *P. mirabilis*. Comparison of the physical maps of pAT9501 and PM2-P1-C8 did not detect any differences in the restriction maps of the overlapping subclones from these constructs. Therefore, there was no evidence of rearrangement of the cosmid-borne sequences downstream of the *terE* coding region. Taken together, the experimental data described in this section lead to the conclusion that if an ORF highly similar to the *terF* gene is encoded within *P. mirabilis* chromosome, it is not a part of the *ter* gene cluster described in this study.

Sequence analysis of the *ter* gene cluster

The physical map and nucleotide sequence of the *P. mirabilis* chromosomal *ter* gene cluster were compiled by analysing subclones from the ten recombinant plasmids described in the previous sections. The schematic outline of the cloning strategy and physical map of this region are depicted in Figures 17 and 18, respectively. Seven large ORFs were identified within this 6120-bp chromosomal DNA region and named *orf3*, *terZ*, *-A*, *-B*, *-C*, *-D*, *-E*. This designation was based on the high degree of similarity between these putative genes and those of the Te^r operons of plasmids R478 and pMER610 (Whelan *et al.*, 1997; Jobling and Ritchie, 1988). The *P. mirabilis* chromosomal *ter* genes are positioned in a tandem arrangement, and by analogy to the plasmid-borne *ter* gene clusters are believed to form an operon. The *orf3* gene of unknown function is divergently transcribed and located 400 bp upstream of the *terZ* locus. The coding region for this gene extends outside of the region sequenced in this

Figure 17. Physical map of the *ter* locus of *P. mirabilis* S2R and constructs used for sequence analysis

The inserts in plasmids pBKS-SP and pBKS-HP were subcloned from the PM2-P1-C8 cosmid; those in pBSK-B2, pBSK-C1.6, pBKS-BS, pBKS-BE and pBSK-C2 were subcloned from the PM2-P8-B4 cosmid; the insert in pBSK-EP was subcloned from the pool of *P. mirabilis* S2 chromosomal DNA fragments generated by digestion with *Eco*RI and *Pst*I, and pBKS-HP was constructed by subcloning a *Hin*dIII fragment from pBSK-EP. All plasmid constructs were generated using either pBluescript KS (pBKS) or pBluescript SK (pBSK) vectors. Thin lines represent recombinant plasmid constructs; the arrowheads indicate the direction and extent of sequencing. The arrows below the restriction map indicate the predicted direction of transcription. Complete open reading frames are indicated by the arrows with solid circles, the simple arrow represents an incomplete ORF (*orf3*). The site of mini-transposon insertion in the chromosome of the *P. mirabilis* S2R-6/39 is indicated by the "lollipop". Restriction enzyme sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sac*I; X, *Xmn*I.

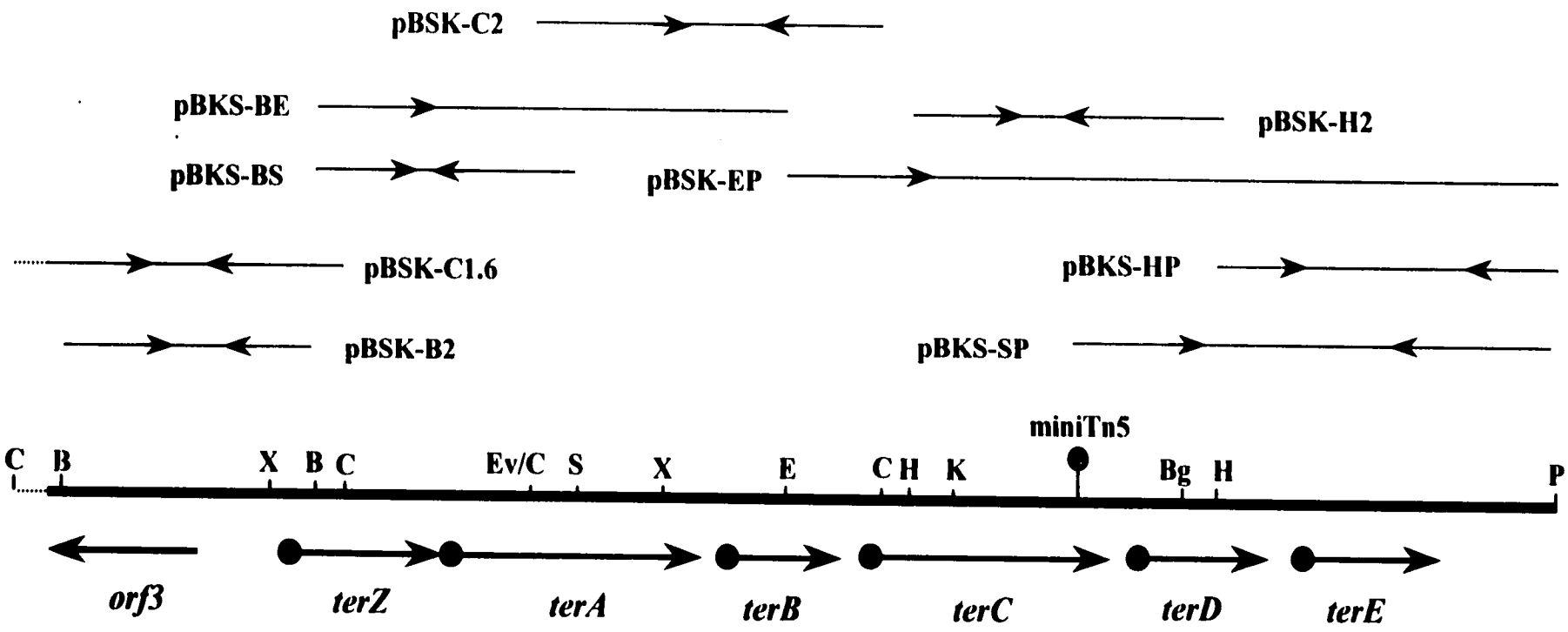
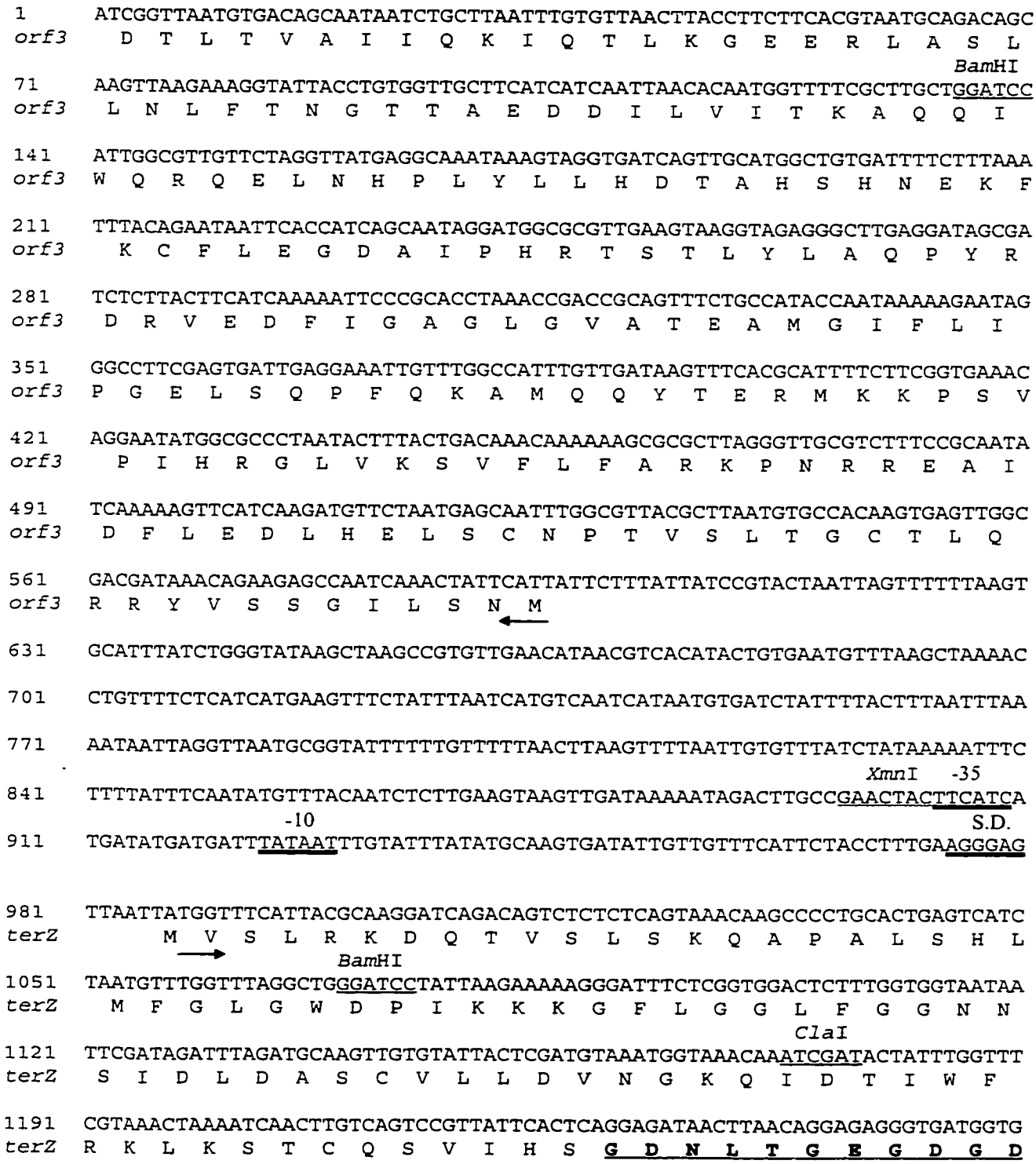
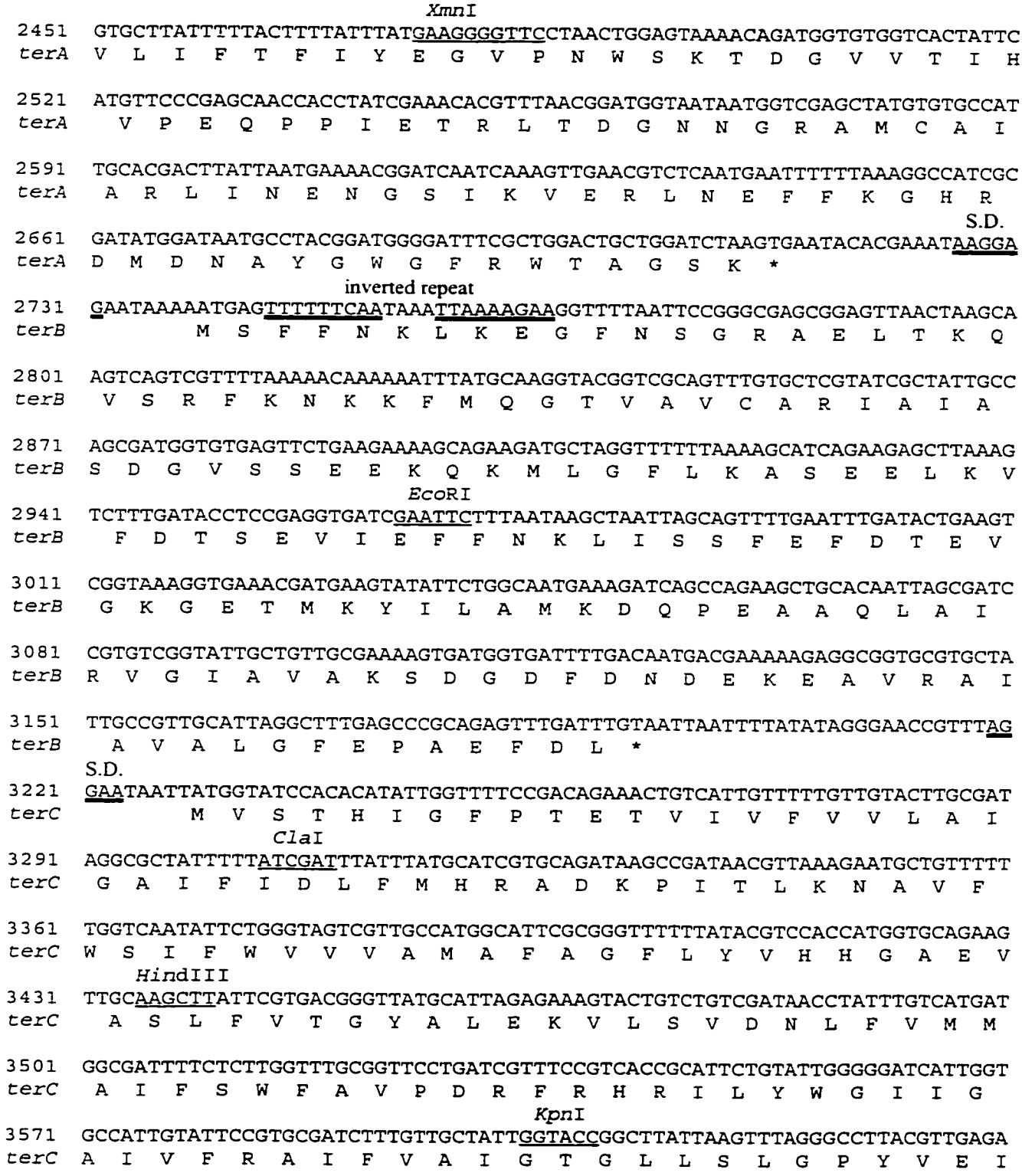

pBSK-C2
pBKS-BE
pBSK-H2
pBKS-BS
pBSK-EP
pBSK-C1.6
pBKS-HP
pBSK-B2
pBKS-SP
miniTn5
C B X B C Ev/C S X E C H K Bg H P
orf3
terZ
terA
terB
terC
terD
terE

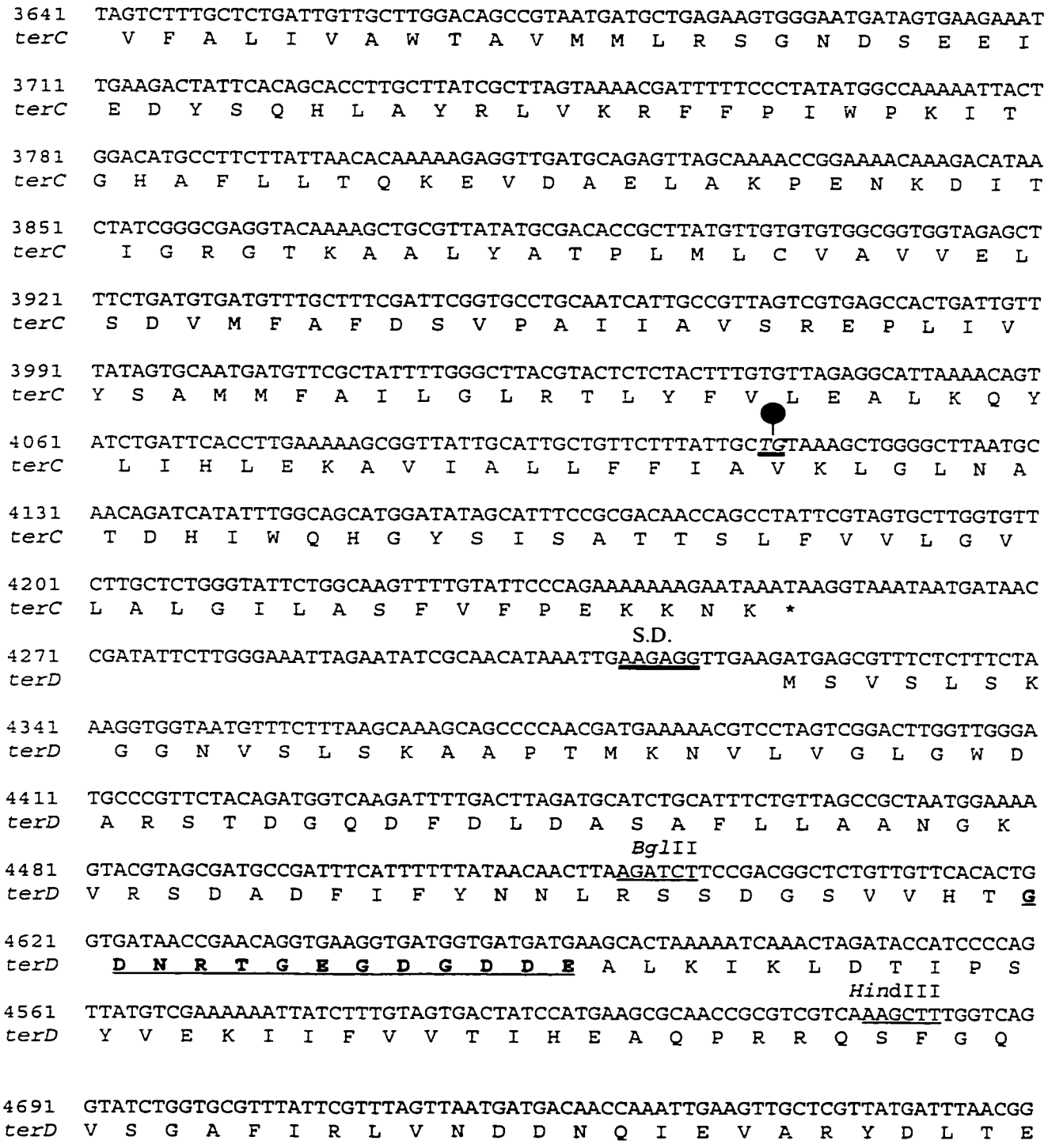
Figure 18. Nucleotide sequence of the *ter* gene cluster of *P. mirabilis* S2R

The deduced amino acid sequences are shown in one-letter code below the DNA sequence. The conserved motif of 13 residues GDN(R/L)TGEG(E/D) GDDE (Whelan *et al.*, 1997) is indicated is bold and underlined. Relevant restriction sites are underlined (single, double or wavy lines). Presumptive -10 and -35 promoter regions (Hoopes and McClure, 1987), putative Shine-Dalgarno (S.D.) sequences (Gold and Stormo, 1987) and inverted repeats suggestive of transcriptional terminators of high probability (Brendel and Trifonov, 1984) are double underlined. Asterisks indicate stop codons. The insertion site of the mini-Tn*5* transposon is double underlined and is indicated by the solid "lollipop". Arrows indicate the direction of transcription of the deduced open reading frames.


1 ATCGGTTAATGTGACAGCAATAATCTGCTTAATTTGTGTTAACTTACCTTCTTCACGTAATGCAGACAGC
orf3 D T L T V A I I Q K I Q T L K G E E R L A S L
BamHI
71 AAGTTAAGAAAGGTATTACCTGTGGTTGCTTCATCATCAATTAACACAATGGTTTTCGCTTGCTGGATCC
orf3 L N L F T N G T T A E D D I L V I T K A Q Q I
141 ATTGGCGTTGTTCTAGGTTATGAGGCAAATAAAGTAGGTGATCAGTTGCATGGCTGTGATTTTCTTTAAA
orf3 W Q R Q E L N H P L Y L L H D T A H S H N E K F
211 TTTACAGAATAATTCACCATCAGCAATAGGATGGCGCGTTGAAGTAAGGTAGAGGGCTTGAGGATAGCGA
orf3 K C F L E G D A I P H R T S T L Y L A Q P Y R
281 TCTCTTACTTCATCAAAAATTCCCGCACCTAAACCGACCGCAGTTTCTGCCATACCAATAAAAAGAATAG
orf3 D R V E D F I G A G L G V A T E A M G I F L I
351 GGCCTTCGAGTGATTGAGGAAATTGTTTGGCCATTTGTTGATAAGTTTCACGCATTTTCTTCGGTGAAAC
orf3 P G E L S Q P F Q K A M Q Q Y T E R M K K P S V
421 AGGAATATGGCGCCCTAATACTTTACTGACAAACAAAAAAGCGCGCTTAGGGTTGCGTCTTTCCGCAATA
orf3 P I H R G L V K S V F L F A R K P N R R E A I
491 TCAAAAAGTTCATCAAGATGTTCTAATGAGCAATTTGGCGTTACGCTTAATGTGCCACAAGTGAGTTGGC
orf3 D F L E D L H E L S C N P T V S L T G C T L Q
561 GACGATAAACAGAAGAGCCAATCAAACTATTCATTATTCTTTATTATCCGTACTAATTAGTTTTTTAAGT
orf3 R R Y V S S G I L S N M
631 GCATTTATCTGGGTATAAGCTAAGCCGTGTTGAACATAACGTCACATACTGTGAATGTTTAAGCTAAAAC
701 CTGTTTTCTCATCATGAAGTTTCTATTTAATCATGTCAATCATAATGTGATCTATTTTACTTTAATTTAA
771 AATAATTAGGTTAATGCGGTATTTTTTGTTTTTAACTTAAGTTTTAATTGTGTTTATCTATAAAAATTTC
XmnI -35
841 TTTTATTTCAATATGTTTACAATCTCTTGAAGTAAGTTGATAAAAATAGACTTGCCGAACTACTTCATCA
-10 S.D.
911 TGATATGATGATTTATAATTTGTATTTATATGCAAGTGATATTGTTGTTTCATTCTACCTTTGAAGGGAG
981 TTAATTATGGTTTCATTACGCAAGGATCAGACAGTCTCTCTCAGTAAACAAGCCCCTGCACTGAGTCATC
terZ M V S L R K D Q T V S L S K Q A P A L S H L
BamHI
1051 TAATGTTTGGTTTAGGCTGGGATCCTATTAAGAAAAAGGGATTTCTCGGTGGACTCTTTGGTGGTAATAA
terZ M F G L G W D P I K K K G F L G G L F G G N N
ClaI
1121 TTCGATAGATTTAGATGCAAGTTGTGTATTACTCGATGTAAATGGTAAACAAATCGATACTATTTGGTTT
terZ S I D L D A S C V L L D V N G K Q I D T I W F
1191 CGTAAACTAAAATCAACTTGTCAGTCCGTTATTCACTCAGGAGATAACTTAACAGGAGAGGGTGATGGTG
terZ R K L K S T C Q S V I H S G D N L T G E G D G D

```
1261  ATGATGAAACTATTTTTGTTGATTTAAATCGTCTTCCTTCATTGGTTGAATATTTGGTTTTCACTGTCAA
terZ   D  E  T  I  F  V  D  L  N  R  L  P  S  L  V  E  Y  L  V  F  T  V  N

1331  TAGTTTCCGTGGGCAGACCTTTAATGAAGTTGAAAATGCGTTTTGTCGAGTTGTCGATAAAACAACCAAT
terZ   S  F  R  G  Q  T  F  N  E  V  E  N  A  F  C  R  V  V  D  K  T  T  N

1401  AAAGAGCTTGCTCATTACAAATTAACGGAACAAGGCGCTCATACAGGGATCGTGATTGCCGCTTTACAAC
terZ   K  E  L  A  H  Y  K  L  T  E  Q  G  A  H  T  G  I  V  I  A  A  L  Q  R

1471  GTAATCAAGGACAATGGGATTTCACGGCTTTTGGCTCACCTTGTAAAGGGCTTGTCATTGACGAGATGAT
terZ   N  Q  G  Q  W  D  F  T  A  F  G  S  P  C  K  G  L  V  I  D  E  M  M

                    S.D.
1541  GCCAGATATTTTGGCGACGGTGGTGCGATAAATGACCCTAACACCTGGCGGAAATATATCCGTACCTGAT
terA   P  D  I  L  A  T  V  V  R  *  M  T  L  T  P  G  G  N  I  S  V  P  D

1611  CAAACGTTAATGGTGCGTATTCACTCAGGCTCACTTGTTGATGTATCTGCATTTAGGCTTTATGCCTCAG
terA   Q  T  L  M  V  R  I  H  S  G  S  L  V  D  V  S  A  F  R  L  Y  A  S  G

1681  GAAAAGTGAACGGTGATGCTGATATGGTGTTTTATGGTCAAACCACTAATGATGATCGTACTATTATTTA
terA   K  V  N  G  D  A  D  M  V  F  Y  G  Q  T  T  N  D  D  R  T  I  I  Y

1751  TGCAACAGCAGGTAATAGCACGTCGTTTACGGTTGACTTAACTCGCTTACGTCCTGATGTCGATAGAATT
terA   A  T  A  G  N  S  T  S  F  T  V  D  L  T  R  L  R  P  D  V  D  R  I

1821  GCTTTTACAGCTACTTGTGAGGGGCAACAAACCATTGCTCATCTACAACATTTGTCTATTCAAGTTGATG
terA   A  F  T  A  T  C  E  G  Q  Q  T  I  A  H  L  Q  H  L  S  I  Q  V  D  A

                                        EcoRV  ClaI
1891  CTAACAATAATGTCGTTGCGAACGGCCATGTTGATATCGATGGACGCACTGAAGCGGCATTAATTTTAGG
terA   N  N  N  V  V  A  N  G  H  V  D  I  D  G  R  T  E  A  A  L  I  L  G

1961  TGAGCTATACCGTCGCAATGGAAGCTGGAAATTTCGGTTTATTGCACAAGGATTTAACGGTGGATTAAAA
terA   E  L  Y  R  R  N  G  S  W  K  F  R  F  I  A  Q  G  F  N  G  G  L  K

                                                                    SacI
2031  CCATTGGCGGAATATTTTGGTGTTGATATTGCTGATCCTGAACCAGCACCAGCACCAGCACCGAGCTCAG
terA   P  L  A  E  Y  F  G  V  D  I  A  D  P  E  P  A  P  A  P  A  P  S  S  V

2101  TGAACTTAAGCAAGGTTTCGCTAACTAAAGAGAAACCGGCAATTAGCTTAACTAAAAAGGATGATTTCGG
terA   N  L  S  K  V  S  L  T  K  E  K  P  A  I  S  L  T  K  K  D  D  F  G

2171  CAAAATCCGCATTAACCTCGATTGGCATCGAGAAAGTAAAAGCGGTGGTTCCGGGTTATTAGGTGGATTA
terA   K  I  R  I  N  L  D  W  H  R  E  S  K  S  G  G  S  G  L  L  G  G  L

2241  TTTGGTGGTAACAAAGGTATTGATTTAGATATTGGCGCCTTTGTTGAACTACAAGATGGTTATAAGTCAG
terA   F  G  G  N  K  G  I  D  L  D  I  G  A  F  V  E  L  Q  D  G  Y  K  S  V

2311  TGATCCAAGCCTTAGGAAATGGATTCGGTGATTTTAATCGCATGCCTTATGTTGAGTTACAAGGTGATGA
terA   I  Q  A  L  G  N  G  F  G  D  F  N  R  M  P  Y  V  E  L  Q  G  D  D

2381  TCGCACTGGTGATGTAGCGGGTGGCGAGTGGATTTTTATCAATGGACGTGAATGGAAAAATATCAAGCAA
terA   R  T  G  D  V  A  G  G  E  W  I  F  I  N  G  R  E  W  K  N  I  K  Q
```


XmnI
2451 GTGCTTATTTTTACTTTTATTTATGAAGGGTTCCTAACTGGAGTAAAACAGATGGTGTGGTCACTATTC
terA V L I F T F I Y E G V P N W S K T D G V V T I H
2521 ATGTTCCCGAGCAACCACCTATCGAAACACGTTTAACGGATGGTAATAATGGTCGAGCTATGTGTGCCAT
terA V P E Q P P I E T R L T D G N N G R A M C A I
2591 TGCACGACTTATTAATGAAAACGGATCAATCAAAGTTGAACGTCTCAATGAATTTTTTAAAGGCCATCGC
terA A R L I N E N G S I K V E R L N E F F K G H R
S.D.
2661 GATATGGATAATGCCTACGGATGGGGATTTCGCTGGACTGCTGGATCTAAGTGAATACACGAAATAAGGA
terA D M D N A Y G W G F R W T A G S K *
inverted repeat
2731 GAATAAAAATGAGTTTTTTCAATAAATTAAAAGAAGGTTTTAATTCCGGGCGAGCGGAGTTAACTAAGCA
terB M S F F N K L K E G F N S G R A E L T K Q
2801 AGTCAGTCGTTTTAAAAACAAAAAATTTATGCAAGGTACGGTCGCAGTTTGTGCTCGTATCGCTATTGCC
terB V S R F K N K K F M Q G T V A V C A R I A I A
2871 AGCGATGGTGTGAGTTCTGAAGAAAAGCAGAAGATGCTAGGTTTTTTAAAAGCATCAGAAGAGCTTAAAG
terB S D G V S S E E K Q K M L G F L K A S E E L K V
EcoRI
2941 TCTTTGATACCTCCGAGGTGATCGAATTCTTTAATAAGCTAATTAGCAGTTTTGAATTTGATACTGAAGT
terB F D T S E V I E F F N K L I S S F E F D T E V
3011 CGGTAAAGGTGAAACGATGAAGTATATTCTGGCAATGAAAGATCAGCCAGAAGCTGCACAATTAGCGATC
terB G K G E T M K Y I L A M K D Q P E A A Q L A I
3081 CGTGTCGGTATTGCTGTTGCGAAAAGTGATGGTGATTTTGACAATGACGAAAAAGAGGCGGTGCGTGCTA
terB R V G I A V A K S D G D F D N D E K E A V R A I
3151 TTGCCGTTGCATTAGGCTTTGAGCCCGCAGAGTTTGATTTGTAATTAATTTTATATAGGGAACCGTTTAG
terB A V A L G F E P A E F D L *
S.D.
3221 GAAATAATTATGGTATCCACACATATTGGTTTTCCGACAGAAACTGTCATTGTTTTTGTTGTACTTGCGAT
terC M V S T H I G F P T E T V I V F V V L A I
ClaI
3291 AGGCGCTATTTTTATCGATTTATTTATGCATCGTGCAGATAAGCCGATAACGTTAAAGAATGCTGTTTTT
terC G A I F I D L F M H R A D K P I T L K N A V F
3361 TGGTCAATATTCTGGGTAGTCGTTGCCATGGCATTCGCGGGTTTTTTATACGTCCACCATGGTGCAGAAG
terC W S I F W V V V A M A F A G F L Y V H H G A E V
HindIII
3431 TTGCAAGCTTATTCGTGACGGGTTATGCATTAGAGAAAGTACTGTCTGTCGATAACCTATTTGTCATGAT
terC A S L F V T G Y A L E K V L S V D N L F V M M
3501 GGCGATTTTCTCTTGGTTTGCGGTTCCTGATCGTTTCCGTCACCGCATTCTGTATTGGGGGATCATTGGT
terC A I F S W F A V P D R F R H R I L Y W G I I G
KpnI
3571 GCCATTGTATTCCGTGCGATCTTTGTTGCTATTGGTACCGGCTTATTAAGTTTAGGGCCTTACGTTGAGA
terC A I V F R A I F V A I G T G L L S L G P Y V E I


3641 TAGTCTTTGCTCTGATTGTTGCTTGGACAGCCGTAATGATGCTGAGAAGTGGGAATGATAGTGAAGAAAT
terC V F A L I V A W T A V M M L R S G N D S E E I
3711 TGAAGACTATTCACAGCACCTTGCTTATCGCTTAGTAAAACGATTTTTCCCTATATGGCCAAAAATTACT
terC E D Y S Q H L A Y R L V K R F F P I W P K I T
3781 GGACATGCCTTCTTATTAACACAAAAAGAGGTTGATGCAGAGTTAGCAAAACCGGAAAACAAAGACATAA
terC G H A F L L T Q K E V D A E L A K P E N K D I T
3851 CTATCGGGCGAGGTACAAAAGCTGCGTTATATGCGACACCGCTTATGTTGTGTGTGGCGGTGGTAGAGCT
terC I G R G T K A A L Y A T P L M L C V A V V E L
3921 TTCTGATGTGATGTTTGCTTTCGATTCGGTGCCTGCAATCATTGCCGTTAGTCGTGAGCCACTGATTGTT
terC S D V M F A F D S V P A I I A V S R E P L I V
3991 TATAGTGCAATGATGTTCGCTATTTTGGGCTTACGTACTCTCTACTTTGTGTTAGAGGCATTAAAACAGT
terC Y S A M M F A I L G L R T L Y F V L E A L K Q Y
4061 ATCTGATTCACCTTGAAAAAGCGGTTATTGCATTGCTGTTCTTTATTGCTGTAAAGCTGGGGCTTAATGC
terC L I H L E K A V I A L L F F I A V K L G L N A
4131 AACAGATCATATTTGGCAGCATGGATATAGCATTTCCGCGACAACCAGCCTATTCGTAGTGCTTGGTGTT
terC T D H I W Q H G Y S I S A T T S L F V V L G V
4201 CTTGCTCTGGGTATTCTGGCAAGTTTTGTATTCCCAGAAAAAAAGAATAAATAAGGTAAATAATGATAAC
terC L A L G I L A S F V F P E K K N K *
S.D.
4271 CGATATTCTTGGGAAATTAGAATATCGCAACATAAATTGAAGAGGTTGAAGATGAGCGTTTCTCTTTCTA
terD M S V S L S K
4341 AAGGTGGTAATGTTTCTTTAAGCAAAGCAGCCCCAACGATGAAAAACGTCCTAGTCGGACTTGGTTGGGA
terD G G N V S L S K A A P T M K N V L V G L G W D
4411 TGCCCGTTCTACAGATGGTCAAGATTTTGACTTAGATGCATCTGCATTTCTGTTAGCCGCTAATGGAAAA
terD A R S T D G Q D F D L D A S A F L L A A N G K
BglII
4481 GTACGTAGCGATGCCGATTTCATTTTTTATAACAACTTAAGATCTTCCGACGGCTCTGTTGTTCACACTG
terD V R S D A D F I F Y N N L R S S D G S V V H T G
4621 GTGATAACCGAACAGGTGAAGGTGATGGTGATGATGAAGCACTAAAAATCAAACTAGATACCATCCCCAG
terD D N R T G E G D G D D E A L K I K L D T I P S
HindIII
4561 TTATGTCGAAAAAATTATCTTTGTAGTGACTATCCATGAAGCGCAACCGCGTCGTCAAAGCTTTGGTCAG
terD Y V E K I I F V V T I H E A Q P R R Q S F G Q
4691 GTATCTGGTGCGTTTATTCGTTTAGTTAATGATGACAACCAAATTGAAGTTGCTCGTTATGATTTAACGG
terD V S G A F I R L V N D D N Q I E V A R Y D L T E

```
4761  AAGATGCATCAACGGAAACGGCGATGTTATTTGGTGAGTTATATCGTCATAACGGTGAGTGGAAATTCCG
terD    D  A  S  T  E  T  A  M  L  F  G  E  L  Y  R  H  N  G  E  W  K  F  R

4831  TGCTGTAGGCCAAGGATATGCAGGGGGCTTAGGCTCAGTATGTGCGCAATATGGTATTAACGCCTCTTAA
terD    A  V  G  Q  G  Y  A  G  G  L  G  S  V  C  A  Q  Y  G  I  N  A  S  *
                                                                    S.D.
4901  TCGTATGTTATGAAATCACAAATTACTGGCAGTTATTCCTAATTGTCAGTTTATTCAACACAGTAGGAGC

4971  TTTGATATGGCTGTTTCCCTTGTTAAAGGTGGTAATGTTTCGCTAACTAAAGAAGCACCAACCATGACGG
terE          M  A  V  S  L  V  K  G  G  N  V  S  L  T  K  E  A  P  T  M  T  V

5041  TGGCTATGGTTGGTTTAGGATGGGATGCCCGCGTGACAGATGGCGCTGAATTTGATTTAGATGCGTCAGT
terE    A  M  V  G  L  G  W  D  A  R  V  T  D  G  A  E  F  D  L  D  A  S  V

5111  ATTCATGGTCGGTGAAGATGGAAAAGTGCTTTCAGATGCAAGTTTTATCTTCTTTAATAATAAAGTGAGC
terE    F  M  V  G  E  D  G  K  V  L  S  D  A  S  F  I  F  F  N  N  K  V  S

5181  CAATGTGGTAGTGTTGAACACCAAGGCGATAATCGTACTGGAGAAGGCGAAGGTGATGATGAACAAGTCA
terE  Q  C  G  S  V  E  H  Q  G  D  N  R  T  G  E  G  E  G  D  D  E  Q  V  K

5251  AAATCACTTTATCCAAAGTTCCTGCTGAAGTGAAAAAACTGGTTTTTGCTGTCACTATTTACGATGCTGA
terE    I  T  L  S  K  V  P  A  E  V  K  K  L  V  F  A  V  T  I  Y  D  A  E

5321  AAACCGCAAACAAAACTTTGGTATGGTGAGCAACAGTTTTATGCGCGTTTATAATAACGACAATAACACT
terE    N  R  K  Q  N  F  G  M  V  S  N  S  F  M  R  V  Y  N  N  D  N  N  T

5391  GAAATTGCACGTTTTGATCTTTCTGAAGATGCCTCTACGGAGACGGCGATGATCTTTGGTGAACTCTATC
terE  E  I  A  R  F  D  L  S  E  D  A  S  T  E  T  A  M  I  F  G  E  L  Y  R

5461  GTCATGGTTCAGATTGGAAATTTAAAGCAGTAGGCCAAGGTTTTGCGGGTGGTTTAGGCGCACTAGCCGC
terE    H  G  S  D  W  K  F  K  A  V  G  Q  G  F  A  G  G  L  G  A  L  A  A

5531  ACAACATGGCGTCAATATTTAATCTTTCGCTAATAAAAGCGCCAAGATGACTAGATAAATAGTTAGTTTA
terE    Q  H  G  V  N  I  *
                    inverted repeat
5601  TGGATAAAGCGGGAAAATACGTTTCCCGCTTTATTTTTATATAATCACTTATGAGTCTTTTTTAAGCGTT

5671  ATTTTAATAGGTGATTTTTTTGACTTATTACTTCCTGTGTGGAAATAGCGCCAATGTGGATCTTGTGCAG
5741  CATAGAGTAATTCATGATCACCACGTGAATCGCCCCACGCTCTTAAGTGGTATTGAGTAAGATCGCCATA
5811  GACACGCTCTAATCGTTTGATTTTTTCGCCACAACGGCAGTTATTGCCAATGATTTTTCCTGTCAGCACA
5881  CCATCAACCACTTCAAGTGTTGTTCCTATTAACTTTATACCAAGTCGATCCGCAAAAGGTTGCAACACCA
5951  TAGCTGGAGAAGCTGAACAAATGGTCACTTCAGCATGACGGTTAATCTCTTCAGCAACGGCTAATAAACC
      PstI                              HindIII
6021  TGCAGGACGCATTAACTTAGTCCAGTAAGCTTTACAAAAGGCTTCGGCTTTTTCTTTGAGCCATTCTTCT
        EcoRV
6091  TTGATATCGGTTAAAAAAGTTTTAATCAGC
```

Table 2. Putative Ter polypeptides and their homologues containing a conserved 13-residue motif.

Organism	Protein	Position of motif (aa number)	Motif	Reference
P. mirabilis	TerZ	82	GDNLTGEGDGDDE	This study
	TerD	77	GDNRTGEGDGDDE	
	TerE	77	GDNRTGEGEGDDE	
plasmid R478	TerZ	82	GDNLTGEGDGDDE	Whelan *et al.*, 1995
	TerD	77	GDNRTGEGDGDDE	
	TerE	77	GDNRTGEGDGDDE	
	TerX	100	GGNRTGAGDGDDE	Whelan *et al.*, 1997
plasmid	TerD	77	GDNRTGEGDGDDE	Jobling and Ritchie, 1988
pMER610	TerE	77	GDNRTGEGDGDDE	
B. subtilis	YceC	87	GDNLTGDGAGDDE	Kumano *et al.*, 1997
	YceD	78	GDNLTGAGEGDDE	
	YceE	78	GDNRTGEGDGDDE	
C. acetobutylicum	OrfC	78	GDNLTGDGDGDDE	Azeddoug and Reysset, 1994
D. discoideum	CABP	208	GDNLTGQGEGDDE	Kaneko *et al.*, 1996
	CABP1-RP	169	GDNLTGAGDGDDE	
	CABP1H-1	135	GDNLTGAGDGDDE	
	CABP1H-3	169	GDNLTGAGDGDDE	
Synechocystis sp	CABP	68	DDNLTGEGEGDDE	Bain *et al.*, 1991
	Consensus		**GDN$^{L}_{R}$TG$^{E}_{A}$GDGDDE**	Whelan *et al.*, 1997

work. No open reading frames were detected immediately downstream of *terE* (within the 1 kbp of DNA analyzed in this study [see page 125]).

Three of the putative polypeptides encoded by the *P. mirabilis ter* locus, TerZ, TerD and TerE, containe a conserved 13-residue motif GDN(R/L)TG(E/A) GDGDDE (Figure 18, Table 2). This motif was originally identified within Ter polypeptides encoded by the R478 Te^r system (Whelan *et al.*, 1997). However, based on CLUSTAL W analysis (Baylor College of Medicine Search Launcher, Houston, Texas), the motif is also present in various polypeptides encoded by both Gram-negative and Gram-positive bacteria, as well as by at least one eukaryotic microorganism. All of the proteins containing the 13-residue motif show overall sequence similarity to Ter proteins (Table 2). Although the biological function of this motif is still unknown, its conservation in numerous proteins synthesized by diverse microorganisms suggests that this motif is critical for the proper function of these polypeptides and merits further investigation.

All ORFs identified in this study, with the exception of the *terC* gene, encode polypeptides predicted by the method of Klein *et al.* (1985) to function in the bacterial cytoplasm. In contrast, several potential hydrophobic helices were detected within TerC by the PSORT (Nakai and Kanehisa, 1991) and TMpred (Hofmann and Stofel, 1993) algorithms. In general, hydrophobic transmembrane segments exist in cytoplasmic membrane proteins only. Thus, the presence of these segments within TerC can be regarded as a sorting signal into the bacterial inner membrane. The hydropathy profile of TerC reveals nine stretches of hydrophobic residues with lengths of 18 to 23 amino acids alternating with

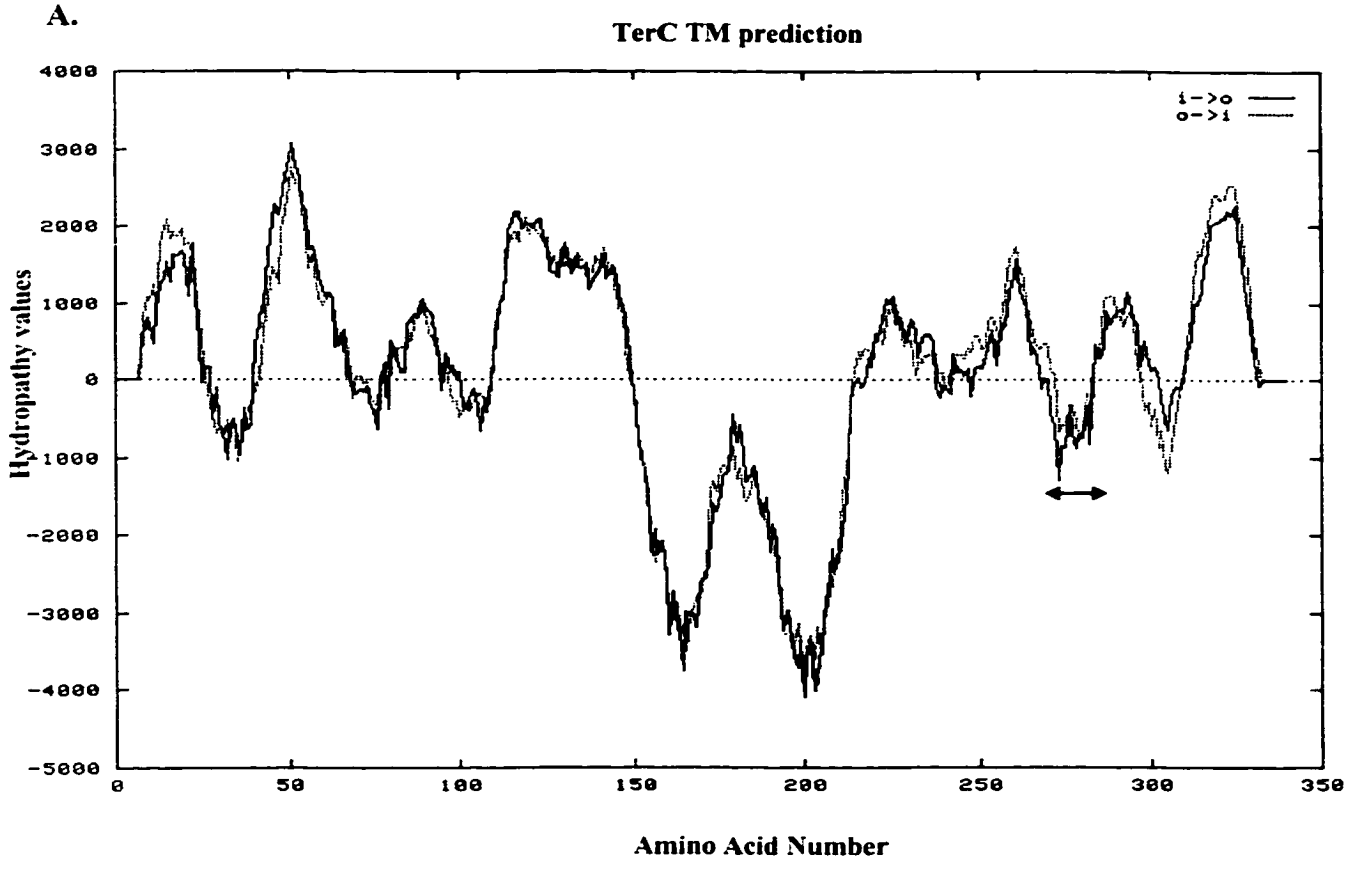
hydrophilic or less hydrophobic portions of the polypeptide (Figure 19A). The TerC N terminus is predicted by the TMpred analysis to extend into the periplasmic space, while the C terminus protrudes into the cytoplasm. In addition, a search for known protein patterns and motifs identified a leucine-zipper motif in the C-terminal region (residues 268-289) of TerC (Figure 19B). Leucine-zipper motifs are characterized by a heptad repeat of leucine residues, and are involved in the generation of dimerization surfaces (Kouzarides and Ziff, 1989). The region spanning the heptad leucine repeats in the TerC C terminus also had a high probability of amphipathic α-helix formation (Figure 19B) according to the algorithms of Chou-Fasman (Chou and Fasman, 1978) and of Robson-Garnier (Robson and Garnier, 1993). This region is located between, and is partially overlapped by, two predicted hydrophobic transmembrane domains (Figure 19A, double-headed arrow). Sequence analysis reveals that the mini-transposon integrated in the 3' part of the *terC* coding region, resulting in an in-frame fusion and synthesis of a TerC-LacZ fusion protein (Figure 18). The fusion junction is located at the alanine-295 residue of TerC.

Based on these observations, decreased levels of potassium-tellurite resistance noted for the mutant strain of *P. mirabilis* can be attributed either to a functional loss of TerC, or to a polar effect of this insertion on the downstream genes (encoding the TerD and TerE polypeptides). The former explanation is more likely, because the mini-Tn*5* construct used in this study does not contain a transcriptional terminator within the kanamycin-resistance cassette (see appendix; De Lorenzo *et al.*, 1990), which would allow transcription to continue,

Figure 19. Hydropathy analysis of TerC

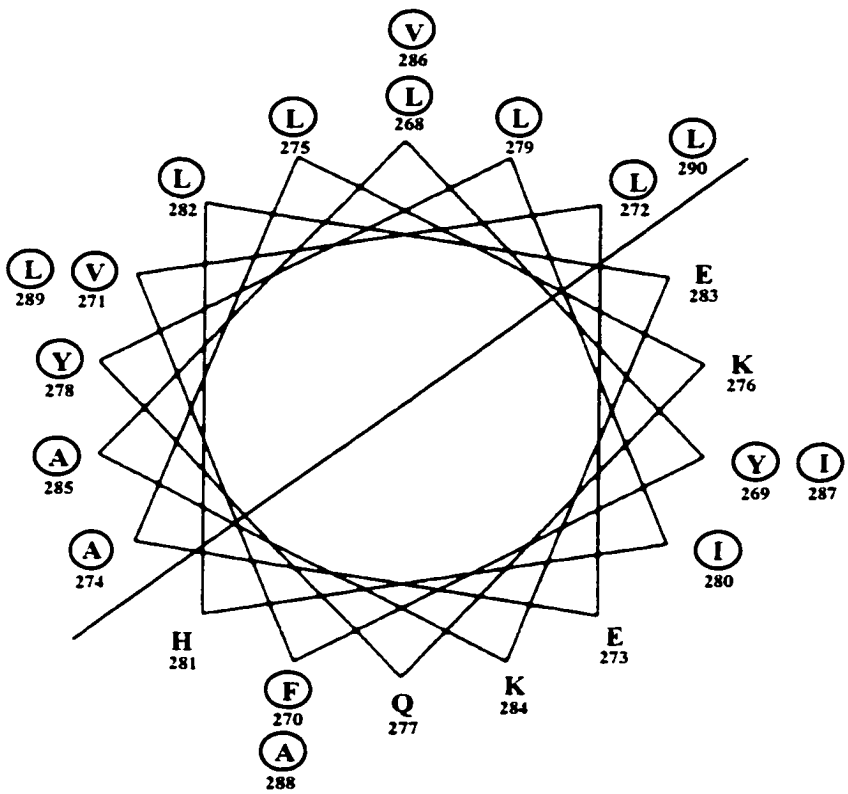
(**A**) TerC prediction graph of possible transmembrane helices and their predicted orientation generated using TMpred (9 aa window) (Hofmann and Stoffel, 1993); only values above 500 are considered significant. Protein sequences that plot above the central dotted line represent hydrophobic domains, whereas protein sequence that plot below the dotted line are hydrophilic. Two lines within the panel designate two possible orientations of the predicted transmembrane helices: the solid line indicates inside→outside orientation, the dotted line indicates outside→inside orientation. The orientation corresponding to the higher TMpred value is favoured. The double-headed arrow indicates the location of the leucine-zipper motif. (**B**) The leucine-zipper motif in the C terminus (residues 268-289) of *P. mirabilis* TerC, aligned with TerC of R478 and of pMER610, and the putative membrane protein MtHP of *Mycobacterium tuberculosis*, indicating leucine heptad repeats in bold (for references see text and Table 2). The α-helical wheel is a projection of *P. mirabilis* TerC C-terminal residues 268-289. Hydrophobic residues are circled.

A.


TerC TM prediction
i->o
o->i
Hydropathy values
4000
3000
2000
1000
0
-1000
-2000
-3000
-4000
-5000
0
50
100
150
200
250
300
350
Amino Acid Number

B.

PmTerC	268	**L**YFVLEA**L**KQYLIH**L**EKAVIA**L**L	290
R478TerC	268	**L**YFVLEA**L**KQYLVH**L**EKAVIV**L**L	290
pMERTerC	268	**L**YFVLEA**L**KQYLSQ**L**EKAVIV**L**L	290
MtHP	244	**L**YFLIDG**L**LDRLVY**L**SYGLAV**I**L	276



downstream of the mini-transposon insertion, into the *terD* and *terE* coding regions. Putative Shine-Dalgarno sequences preceding both of these genes would allow subsequent binding of ribosomes and translation of TerD and TerE proteins. At this point, this hypothesis remains to be confirmed by future experiments.

In addition to many similarities, there are several differences between the *P. mirabilis* chromosomal *ter* gene cluster and plasmid-borne Te^r loci. The *ter* locus of *P. mirabilis* contains six genes (*terZ*, *-A* through *-E*) instead of the seven genes found in other Te^r gene clusters (see preceding sections). The stop codon of the *terZ* coding region is followed immediately by the start codon of *terA* (Figure 18). The putative TerA polypeptide has an additional 44 aa residues (Figure 18) compared to its plasmid-encoded counterparts, which have the methionine residue at position 45 as the first amino acid (Whelan *et al.*, 1995). A BLAST search of the TerA N-terminal stretch of 44 extra aa residues showed high similarity of this polypeptide to TerD proteins. Interestingly, in R478, these genes are separated by a 150-bp intergenic region, which contains a possible transcription terminator. On the other hand, no potential terminator sequences are evident immediately downstream of the *terZ* coding region. The *terA* and *terE* coding regions are flanked at the 3' end by inverted repeats, suggestive of possible transcriptional terminators (Yager and von Hippel, 1987). Taken together, these observations support the physical organization of the *ter Z* and *terA* genes as presented in Figure 18 (and unlikely to be attributed to sequencing mistakes).

Similarities within the ORFs of the *P. mirabilis ter* gene cluster

Comparisons of the predicted Ter polypeptides show widespread relatedness between pairs of ORFs and identify specific regions of conserved residues within the groups of ORFs. Similar to the situation for two plasmid-borne Ter^r systems (Whelan *et al.*, 1995; Jobling and Ritchie, 1988), the putative TerD and TerE proteins are similar in size and show 64% identity (Figure 20A). Furthermore, both proteins had nearly identical hydropathy profiles, with a highly hydrophilic central core and a short potential membrane-spanning hydrophobic helix at the C terminus (Figure 20B). These observations are in agreement with the earlier reports that pMER610-derived *terE* and *terD* are functionally interchangeable and are encoded on a single transcript (Jobling and Ritchie, 1987,1988).

Alignment of all six Ter polypeptides by BLOCK MAKER (Henikoff *et al.*, 1995) identified several regions (blocks) of the most highly conserved regions (Figure 21). Overall, the putative TerB polypeptide is the most dissimilar to the others. When the TerB sequence is included in the alignment, the region of conservation is reduced to a 16-aa stretch in the N-terminal regions of TerZ, TerA and TerB and in the C-terminal region of TerD and TerE. However, when TerB is excluded from the analysis, three additional regions of conservation are identified. The highest level of relatedness between multiple proteins is limited to the N-terminal half of TerD, TerE and TerZ sequences (Figure 21). Therefore, the N-terminal parts of these polypeptides are likely to contain functionally

important domains and/or motifs, and future function-structure analysis of these polypeptides should target these regions.

Similarity searches of coding sequences in GenBank demonstrated that all genes from the *P. mirabilis ter* cluster are related to the *yce* genes of *B. subtilis* (Table 3). Two of the four predicted Yce polypeptides share more than 50% identity with the TerD and TerE proteins. The biological role of the putative Yce proteins remains unknown, but their sequence similarity to the Ter systems has suggested a probable role in tellurite resistance (Kumano *et al.*, 1997). All of the Ter polypeptides, with the exception of TerC, are related to the *Clostridium acetobutilicum* genes implicated in sulfonate, mytomicin and UV-resistance, as was previously reported for the R478-encoded *ter* gene cluster (Whelan *et al.*, 1995). Similarity between some of the plasmid-borne Ter proteins and cyclic-AMP binding proteins of the slime mould *Dictyostelium discoideum* has already been established for R478 and pMER610 (Whelan *et al.*, 1995; Jobling and Ritchie, 1988, 1987). GenBank searches conducted in this study extend this relatedness to the Ter proteins of *P. mirabilis*. In addition, two of the Ter proteins are related to another cyclic-AMP binding protein produced by the *Synechocystis* spp. (Table 3).

The putative TerC protein shows almost no similarity to the polypeptides described in the above paragraph. However, the *terC* gene product shares approximately 30% identical residues with hypothetical proteins encoded by four bacteria: *Myxococcus xanthus*, *E. coli*, *Rickettsia prowazekii* and *Mycobacterium tuberculosis* (Table 3). The biological role for any of these proteins has not been

Figure 20. Sequence analysis of the putative TerE and TerD polypeptides

(**A**) CLUSTAL W alignment of TerD and TerE sequences; identical residues are shaded. (**B**) Hydropathy profiles of TerD and TerE generated using TMpred (9 aa window) (Hofmann and Stoffel, 1993); only values above 500 are considered significant. Protein sequences that plot above the central dotted line represent hydrophobic domains, whereas protein sequence that plot below the dotted line are hydrophilic. Two lines within each panel designate two possible orientations of the predicted transmembrane helices: the solid line indicates inside→outside orientation, the dotted line indicates outside→inside orientation. The orientation corresponding to the higher TMpred value is favoured.

A.

```
PmTerD    MSVSLSKGGNVSLSKAAPTMKNVLVGLGWDARSTDGQDFDLDASAFLLAANGKVRSDADF   60
PmTerE    MAVSLVKGGNVSLTKEAPTMTVAMVGLGWDARVTDGAEFDLDASVFMVGEDGKVLSDASF   60

PmTerD    IFYNNLRSSDGSVVFTGDNRTGEGDGDDEALKIKLDTIPSYVEKIIFVVTIHEAQPRRQS   120
PmTerE    IFFNNKVSQCGSVEHQGDNRTGEGEGDDEQVKITLSKVPARVKKLVFAVTIYDAENRKQN   120

PmTerD    FGQVSGAFIRLVNDDNQIEVARYDLTEDASTETAMLFGELYRHNGEWKFRAVGQGYAGGL   180
PmTerE    FGMVSNSFMRVVNNDNNTEIARFDLSEDASTETAMIFGELYRHGSDWKFRAVGQGFAGGL   180

PmTerD    GSVCAQYGINAS     192
PmTerE    GALAAQHGVNI-     191
```

B.

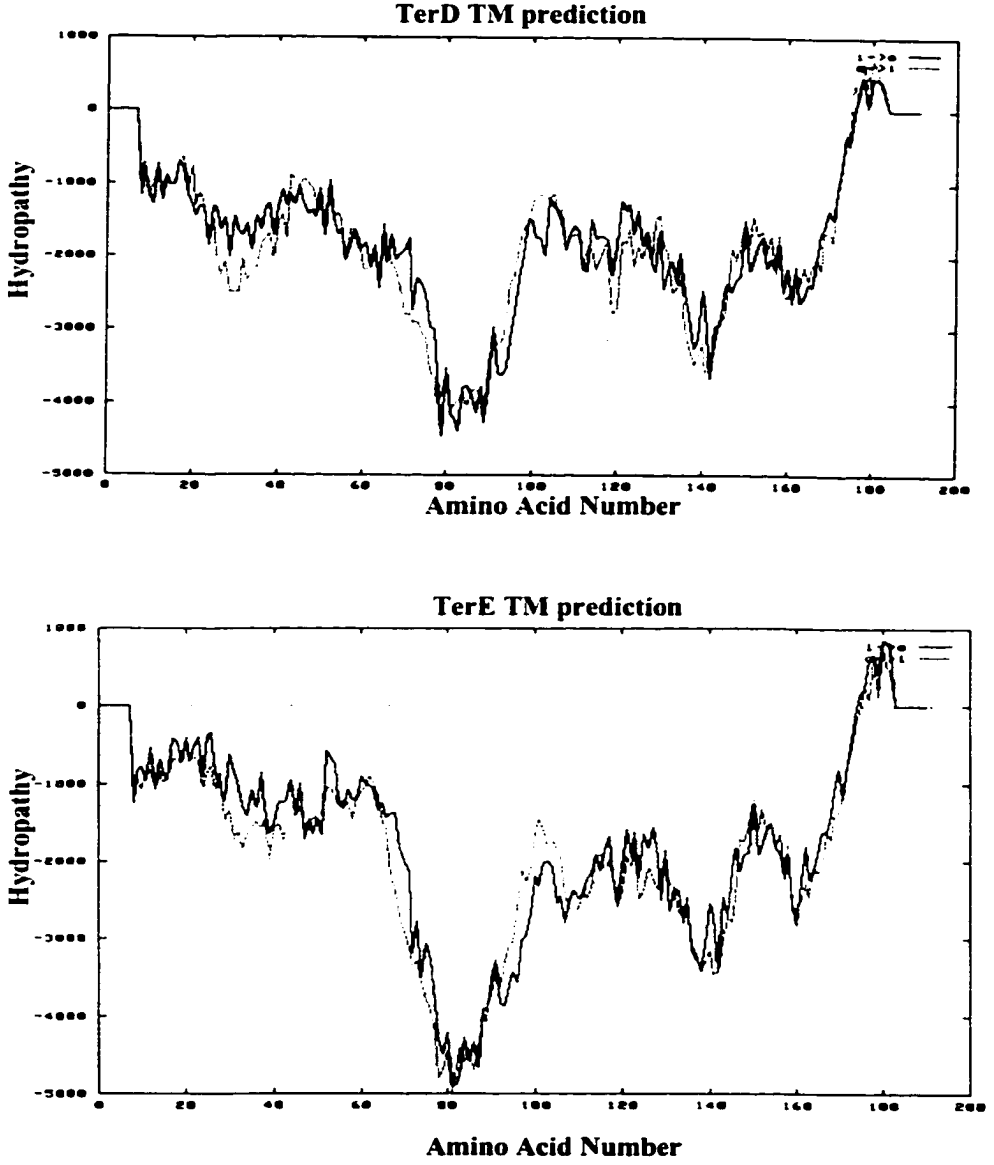


Figure 21. BLOCK MAKER analysis of the internal similarities in the ORFs of the *P. mirabilis ter* gene cluster.

Each block represents a stretch of short multiply aligned ungapped segments corresponding to the most highly conserved residues. Identical residues are shaded.

Protein	Position of block (aa number)	block	Position of block (aa number)	block
TerA	138	VSLTKEKPAIS	176	LGGLFGGNKGIDLD
TerC	2	VSTHIGFPTET	21	IGAIFIDLFMHRAD
TerD	11	VSLSKAAPTMK	27	LGWDARSTDGQDFD
TerE	11	VSLTKEAPTMT	27	LGWDARVTDGAEFD
TerZ	10	VSLSKQAPALS	37	LGGLFGGNNSIDLD

TerA	322	DNAYGWGFR
TerC	206	DITIGRGTK
TerD	78	DNRTGEGDG
TerE	78	DNRTGEGEG
TerZ	83	DNLTGEGDG

TerA	93	GSWKFRFIAQGFNGGL
TerB	23	SRFKNKKFMQGTVAVC
TerC	101	DRFRHRILYWGIIGAI
TerD	165	GEWKFRAVGQGYAGGL
TerE	165	SDWKFKAVGQGFAGGL
TerZ	29	DPIKKKGFLGGLFGGN

TABLE 3. Proteins similar to *P. mirabilis* putative Ter proteins.

Protein identity (%) (protein X :*P. mirabilis* protein)*	Function and references
SmTerZ:TerZ 73 :TerD 36 :TerE 33 **SmTerA**:TerA 66 **SmTerB**:TerB 81 **SmTerC**:TerC 87 **SmTerD**:TerZ 36 :TerD 92 :TerE 63 :TerE 88 **SmTerE**:TerZ 33 :TerD 63 **SmTerF**:TerD 25	*Serratia marcescens* plasmid R478. Phage inhibition, colicin resistance, tellurite resistance (Whelan *et al.*, 1995)
TerX:TerD 39 :TerE 36	Protection from the toxic effects specified by the *terZ* locus (Whelan *et al.*, 1997)
AsTerA:TerA 66 **AsTerB**:TerB 78 **AsTerC**:TerC 87 **AsTerD**:TerZ 36 :TerD 90 :TerE 63 **AsTerE**:TerZ 34 :TerD 63 :TerE 81	*Alcaligenes* sp. PMER610. Plasmid-borne tellurite resistance (Jobling and Ritchie, 1988; Whelan *et al.*, 1995)
YceC:TerZ 37 :TerD 36 :TerE 43 **YceD**:TerZ 36 :TerD 55 :TerE 52 **YceE**:TerZ 35 :TerD 52 :TerE 50 **YceF**:TerC 21	*Bacillus subtilis*. Probable tellurite-resistance related proteins (Kumano *et al.*, 1997)

stkORF319:TerC 30	*Myxococcus xanthus*. (Kupfer *et al.*, 1996).
YGJT:TerC 30	*Escherichia coli*, putative inner membrane protein in EBGC-UXAA intergenic region (Blattner *et al.*, 1997)
RPYGJT:TerC 27	*Rickettsia prowazekii* hypothetical protein (Andersson and Andersson, 1997)
Rv2723:TerC 26	*Mycobacterium tuberculosis*. Probable membrane protein of unknown function (Cole *et al.*, 1998)
OrfB:TerZ 33 :TerD 55 :TerE 48 **OrfC**:TerZ 35 :TerD 52 :TerE 50	*Clostridium acetobutilicum*. Methyl methane sulfonate, mytomicin C, UV resistance (Azzeddoug and Reysset, 1994)
CABP:TerZ 29 :TerE 30	*Synechocystis* sp. Cyclic-AMP binding protein (Kaneko *et al.*, 1996)
CABP1:TerZ 32 :TerD 48 :TerE 50	*Dictyostelium discoideum*. Cyclic-AMP binding protein (Bain *et al.*, 1991)

*Percentage of identical amino acids estimated by CLUSTAL W analysis. Only scores above 20% are shown.

established, but, interestingly, like TerC, they all are predicted to be integral membrane proteins. The similar subcellular location of these similar proteins suggests that they have similar functions, perhaps forming a membrane-associated complex with other proteins.

Transcript analysis of *P. mirabilis ter* gene cluster by northern blot analysis

This study investigated transcriptional expression of the Te^r determinants of *P. mirabilis* using northern blot analysis. This approach was chosen because it would allow direct detection of the mRNA transcript(s) encoded by the *ter* gene cluster. Additionally, analysis of cells grown with and without tellurite would allow assessment of the effect of tellurite on transcription of the *ter* genes. Northern blot hybridization analysis, with two different DNA probes internal to the *ter* locus coding region, revealed the presence of multiple hybridization bands in RNA extracted from WT as well as the mutant strain described here (Figure 22A and B). However, in both strains hybridization bands were detectable only when RNA was extracted from bacteria grown in the presence of potassium tellurite.

Five transcripts of 5.0, 3.0, 2.7, 2.0 and 1.5 kb were detected when a 1.3-kbp *Hin*dIII fragment (partial *terC-terD* coding region) was used as a probe (Figure 22A). Only three clear signals of 5.0, 2.5 and 2.0 kb were detected when a 0.9-kbp *Bam*HI – *Eco*RI fragment (partial *terZ-terA* coding region) was used as a probe (Figure 22B). The sharpness and intensity of the 5-kb band detected with both probes suggested that this corresponds to the multicistronic product of the entire *ter* locus. The identities of the other bands were more difficult to

establish because some of the smaller signals could have been degradation products of larger transcripts. Furthermore, extensive sequence similarity among individual *ter* genes (see Figures 20 and 21) could have resulted in non-specific hybridization, giving false positive signals. For example, the 0.9-kbp *Bam*HI-*Eco*RI probe containing the partial *terZ-terA* coding region may hybridize not only to transcripts of the *terZ* and *terA* genes, but also to mRNA transcribed from the *terD*, *terE* and, possibly, the *terC* genes.

The intensities of several bands, especially that corresponding to the 5-kb transcript, appeared to be significantly less in the mutant strain as compared to WT, suggesting that the mutant strain is impaired in the ability to transcribe the *ter* locus. Alternatively, the mini-transposon insertion in the *terC* coding region of the mutant strain could have decreased the stability of mRNA corresponding to the *ter* gene cluster. It is reasonable to suggest that the 5-kb band in the mutant strain corresponds to a partial *ter*-locus transcript fused to the mRNA for the reporter gene (*lacZ*). Such "hybrid" transcript would be expected to differ in size from the "full-length" transcript encoding an entire *ter* locus in the WT strain. However, the expected difference in size was not apparent on these northern blots, perhaps because the size differences of transcripts of such large sizes would not have been resolved. The putative "hybrid" transcript from the mutant strain could have been less stable, thus providing an alternative explanation for reduction in intensity of the hybridization signal detected for this strain.

In addition to possible induction by tellurite, another possibility still remained that no *ter*-specific signal was observed in the absence of potassium

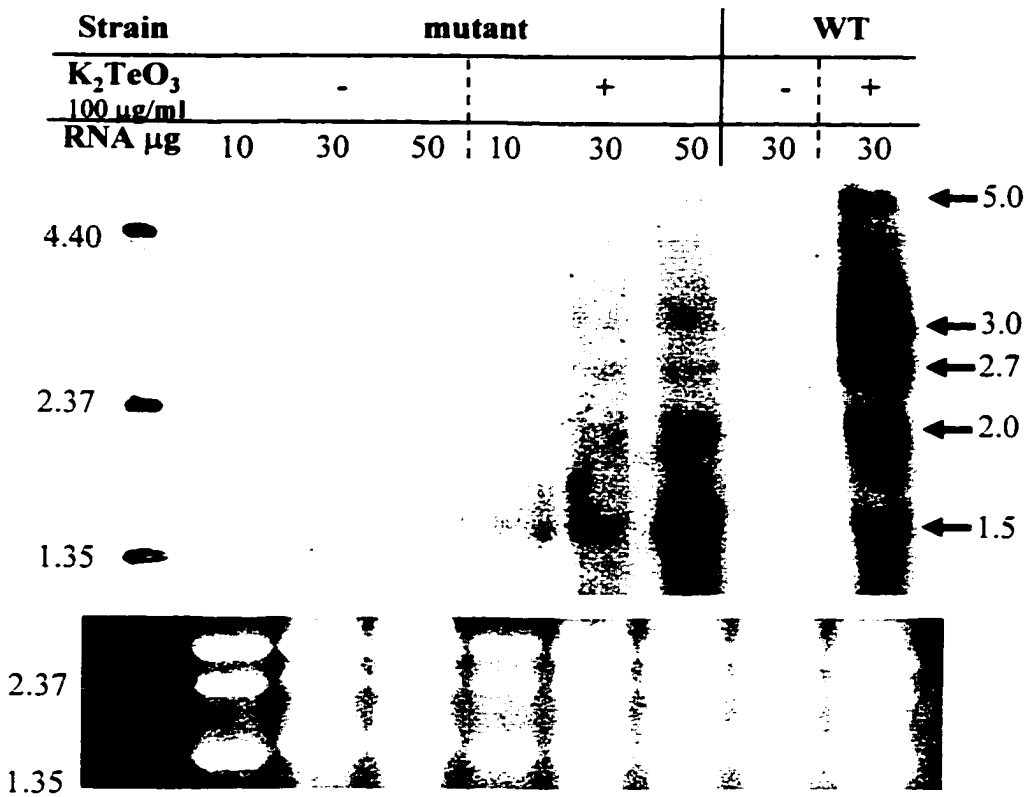
tellurite due to degradation of the RNA (despite the presence of intact ribosomal RNA as shown on the bottom panels in Figure 22A and B). To address this possibility, an excess of RNA from the WT and the mutant strains grown without potassium tellurite was hybridized with the *terC – terD* probe, and the resulting blot was exposed for a longer period of time. Four hybridization signals of 5.0, 3.0, 2.5 and 1.5 kb were detected for both strains (Figure 22C). Interestingly, all the signals were less intense in the mutant strain, in agreement with the previous experiment. Taken together, the results obtained by Northern blot analysis with the *ter*-specific probes indicate that potassium tellurite has a positive affect on expression of the *ter* genes in *P. mirabilis*. These experiments also raised a possibility that multiple internal transcription start sites exist within the *ter* gene cluster, in addition to the one upstream of *terZ*. However, defining the number and location of such transcription sites would require further mapping by primer-extension analysis.

The positive effect of potassium tellurite appeared to be specific to the expression of *ter* genes. When a 1.6-kbp *Bam*HI PCR amplicon encoding *P. mirabilis ureR* was used as a hybridization probe, potassium tellurite appeared to have no effect on hybridization signals detected in both strains (Figure 23). Specifically, a strong band of the expected size at approximately 2 kb (indicated by the arrow) was present in all lanes. The hybridization signal in this experiment was not affected by the presence of potassium tellurite, indicating that potassium tellurite does not non-specifically stimulate transcription levels in

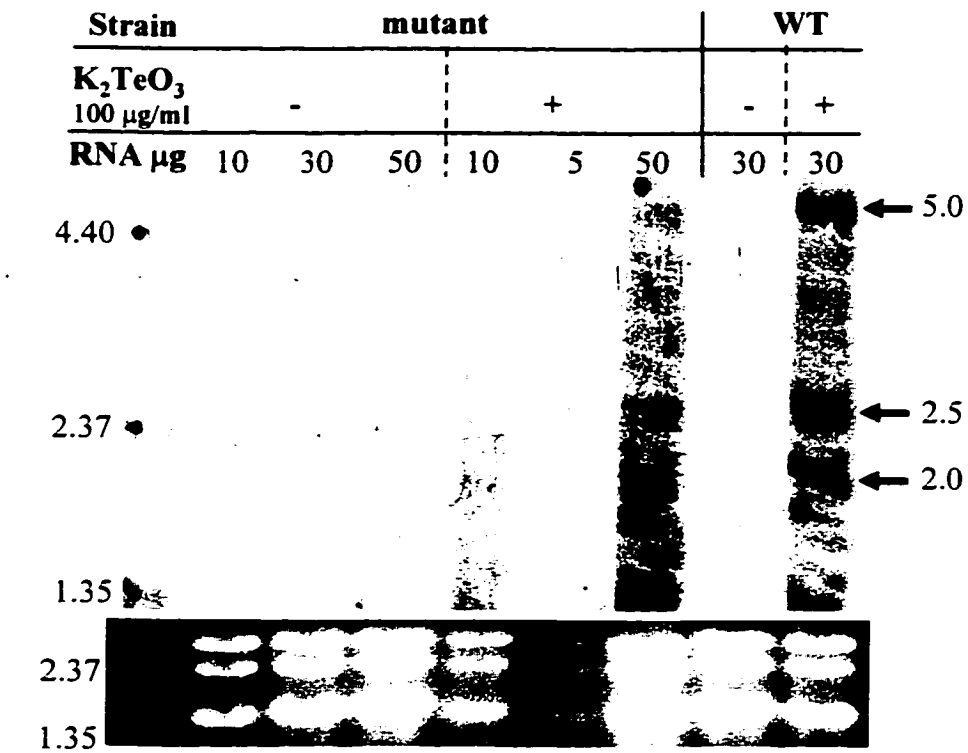
Figure 22. Northern blot hybridization with *ter*-specific probes

RNA was isolated from *P. mirabilis* strains S2R (WT) and S2R- 6/39 (mutant) grown either in the presence or absence of potassium tellurite (indicated by the plus or minus signs). The bottom panels show ribosomal RNA in the gel before transfer, to indicate amounts loaded. Positions of molecular-weight standards are shown on the left of each panel (in kb). (**A**) and (**C**) A 1.3-kbp *Hin*dIII fragment containing sequences for the *terC-terD* coding region was used as a probe; (**B**) A 0.9-kbp *Bam*HI-*Eco*RI fragment contacting sequences for the *terZ-terA* coding region was used as a probe.

A.


Strain
mutant
WT
K_2TeO_3 100 µg/ml
-
+
-
+
RNA µg
10
30
50
10
30
50
30
30
4.40
2.37
1.35
5.0
3.0
2.7
2.0
1.5
2.37
1.35

B.


Strain
mutant
WT
K_2TeO_3
100 µg/ml
-
+
-
+
RNA µg
10
30
50
10
5
50
30
30
4.40
2.37
1.35
5.0
2.5
2.0
2.37
1.35

C.

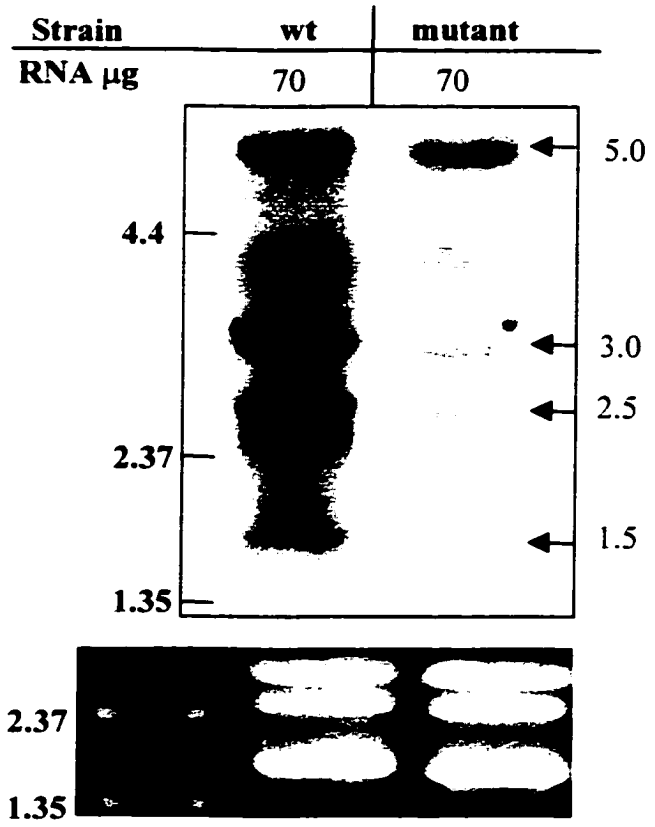
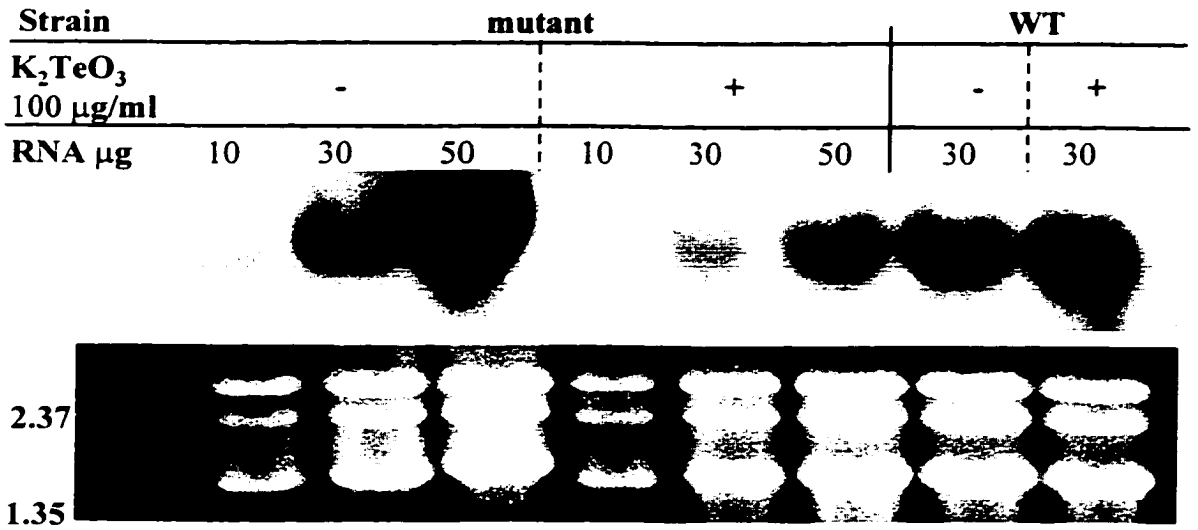


Figure 23. Northern blot hybridization with the *ureR*-specific probe

RNA was isolated from *P. mirabilis* strains S2R (WT) and S2R-6/39 (mutant) grown either in the presence or absence of potassium tellurite (indicated by the plus or minus signs, respectively). The bottom panel shows ribosomal RNA in the gel before transfer, to indicate amounts loaded. Positions of molecular-weight standards are indicated on the left. A 1.6-kbp *Bam*HI fragment encoding *P. mirabilis ureR* was used as a probe.


Strain
mutant
WT
K_2TeO_3
100 µg/ml
-
+
-
+
RNA µg
10
30
50
10
30
50
30
30
2.37
1.35

P. mirabilis. This observation provides addition evidence for the idea that potassium tellurite specifically activates expression of the *terZ* locus.

Primer extension analysis

Primer-extension studies were carried out to map the location of the promoter region located upstream of the *terZ* and to confirm the results of northern blot hybridization. The analysis identified a single transcription start site at positions -51 and -52 relative to the *terZ* translation start site (Figure 24, indicated by the arrowheads). The intensity of the signal at the -52 position was somewhat greater, indicating that transcription may initiate more frequently at this position than at position -51. The locations of the presumptive -10 and -35 promoter sequences are in agreement with the identified transcriptional start sites (Figure 24 and 25). The putative –10 (TATAAT) and –35 (TTCATC) promoter sequences of the *terZ* locus are identical to the *E. coli* $E\sigma^{70}$ consensus sequences (Hoopes and McClure, 1987). Sequence searches for oligonucleotide repeats (Solovyov *et al.*, 1984) within the *terZ* promoter region identified a pair of 7-base pair inverted repeats (TCATCAT) between the –35 and –10 sequences, and a pair of 7-base pair direct repeats (ATTTATA) overlapping the –10 consensus sequence and transcription start sites (Figure 25). These repeats warrant further analysis, because they may represent binding sites for putative transcriptional regulatory proteins (which remained to be identified).

Similar to the results obtained by northern blot hybridization, the primer-extension signals were only detectable using RNA from bacteria grown in the

Figure 24. Primer-extension analysis of the transcription start sites for the *terZ* locus

Total RNA was extracted from WT *P. mirabilis* grown in the presence or absence of potassium tellurite and hybridized to the radiolabelled TERZR oligonucleotide (Table 1). The arrowheads mark the transcription start sites, and the corresponding major extension products. The putative -10 promoter region is underlined. The sequencing ladder was generated using the same oligonucleotide and pBSK-C1.6 as a template.

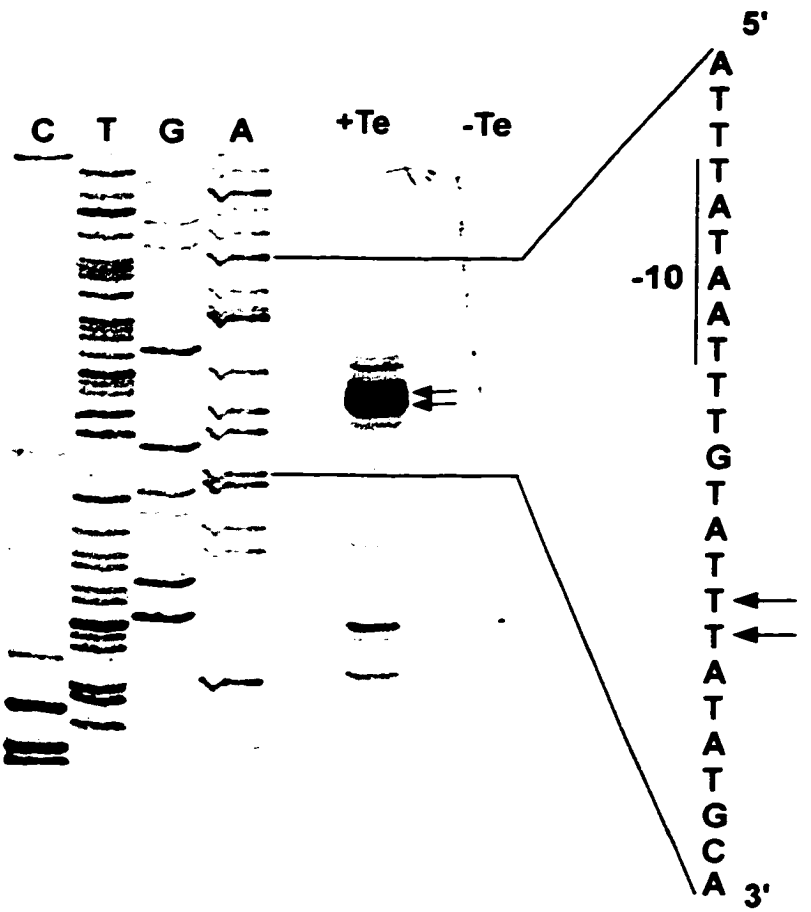
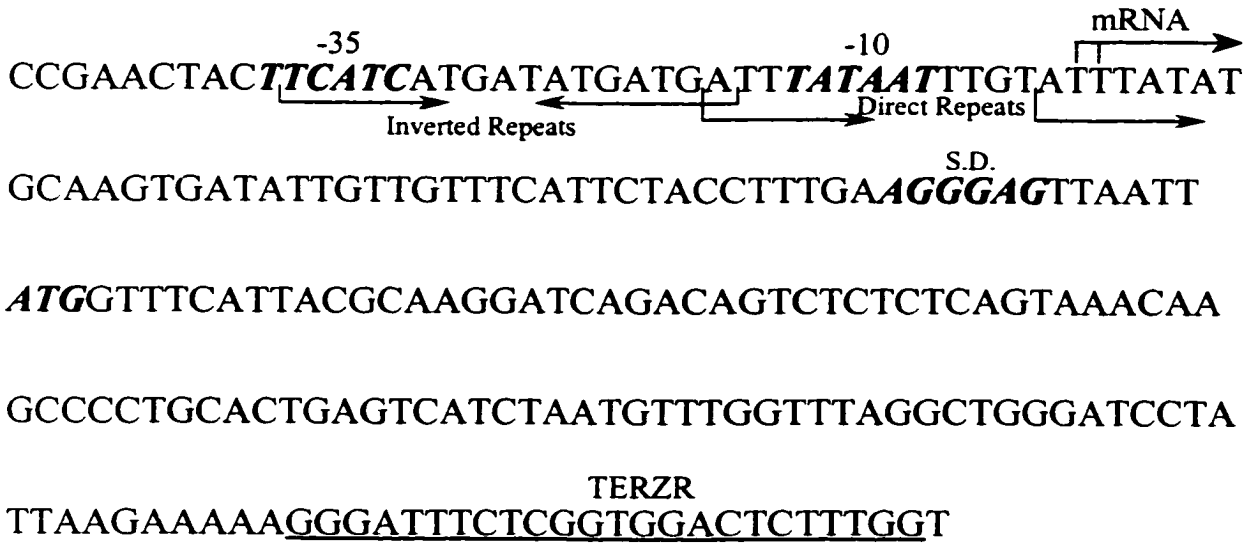

C
T
G
A
+Te
-Te
5'
A
T
T
T
A
T
A
A
T
T
T
G
T
A
T
T
T
A
T
A
T
G
C
A
3'
-10

Figure 25. Sequence analysis of the *terZ* promoter region

The arrow above the promoter region sequence indicates the transcription start site (TT). The arrows below the sequence indicate direct and inverted repeats identified within this region. Presumptive promoter sequences (-10 and -35), ribosome binding site (S.D.) and translational start site (ATG) are shown in bold italic font. The underlined sequences indicate the TERZR oligonucleotide annealing site.


mRNA
-35
-10
CCGAACTACTTCATCATGATATGATGATTTATAATTTGTATTTATAT
Inverted Repeats
Direct Repeats
S.D.
GCAAGTGATATTGTTGTTTCATTCTACCTTTGAAGGGAGTTAATT
ATGGTTTCATTACGCAAGGATCAGACAGTCTCTCTCAGTAAACAA
GCCCCTGCACTGAGTCATCTAATGTTTGGTTTAGGCTGGGATCCTA
TERZR
TTAAGAAAAAGGGATTTCTCGGTGGACTCTTTGGT

presence of tellurite (Figure 24). These results confirmed that the expression of the *ter* locus is inducible by potassium tellurite (at least for a single promoter defined in this part of the study).

DISCUSSION

Over the last decade, transposon mutagenesis has been used extensively to characterize genes involved in swarmer cell differentiation in *P. mirabilis* (Allison and Hughes, 1991; Belas *et al.*, 1991; 1991a). While many genes have been identified, the developmental program remains poorly understood. The statement made by J. Kvittingen (1949) reflects the level of our current understanding of this phenomenon: "In the course of the time this work on the life-style of *Proteus* has been going on, and as new details have been clarified, the feeling has increased that the full understanding of the biology and sociology of the microbe becomes increasingly evasive".

I used transposon mutagenesis (Herrero *et al.*, 1990) in an attempt to generate promoter-reporter gene (*lacZ*) fusions in developmentally regulated genes. One of the recovered transconjugants (*P. mirabilis* strain S2-6/39) exhibited negative regulation during swarming. Namely, the short vegetative (swimmer) cells of this strain showed a four-fold increase in the activity of the reporter gene as compared to the swarmer cells (Figure 2B). A similar decrease in reporter-gene activity was observed as the cell density of the mutant strain increased in broth cultures (Figure 2A). Cell density has been demonstrated to be one of the signals triggering swarmer cell differentiation in *P. mirabilis* (Belas *et al.*, 1998; Rauprich *et al.*, 1996). Therefore, it was reasonable to hypothesize that the mini-transposon insertion occurred in a developmentally repressed locus. Characterization of the site of the mini-transposon insertion identified a

chromosomal *ter* locus encoding tellurite resistance, which in other bacteria also confers colicin resistance and phage inhibition. Transcript analysis demonstrated that the *ter* locus is inducible by potassium tellurite. Attempts to determine the molecular basis of the developmental regulation of the *ter* genes gave inconclusive results. However, I have obtained evidence that both potassium tellurite and the developmental program control expression of the *ter* genes.

Characterization of the site of the mini-transposon insertion

In the mutant strain that I characterized, the mini-transposon had integrated into an open reading frame that was highly similar to the plasmid-borne *terC* genes of Gram-negative bacteria (Jobling and Ritchie, 1988; Whelan *et al.*, 1995). In two other bacterial systems, *terC* is the fourth gene of the seven-gene cluster (*terZABCDEF*). Transposon mutagenesis analysis indicates that the three phenotypes mediated by the *ter* locus are genetically linked, and require a functional *terC* gene (Whelan *et al.*, 1995). The *P. mirabilis terC* mutant characterized in this study displayed a decreased tolerance to potassium tellurite (above 150 μg/ml), confirming the role of this gene in tellurite resistance (Figure 6). Interestingly, the *terC* mutant is significantly less sensitive to tellurite than are the R478 plasmid *terC* mutants generated by Whelan and co-workers. In the R478 plasmid-borne Te^r system, insertion of the Tn*1000* transposon in any position within the *terC* coding region renders the resulting strain sensitive to tellurite concentrations above 20 μg/ml (Whelan *et al.*, 1995). Jobling and Ritchie

reported similar results for mutagenic analysis of the pMER610 Te^r system: disruption of *terC* produced mutants fully sensitive to tellurite (Jobling and Ritchie, 1987, 1988). Different levels of sensitivity of the *terC* mutants in chromosomal and plasmid-borne Te^r systems have several possible explanations. In the *P. mirabilis* mutant strain, the mini-transposon insertion into the C terminus of the *terC* gene might still permit expression of a partly functional protein. Alternatively, the downstream *terD* and *terE* genes may be expressed by read-through from the promoter of the inserted kanamycin-resistance cassette, or from an internal promoter in the *terC-terD* intergenenic region that is not present in the R478 Te^r system. Interestingly, Northern blot analysis suggested the existence of multiple promoters within the pMER610 *ter* locus, with one of the promoters located in the *terC-terD* intergenic region (Hill *et al.*, 1993). However, our current knowledge of transcriptional organization and regulation of the Te^r systems in insufficient to provide a conclusive explanation.

Complementation analysis

The complementation studies described here demonstrated that a partial R478 *ter* locus (a 5.8-kbp *Bam*HI-*Sal*I fragment) could restore the wild-type level of tellurite resistance in the *terC* mutant of *P. mirabilis* (Figure 5 C,D). Furthermore, the 5.8-kbp *Bam*HI-*Sal*I fragment contains an entire tellurite resistance determinant, since it is sufficient to allow otherwise fully sensitive *E. coli* JF626 cells to grow in the presence of potassium tellurite (100 μg/ml). This observation is in agreement with the earlier report from Jobling and Ritchie

(1988) that pMER610 *terA-terD* products are necessary and sufficient for tellurite resistance. Taken together, the results of my complementation and sequence analysis of the *P. mirabilis ter* gene cluster suggest that the *terZ* and *terF* genes may not be required for expression of a tellurite-resistance phenotype. Alternatively, the *terZ* and *terF* polypeptides may have a role in tellurite resistance, but over-expression of the remaining *ter* genes from the recombinant plasmid might compensate for the lack of functional TerZ and TerF polypeptides in the *E. coli* cells. The latter explanation would require that a certain degree of functional redundancy exists among the Ter polypeptides. Such functional redundancy was proposed for the TerD and TerE proteins (Jobling and Ritchie, 1987). Clearly, additional studies involving expression of the *ter* coding sequences from a single-copy chromosomal insertion (to eliminate the potential gene-dosage effect) are required to investigate this possibility further.

Characterization of the *terC* gene

The TerC protein of *P. mirabilis* shows strong sequence similarity to the plasmid-encoded TerC proteins as well as to polypeptides of *E. coli*, *Mycobacterium tuberculosis*, *Rickettsia prowazekii*, *Myxococcus xanthus* and *Bacillus subtilis* (Figure 28A; see references in Table 3). Jobling and Ritchie predicted that the TerC polypeptide of pMER610 functions as an integral inner-membrane protein, and identified nine putative hydrophobic transmembrane helices within the protein sequence (Jobling and Ritchie, 1988). The same inner-

membrane location is predicted here for the TerC protein of *P. mirabilis*, using an algorithm of Nakai and Kanehisa (1992).

Secondary-structure analysis of the TerC protein

A two-dimensional model of the predicted TerC topology is shown in Figure 26. The hydropathy analysis of TerC revealed nine hydrophobic segments with lengths of 17 to 22 residues. Each of these regions has TMpred values of over 1000 (Figure 19A). Thus, these segments meet the requirements used in the TMpred algorithm for transmembrane helices (Hofmann and Stoffel, 1993). According to this model, segments of the TerC polypeptide are exposed on each side of the cytoplasmic membrane. The topological model in Figure 26 is consistent with the "positive inside" rule of von Heijne and Gavel (1988), which states that cytoplasmic domains of membrane proteins are enriched in lysine and arginine residues. However, the TerC topology model is only a prediction, and the exact length and position of membrane-spanning domains has to be defined by structure-function analysis. Generation of a series of *terC* translational fusions to the *E. coli phoA* or *lacZ* reporter genes might be used to confirm that TerC functions as an integral inner-membrane protein, as well as to define the length and position of the membrane-spanning domains (Haardt and Bremer, 1996). Additionally, regulation of *terC* expression during the developmental cycle of *P. mirabilis* could be monitored using green fluorescent protein (GFP) translational fusions. Such GFP fusions are usually non-toxic to the host bacterium and have been used extensively in recent years in diverse microorganisms (Gerdes and Kaether, 1996). Therefore, such fusions may

overcome some potential difficulties connected with expression and assembly of a rather large and bulky β-galactosidase tetramer encoded by the *lacZ* reporter gene.

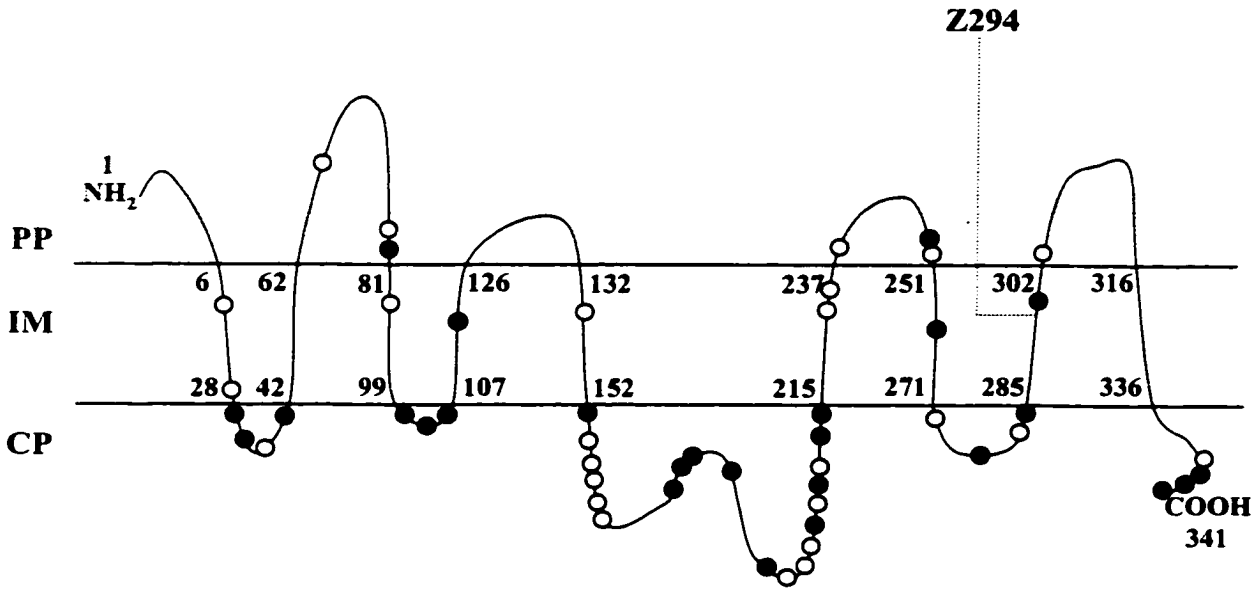
Hydropathy analysis and secondary-structure predictions indicate that, with the exception of TerC, all of the Ter polypeptides are likely to function in the bacterial cytoplasm. Therefore, I hypothesize that the TerC protein functions as a membrane-associated transporter. This hypothesis can be tested by assessing uptake of tellurite ions by TerC-containing vesicles (Kaback, 1971). Of note, seven of the nine predicted TerC transmembrane helices contain charged residues, which is unusual for typically apolar transmembrane regions (von Heijne and Gavel, 1988). Cox *et al.* proposed that charged amino acids would be found embedded within membrane-spanning domains of transport proteins only if they are functionally important in the transport processes (Cox *et al.*, 1988; 1989). Therefore, site-directed mutagenesis of the charged residues within the membrane-spanning domains would be instrumental in determining whether TerC functions as a membrane-associated transporter.

Comparative homologies of the TerC polypeptides and functional implications

P. mirabilis TerC and all of its homologues are predicted to be integral inner-membrane proteins (Nakai and Kanehisa, 1992). Moreover, four of the TerC homologues (hypothetical proteins from *E.coli, R. prowazekii, M. tuberculosis* and ORF319 from the *stk* locus of *M. xanthus*) have remarkably similar hydropathy profiles (variable only in the carboxyl termini of the polypeptide chains) despite limited overall sequence similarity (Figure 27).

Figure 26. Model for the topological organization of TerC

A model for the two-dimensional topology of TerC within the inner membrane is shown. The first and the last residues within predicted transmembrane segments are indicated. The dotted line and letter Z indicate the position of the ß-galactosidase fusion due to mini Tn5 insertion. The numbers following letter Z identify the *terC* codon carrying the fusion junction. The solid circles indicate positively charged residues (R, K); the open circles indicate negatively charged residues (D, E); IM, inner membrane; PP, periplasm; CP, cytoplasm.


Z294
1
NH2
PP
IM
CP
6
62
81
126
132
237
251
302
316
28
42
99
107
152
215
271
285
336
COOH
341

The conservation of predicted subcellular localization and secondary structure for these proteins might reflect their common function, perhaps as membrane-associated transport proteins. At the same time, limited sequence similarities detected among these polypeptides may be due to differences in their substrate specificities. Taken together, these observations and speculations suggest that TerC and its homologues may represent a novel family of bacterial transporter proteins.

The third cytoplasmic domain predicted within TerC (aa 152-215) is particularly interesting (Figure 26), because this 63-residue domain is highly hydrophilic and contains a large number (20) of both positively (lysine, arginine) and negatively (aspartate, glutamate) charged residues. This domain is predicted to form a major loop protruding into the bacterial cytoplasm, where it might interact with other proteins mediating tellurite resistance (TerE/TerD) and /or additional yet-unknown proteins (Figure 19A and 26). The four aforementioned TerC homologues all contain large highly hydrophilic domains between aa 150 and 200 comprising 10 to15 charged residues, but which otherwise show no significant sequence similarities (see Figure 27 for references). These domains may be critical for the biochemical function of TerC and its homologues. Future research using linker-insertion and site-directed mutagenesis is required to evaluate the involvement of this large hydrophilic domain (especially its charged residues) in proposed protein-protein interactions.

Sequence analysis performed in this study identified homology between the putative TerC protein and ORF319 from the *stk* locus of *M. xanthus* (Kupfer

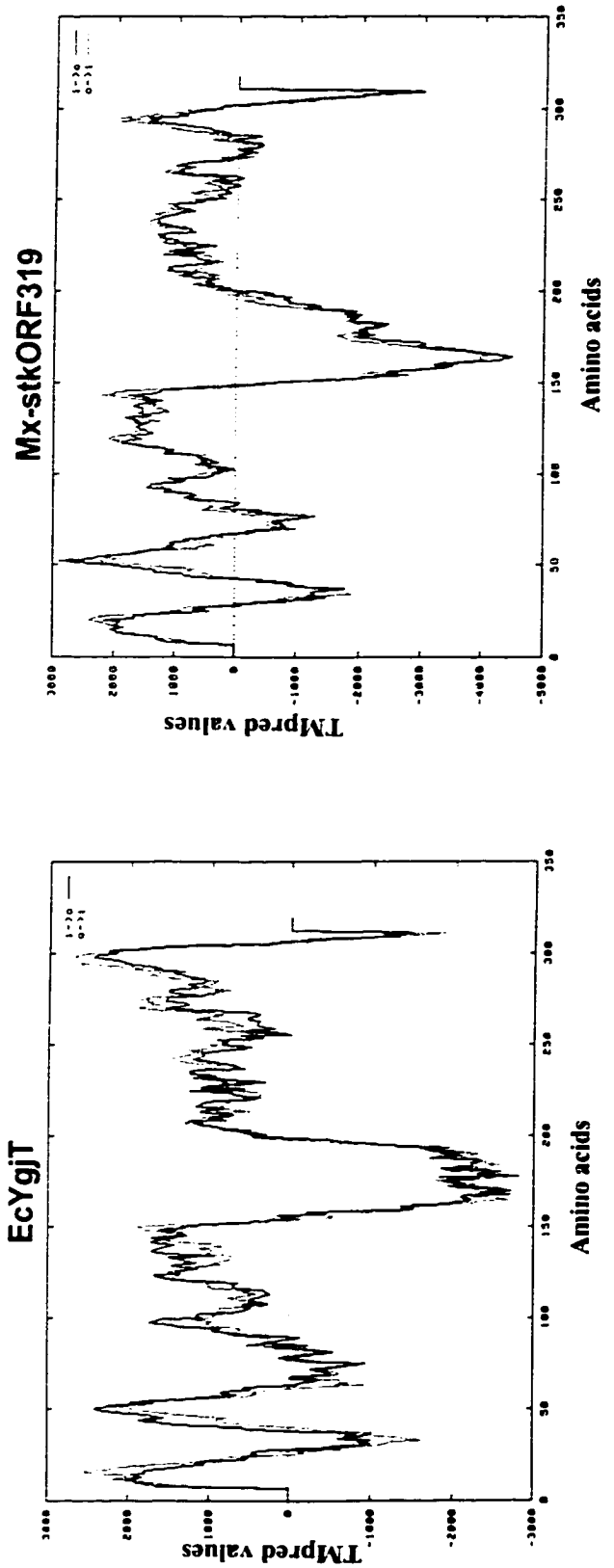
et al., 1996). Transposon mutagenesis analysis implicated the *stk* locus in the so-called adventurous motility that is utilized by individual cells of *M. xanthus* (Dana and Shimkets, 1993). Characterization of the *stk* null mutation in otherwise wild-type cells and in social (group) motility mutants led to the suggestion that this locus may act as a negative regulator of fibril synthesis. Fibrils are generally observed on myxobacteria exhibiting social (group) motility, and are believed to be required for cell-cell contact and/or signaling (Behmlander and Dworkin, 1991). Roles for both cell-cell contact and intercellular communication have also been suggested in *P. mirabilis* swarming (Allison *et al.*, 1992a; Belas *et al.*, 1998; Rauprich *et al.*, 1996). The biochemical mechanism of action of the *stk*-encoded product(s) remains unknown. Currently, the significance of primary-sequence homology between the TerC and *stk*-encoded polypeptide(s) is unclear. However, it does raise the possibility that TerC may be a part of a multi-protein complex (including TerC, TerD, TerE and possibly additional yet-unknown proteins) that is homologous to the *M. xanthus* fibril biogenesis apparatus. A similar relationship has been established between the contact-induced type III protein secretion machinery and flagellar biosynthetic proteins of Gram-negative bacteria (Hueck, 1998).

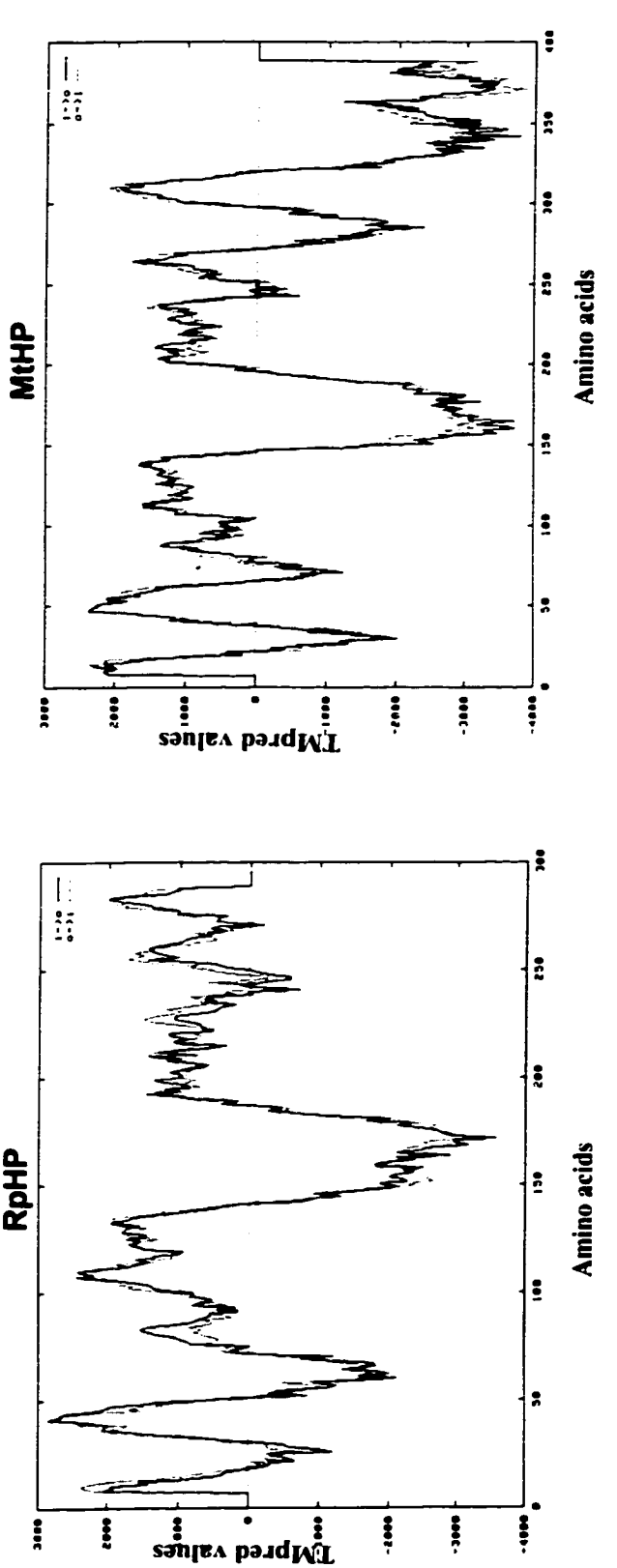
Leucine-zipper motif

A search for protein patterns and motifs identified a leucine-zipper motif in the carboxyl terminus of TerC (Figure 19B). A copy of this motif was also identified in the plasmid-encoded TerC proteins (Figure 19B). This similarity was expected, given very high degree of overall sequence similarity between the

Figure 27. Hydropathy analysis of the putative bacterial polypeptides homologous to TerC

The prediction graphs were generated using TMpred (Hofmann and Stoffel, 1993) for the hypothetical proteins from *E. coli* (EcYgjT) (Blattner *et al.*, 1997). *R. prowazekii* (RpHP) (Andersson and Andersson, 1997), *M. tuberculosis* (MtHP) (Cole *et al.*, 1998), and ORF319 in the *stk* locus of *M. xanthus* (Kupfer *et al.*, 1996). Each panel contains two lines, designating two possible orientations for the predicted transmembrane helices: the solid line indicates inside→outside orientation, the dotted line indicates outside→inside orientation. The orientation corresponding to the higher TMpred value is preferred. TMpred value is plotted on the vertical axis, while amino acid position is plotted on the horizontal axis.


Mx-stkORF319
TMpred values
Amino acids


MtHP
TMpred values
Amino acids

EcYgjT
TMpred values
Amino acids

RpHP
TMpred values
Amino acids

plasmid-encoded TerC proteins and their counterpart from *P. mirabilis* (87% identity [Table 3]). Surprisingly, a copy of the motif is also present in the hypothetical protein of *M. tuberculosis*, though overall sequence similarity to TerC proteins is low (Figure 19B). The biological function of this protein is unknown, and its possible role in tellurite resistance, as suggested by the similarities to the TerC, remains to be confirmed by further experimental work.

Secondary-structure predictions indicate that the leucine-zipper region (aa 268-289) forms an amphipathic α-helix (Chou and Fasman, 1978; Robson and Garnier, 1993). I speculate that several TerC polypeptides may use the leucine-zipper motifs as dimerization surfaces (Kouzarides and Ziff, 1989) to form pore-like structures within the bacterial membrane, which somehow mediate Te^r phenotypes. Other putative Ter proteins (e.g., TerD and TerE) may also participate in the formation of these structures (see below). Alternatively, TerC may interact with a component of the TonB-ExB-ExD uptake system, which is involved in the uptake of several pore-forming colicins and several phages (Braun and Herman, 1993; Postle, 1990). This hypothetical interaction may represent a tentative link between the TonB system and the Te^r determinants, as was suggested by Whelan *et al.* (1995).

Recently, Dinsmore *et al.* reported that a leucine-zipper motif in the AbiA protein of *Lactococcus lactis* is required for the resistance of this bacterium to a variety of bacteriophages (Dinsmore *et al.*, 1998). The AbiA protein plays a role in an abortive bacteriophage infection mechanism that is widespread in lactococci. The protein inhibits phages early in the infection process, since no

replication of phage DNA is observed in strains harboring the plasmid-encoded AbiA protein (Hill *et al.*, 1991). The biochemical mechanism of the abortive bacteriophage infection is still unknown. Therefore, any analogy to the bacteriophage attachment inhibition associated with the Te^r determinants is premature. However, the observation that two bacterial proteins involved in diverse bacteriophage resistance mechanisms contain the same protein motif suggests an interesting venue for further investigation.

Similarities of the putative TerZ, TerA, TerD and TerE proteins, and functional implications

Currently there are seven characterized systems which contain genes highly similar to the *ter* genes (*terZ, -A, -D* and *-E*) of *P. mirabilis* (Figure 28B, pp.192).

Plasmid-borne *ter* loci

Two large conjugative IncH plasmids, pMER610 and R478, each contain a cluster of seven genes that specify phage inhibition, tellurite and colicin resistance (Jobling and Ritchie, 1988; Whelan *et al.*, 1995). The open reading frames identified in this study were designated *terZ*, *terA-terE* due to their strong similarities (up to 92% amino acid sequence identity) to the corresponding plasmid-borne *ter* genes. Only one of the three phenotypes that may be encoded by this gene cluster, tellurite resistance, was investigated in this study. The remaining two phenotypes of phage attachment inhibition and colicin resistance are also expected to be encoded by the *P. mirabilis ter* locus, because

of the strong similarity with the known Te^r systems. Almost one hundred bacteriophages have been isolated on strains of *P. mirabilis*, *P. vulgaris* and *P. rettgeri* (Hickman and Farmer, 1976; Alisky *et al.*, 1998), so it is reasonable to expect Ter proteins to confer resistance to bacteriophages. Investigation of these phenotypes would provide interesting information regarding the effects of the transposon insertion in the *terC* coding region. Additionally, such experiments would determine what effect, if any, the lack of a *terF* homologue has on levels of colicin resistance and phage attachment inhibition in *P. mirabilis*.

The *terXYW* gene cluster

The R478 plasmid contains an additional *ter* gene cluster that protects the host *E. coli* cell from the toxic effect specified by the *terZ* locus (Whelan *et al.*, 1997). This toxicity is seen for *E.coli* cells transformed with a recombinant plasmid containing the R478-derived *terZ* coding region, which undergo filamentation consistent with a block in cell septation. Of note, the Te^r locus identified in this work also appears to be toxic to *E. coli* cells, making it impossible to maintain recombinant cosmid or plasmid constructs containing an entire *P. mirabilis terZ* locus. The disruption of the subcloned R478 *terZ*, *-A*, *-B* and *-C* genes by transposon mutagenesis resulted in the loss or alleviation of the filamentation phenotype (Whelan *et al.*, 1997). Alternatively, host cells were protected from the filamentation phenotype when an additional R478-derived fragment was co-cloned along with the *terZ* coding region. This second plasmid DNA fragment contains an additional *ter* gene cluster, *terX*, *-Y* and *-W*. The TerX polypeptide is similar to the TerZ, TerE and TerD proteins and contains a

conserved 13-residue motif identified in these proteins. In contrast, *terW* and *terY* show no similarity to known DNA sequences or to each other (Whelan *et al.*, 1997, and see Table 3). Complementation analysis demonstrated that only *terW* is required for protection of cells from the toxicity of the *terZ* locus. The roles of *terX* and *terY* remain unclear.

My study suggests that *P. mirabilis* strain S2 does not contain sequences similar to the *terW* gene cluster. However, it is still possible that homologues are present in *P. mirabilis,* but show insufficient sequence similarity with the *terX* locus to be detected by the hybridization screen that I used. Given the strong similarities between the chromosomal and plasmid-borne *ter* genes currently characterized, this possibility seems unlikely. Furthermore, inhibition of cell septation is the hallmark of swarmer cell differentiation, so I propose that *P. mirabilis* does not need protection from the filamentation phenotype specified by the *terZ* locus, because this process is incorporated into the developmental behaviour of this bacterium. However, this hypothesis is likely to be too simplistic, since *E.coli* also undergoes swarmer cell development on suitable rich solid medium (Harshey and Matsuyama, 1994). It would be informative to determine during future investigations what effect, if any, the expression of the plasmid-borne Te^r system has on the ability of *E. coli* to swarm.

Interestingly, the filamentation phenotype associated with overexpression of the R478 *terZ* locus from a recombinant plasmid appears to be different from the filamentation induced as part of SOS response, heat-shock response and many other stress-induced events (Donachie, 1993). During the terZ-locus

induced filamentation, DNA replication is not affected, since DAPI staining shows nucleoids evenly distributed throughout the length of the bacterial filament (Whelan *et al.*, 1997). In contrast, in many cases of stress-induced filamentation, the nucleoids show aberrant distribution (Donachie, 1993). Perhaps the R478-encoded Ter polypeptides, including TerW, form a stoichiometric functional complex, and selective overexpression of some of the components, but not TerW, results in disruption of the stoichiometric ratio with subsequent filamentation. Concurrent overexpression of TerW would eliminate the filamentation phenotype by restoring the appropriate ratio. Indirect support for this hypothesis is provided by the observation that transformation of the partial R478 *terZ* locus on a recombinant plasmid into WT *P. mirabilis* cells results in the reduced fitness of the strain and decreased levels of tellurite resistance (Figure 7B). As the next step, it would be informative to determine whether co-cloning of the plasmid-borne *terW* and *terZ* loci can restore WT levels of tellurite resistance in the *P. mirabilis* S2 genetic background. Association of the TerW protein with a filamentation phenotype of the host cell also raises a possibility that overexpression of *terW* in *P. mirabilis* may abrogate swarming by interfering with the elongation of differentiating swarmer cells. Thus, overexpression of TerW under the control of an inducible promoter may be a novel molecular tool for clarification of the biochemical mechanisms controlling the early stages of swarm cell development.

The sex factor genes of *Lactococcus lactis*

The sex factor (plasmid) of *L. lactis* contains a cluster of genes impiicated in tellurite resistance, and the encoded proteins show up to 55% amino acid identity with the pMER610 Te^r polypeptides (Pilliadge *et al.*, 1994). Unfortunately, the nucleotide sequences of the *L. lactis* tellurite resistance gene cluster have not been deposited in GeneBank, making it impossible to compare them directly to those of the *P. mirabilis* locus described in this study.

The *yce* locus of *Bacillus subtilis*

Kumano *et al.* identified a *Bacillus subtilis* chromosomal *yce* gene cluster that is homologous to the Te^r systems (Kumano *et al.*, 1997). Those authors included seven open reading frames (*yceC* through *yceI*) in this locus. However, while the predicted amino acid sequences of YceC, YceD and YceE show significant similarity to the plasmid-encoded Ter proteins, the remaining Yce polypeptides show no similarity to the products of any Te^r system. Therefore, a possible role of YceG, YceH and YceI in tellurite resistance, if any, remains to be elucidated.

Dictyostelium discoideum cyclic-AMP binding proteins

Jobling and Ritchie (1987; 1988) showed that the products encoded by *ter* open reading frames of pMER610 are related to the subunits of cyclic-AMP binding protein 1 (CABP) and related polypeptides of the slime mold *D. discoideum* (Bain *et al.*, 1991; Grant *et al.*, 1990). The work of Whelan *et al.* extended this similarity further to the products of the R478-encoded *ter* gene cluster (Whelan *et al.*, 1995). As expected, the *P. mirabilis terZ*, *-D* and *-E* gene

products described here are also highly similar to the *D. discoideum* CABP. The carboxyl termini of CABP1 subunits demonstrate 50% similarity to TerD and TerE proteins, and contain the 13-residue conserved motif GDN(L/R)TG(E/A)GD GDDE found in TerZ, TerD and TerE proteins and their homologues (Table 2). The significance of this homology is not clear at present. Interestingly, the subunits of *D. discoideum* CAPB bind to each other to form a functional complex; at the same time, mutational analysis of pMER610 suggests that TerD and TerE bind to each other and may interact with TerC to form a functional unit associated with the bacterial membrane (Jobling and Ritchie, 1988).

The ability of Ter polypeptides to bind cyclic AMP has not been studied, but this research avenue may provide some information about the biochemical roles of TerD and TerE, and merits further investigation. Furthermore, the 13-residue conserved motif resembles the consensus phosphate-binding loop (P-loop or Walker A sequence), which occurs in adenylate kinase and uridylate kinase and binds the phosphate moiety of the nucleotide (Saraste *et al.*, 1990; Walker *et al.*, 1982). Typically, the P-loop folds to form a small "ledge" perpendicular to the plane of the central β-sheet (Smith and Rayment, 1996). The P-loop consensus sequence is GxxxxGK(T/S), where x denotes various amino acids. However, variations on the theme include GE(S/T)G(T/S/V)G K(E/D) found in over 60 members of the σ^{54}-dependent protein family of transcriptional regulators (Gao *et al.*, 1998) and the GTNGKGS nucleotide-binding site of folylpolyglutamate synthetase (which is responsible for the addition of a polyglutamate tail to folate and folate derivatives) from *Lactobacillus*

casei (Sun *et al.*, 1998). Therefore, despite the lack of delectable P-loop consensus sequence, the 13-residue conserved motif may have a nucleotide-binding function, similar to the P-loop.

The Te^r-related sequences are not found in the yeast genome, and *D. discoideum* appears to be the only eukaryotic organism described so far that contains these sequences. This observation, together with the data presented in the homology tree (Figure 28B), suggests a possibility that *D. discoideum* acquired the *ter*-related CAPB coding sequences from eubacteria. However, the number of eukaryotic organisms containing the *ter*-related sequences may increase as more complete genome sequences become available.

Synechocystis spp. cyclic-AMP binding protein

Synechocystis spp. cyclic-AMP binding protein (Kaneko *et al.*, 1996) shows limited sequence similarity (30% identity or less) to the putative Ter polypeptides, and contains 13-residue conserved motif (Table 2). Unlike *D. discoideum* CABP, this protein appears to be more closely related to TerA than to TerD or TerE (Figure 28B). This relationship may be a reflection of different evolutionary origins of CAPB of *Synechocystis* and *D. discoideum*.

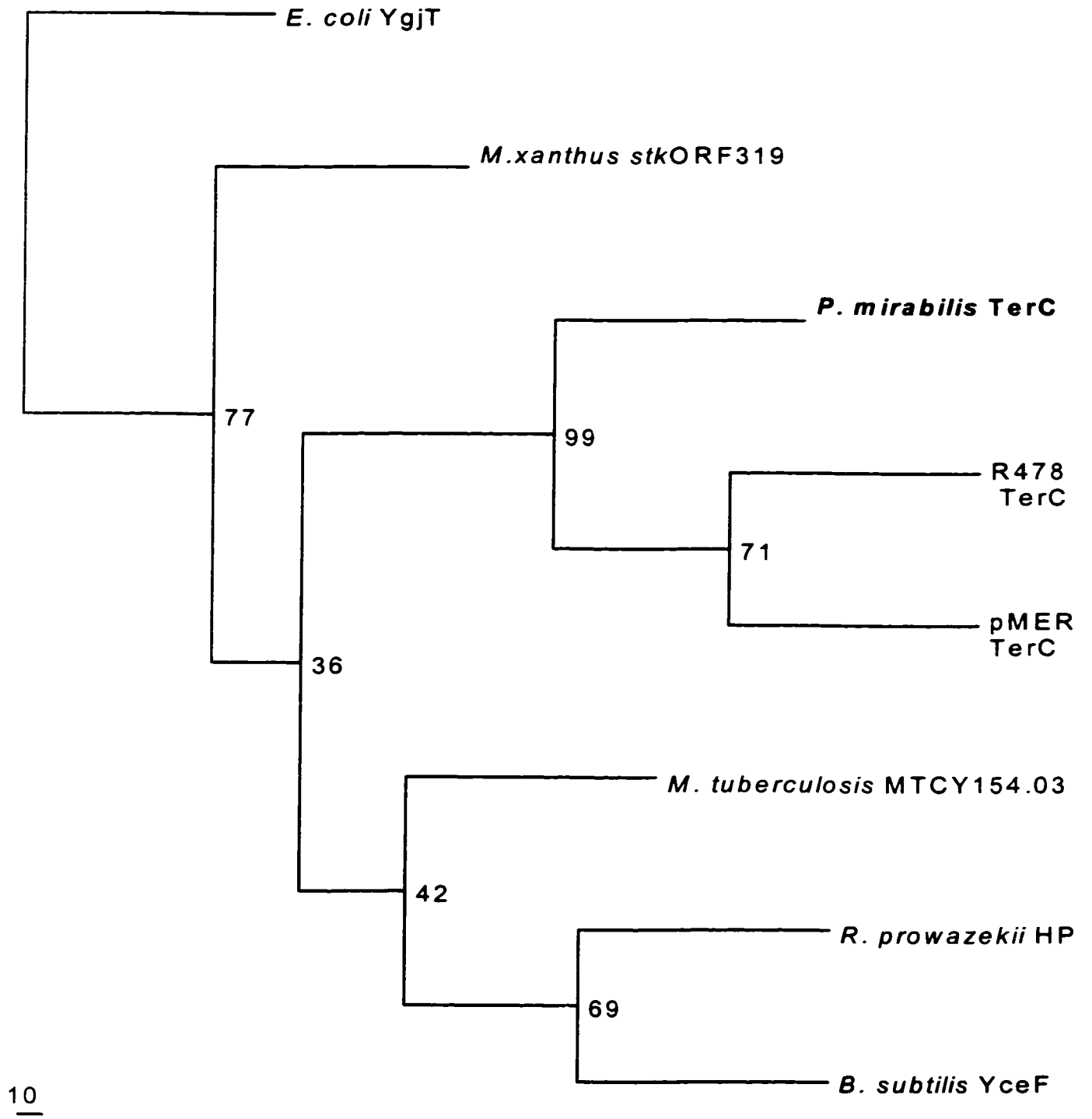
Methyl methane sulfonate, mitomycin C and UV resistance encoding genes of *Clostridium acetobutylicum*

Previous characterization of the plasmid-borne *ter* loci established similarity between the *ter* genes and two genes that confer methyl methane sulfonate, mitomycin C, and UV resistance to *C. acetobutylicum* (Azeddoug and Reysset, 1994; Whelan *et al.*, 1995). My work extends this similarity to the *P.*

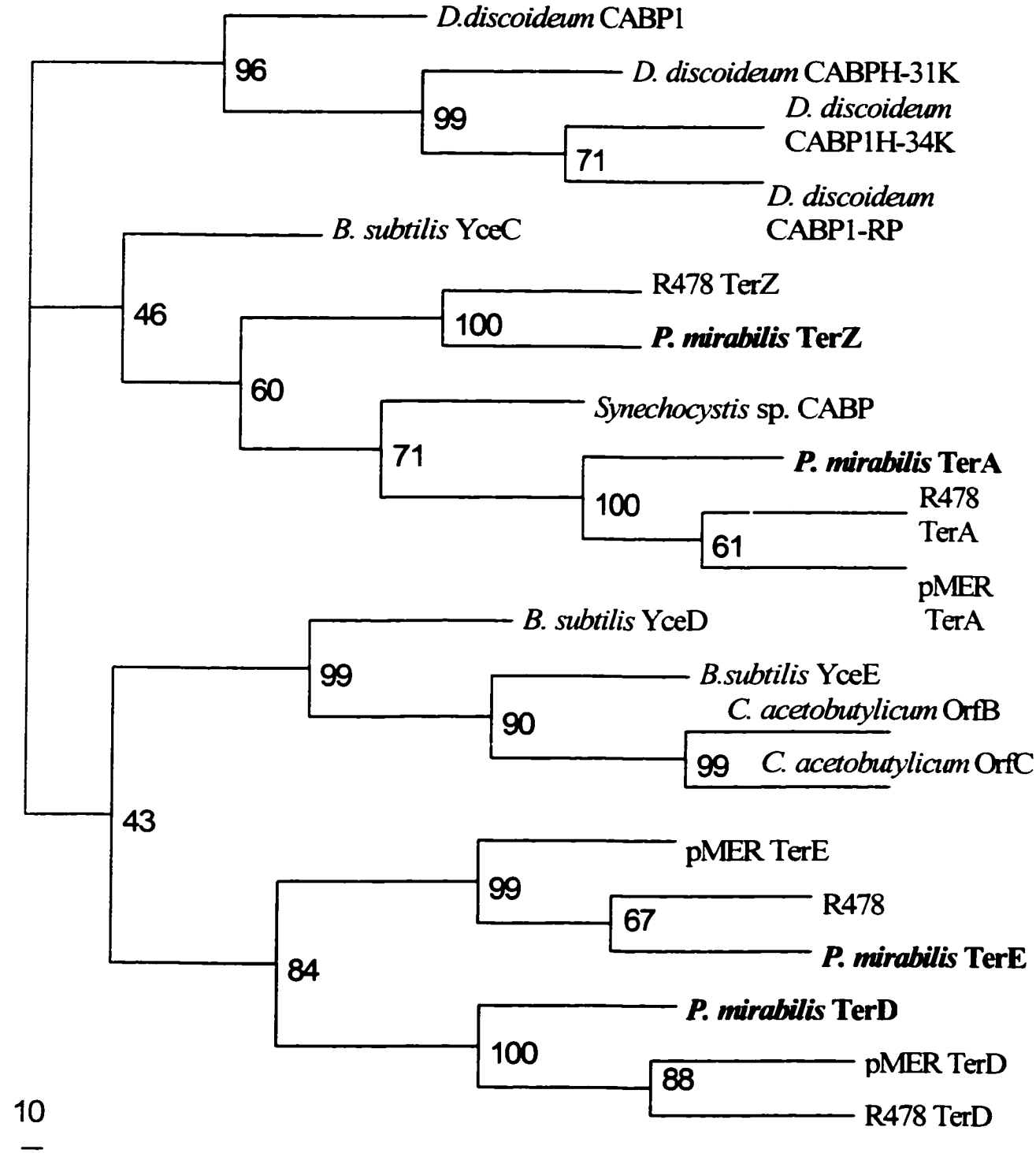
Figure 28. Homology tree showing relationships of the *P. mirabilis* putative TerC polypeptide (A) and TerZ, TerD and TerE polypeptides (B) to homologous proteins from other bacteria and from *Dictyostelium discoideum*

The trees were constructed by the PHYLIP package using PROTDIST, NEIGHBOR, DRAWTREE and SEQBOOT programs using parsimony analysis (Department of Genetics, University of Washington). Bootstrapping values (the probability of obtaining the true tree from the sampled sequences) are shown at the nodes of the branches. Bootstrapping is a conservative approach for estimating the reliability of an inferred phylogeny; bootstrap estimation is formulated as a two-step sampling procedure (1) sampling of sequences from the evolutionary process and (2) resampling of the original sample (Zharkikh and Li, 1992). The length of branches corresponds to the number of inferred changes that occurred since the divergence of individual branches. The bar at the bottom of the panel indicates the length of a branch corresponding to 10 inferred changes. See text and Table 2 for references.

A.


E. coli YgjT
M.xanthus stkORF319
P. mirabilis TerC
77
99
R478 TerC
71
pMER TerC
36
M. tuberculosis MTCY154.03
42
R. prowazekii HP
69
B. subtilis YceF
10

B.


D.discoideum CABP1
96
D. discoideum CABPH-31K
99
D. discoideum CABP1H-34K
71
D. discoideum CABP1-RP
B. subtilis YceC
46
R478 TerZ
100
P. mirabilis TerZ
60
Synechocystis sp. CABP
71
P. mirabilis TerA
100
R478 TerA
61
pMER TerA
B. subtilis YceD
99
B.subtilis YceE
C. acetobutylicum OrfB
90
99
C. acetobutylicum OrfC
43
pMER TerE
99
R478
67
P. mirabilis TerE
84
P. mirabilis TerD
100
pMER TerD
88
R478 TerD
10

mirabilis ter gene cluster. The existence of these similar proteins suggests that they may perform a similar essential protection function in the three unrelated organisms. However, underlying biochemical mechanisms remain to be clarified.

Functional implications

Despite the fact that proteins related to the Ter polypeptides are synthesized by a rather large number of bacterial species and by the slime mold *D. discoideum*, the biochemical function of these polypeptides still remains a mystery. In this work, extensive sequence analysis failed to detect functional motifs or patterns of known function in the putative Ter polypeptides (the function of the 13-residue motif is yet-unknown). TerZ, TerA and TerB are predicted to function in the bacterial cytoplasm, while TerD and TerE carboxyl termini contain predicted short transmembrane helices in addition to highly hydrophilic central cores (Figure 20B). These short transmembrane helices may function as anchoring domains, attaching the putative TerD and TerE proteins to the inner surface of the cytoplasmic membrane. This subcellular localization may facilitate formation of the putative TerC/TerD/TerE complex, as suggested by Jobling and Ritchie (1988). The presence of the conserved 13-residue motif was first reported by Whelan and co-workers in the R478 Ter polypeptides (Whelan *et al.*, 1997). My work extends this finding to include the putative TerZ, TerD, and TerE polypeptides of *P. mirabilis* and their homologous proteins from other microorganisms (Table 2). Delineation of the biological role of this motif would

provide information about function common to all the proteins listed in Table 2. Binding of nucleotides (e. g., cyclic AMP) represents one such possible function.

Regulation of the *ter* locus expression by potassium tellurite

During the course of this study it was observed that prior exposure to moderate (50-100 μg/ml) concentrations of potassium tellurite induced increased resistance levels in *P. mirabilis* and *E. coli* strains harboring either a complete or partial Te^r locus. This observation prompted further investigation into the molecular mechanism of the induction.

Expression of the *terC-lacZ* translational gene fusion

No differences in levels of β-galactosidase activity were observed for cultures of the mutant strain of *P. mirabilis* grown in the absence of tellurite and those grown in the presence of tellurite. These unexpected results did not accord with the observed induction of tellurite resistance in the same strain grown in tellurite-supplemented culture medium. Furthermore, these results implied that the observed induction of resistance in cultures was not linked directly to the expression of the resistance determinant (*terC*). An alternative explanation for the conflicting data may be provided by a suggestion of an inhibitory effect of potassium tellurite on β-galactosidase activity. This possibility could be clarified by determining whether potassium tellurite inhibits commercial preparations of β-galactosidase. Alternatively and more likely, the TerC-LacZ

hybrid protein in the bacterial membrane might remain functionally inactive by failing to form a tetrameric complex (β-galactosidase functions as a tetramer). While the second theory remains to be tested, the results of my analysis provide indirect evidence in its favour. The subcellular location of the TerC-LacZ fusion protein may be determined by ultracentrifugation of bacterial lysates with subsequent assas of the resulting supernatant and pellet for the reporter-gene activity (β-galactosidase). These assays would allow differentiaton between cytoplasmic (supernatant –associated β-galactosidase activity) and membrane-associated (pellet-associated β-galactosidase activity) localization for the TerC-LacZ fusion protein.

Transcript analysis by northern blotting

Northern blot analysis using two non-overlapping DNA probes demonstrated that transcription of the *ter* genes is strongly induced in both strains by potassium tellurite (Figure 22). This activation appears to be specific for the *ter* gene cluster, since transcription of the *ureR* gene is not affected by the presence of tellurite in the medium (Figure 23).

Northern blotting analysis detected multiple signals that corresponded either to multiple transcripts or to degradation products of the largest (5-kb) transcript, which is likely to be a polycistronic message encompassing an entire *ter* locus (Figure 22). The intensity of several bands (e.g., 5-kb transcript) was significantly decreased in the mutant strain compared to WT, suggesting that the mutant is impaired in its ability to transcribe the *ter* locus. Additionally, it is

reasonable to suggest that the 5-kb band in the mutant strain corresponds to a partial *ter* locus transcript fused to the mRNA for the reporter gene (*lacZ*). Such "hybrid" transcript would be expected to be different in size from the "full-length" transcript encoding an entire *ter* locus in the WT strain. However, the expected difference in size was not apparent on my northern blots, perhaps because the different sizes of the transcripts of such large size would not be resolved by gel electrophoresis. The putative "hybrid" transcript from the mutant strain may also be less stable, thus providing an alternative explanation for reduction in intensity of the hybridization signal detected in this strain.

The results of northern blot analysis were confirmed by primer-extension transcriptional start-site mapping. Using RNA isolated from the WT *P. mirabilis* strain, a single strong signal was detected only in bacteria exposed to tellurite (Figure 24). Moreover, a partial R478 *ter* locus expressed from a recombinant plasmid not only enabled the host *E. coli* cells to tolerate otherwise toxic levels of potassium tellurite, but demonstrated that this phenotype was inducible by prior exposure to tellurite. Taken together, the experimental data discussed in this section uncover a molecular basis for the increased expression of tellurite resistance in *P. mirabilis* in the presence of potassium tellurite. Additionally, at least one of the plasmid-borne Te^r systems (R478) appears to be similarly activated by exposure to tellurite. However, it remains to be determined whether the activation of the R478-encoded *ter* genes occurs at the transcriptional level.

Initial characterization of the pMER610-encoded Te^r determinants demonstrated that the tellurite resistance is inducible in the *E. coli* genetic

background by prior exposure to subtoxic levels of tellurite (Jobling and Ritchie, 1987). However, subsequent transcript analysis by the same laboratory of the pMER610 Te^r determinant (northern blot) failed to detect any effect of potassium tellurite on transcription levels. Similarly, the activity of reporter-gene fusions with pMER610 *ter* genes was uninducible by prior exposure to tellurite. Given the inconclusive results of these two studies, the authors left the nature of induction open to speculation (Hill *et al.*, 1993). Whelan and co-workers did not address the issue of tellurite-resistance induction during characterization of the R478-encoded *ter* locus (Whelan *et al.*, 1995; 1997). Two possible explanations can be offered to explain why Hill and co-workers with pMER610 analysis did not observe induction of tellurite resistance. The expression of the pMER610 Te^r determinant may be constitutive, and the induction reported by Jobling and Ritchie (1987) may be spurious or unrelated to expression of the resistance determinant. Alternatively, in that system the induction may occur at concentrations of tellurite above those tested (2 mM). Interestingly, Lee *et al.* (1990) made the following prediction regarding induction of bacterial heavy-metal resistance systems: cations that are required as trace elements but are toxic at higher concentration (Co^{2+}, Zn^{2+}, Cu^{2+}) should show gradual induction of resistance determinants (Brown *et al.*, 1991; Brown *et al.*, 1992), while resistance systems for purely toxic cations (Hg^{2+}, Te^{4+}) might show a sharp induction over a very narrow concentration range (Ralston and O'Halloran, 1990). The pattern of transcriptional activation of the *P. mirabilis ter* locus by tellurite is consistent with this prediction.

Analysis of the *terZ* promoter region

Hill *et al.* (1993) suggested the presence of multiple promoters within the pMER610 *ter* locus, based on northern blotting analysis. The putative promoter (upstream of *terZ*) and terminator sequences (downstream of both *terA* and *terE*) identified in this study indicate that at least two transcripts could be synthesized (Figure 18). Generally, detection of large bacterial transcripts using bacterial RNA is difficult due to degradation. Therefore, detection of a distinct hybridization signal corresponding to a 5-kb transcript with two non-overlapping DNA probes suggests that a strong promoter may be located in the *orf3-terZ* intergenic region. Primer-extension analysis mapped a single transcriptional start site that is preceded by motifs matching the *E.coli* $E\sigma^{70}$ promoter consensus (Figure 24 and 25). Interestingly, no additional reasonable matches to this promoter consensus that would correspond to internal promoter(s) within the *ter* locus were identified. However, the existence of additional promoters within the *ter* locus cannot be ruled out without further investigation.

Sequence searches for oligonucleotide repeats (Solovyov *et al.*, 1984) within the *terZ* promoter region identified a 7-basepair inverted repeat TCATCAT . . . ATGATGA located between the –35 and –10 consensus promoter sequences. Additionally, a pair of 7-bp direct repeats, ATTTATA, overlaps the –10 consensus sequence and transcriptional start site (Figure 25). These repeats could potentially play regulatory roles and are prime candidates for binding sites of putative transcription regulatory factors. Characterized bacterial toxic ion-resistance systems are typically activated by ion binding, which, in turn, leads to

the stimulation of mRNA synthesis rather than to repression (Silver and Walderhaug, 1992). For example, transcription of the *Streptomyces lividans mer* genes encoding broad-spectrum mercury resistance is repressed by the MerR transcriptional regulator (Brunker *et al.*, 1996). Addition of mercuric ions prevents the binding of the MerR repressor to DNA. Therefore, any putative transcriptional regulatory factor for the *ter* locus is likely to function as a repressor that shuts down expression of this locus in the absence of tellurium ions. Since tellurite resistance is only one of three phenotypes mediated by bacterial *ter* loci, transcriptional regulation of these genes is likely to be complex, integrating input information from several sensor systems.

Regulation of *ter* locus expression by global transcriptional regulator(s)

The induction of tellurite resistance in *E. coli* strain JF626 containing the partial R478 *ter* locus (pAT9804) raises an interesting question regarding the identity of putative transcriptional factor(s) mediating this activation. The TerZ or TerW polypeptides are unlikely candidates for the role of a transcriptional regulator, since these polypeptides are not encoded by the pAT9804 construct. This finding raises a possibility that transcription activation of the *ter* loci is under control of yet-unknown global transcriptional regulators. It has been hypothesized that bacteria might benefit from global regulation in response to toxic heavy metals (Silver and Walderhaug, 1992). For instance, it would be advantageous for bacteria to coordinate expression of heavy-metal resistance determinant(s) and DNA-repair mechanisms, since some of the heavy metals

cause DNA damage (Bose *et al.*, 1998; Braun, 1997). However, currently there are no experimental data supporting this hypothesis and additional research is needed.

Concluding remarks

This work describes the molecular basis of the tellurite-resistance phenotype *in P. mirabilis*, which was originally reported over six decades ago by Alexander Fleming (1932). In addition to a number of conclusions and hypotheses discussed in the above sections, the results of this study suggest a possibility that *P. mirabilis* may be the evolutionary origin of the plasmid-borne *ter* loci in Gram-negative bacteria. Homology trees constructed in this study (Figure 28) are consistent with this hypothesis. High bootstrapping values clearly indicate a high degree of relatedness between *P. mirabilis* Ter proteins and those derived from large conjugative plasmids. The high degrees of relatedness between homologous Ter proteins can be attributed to the relatively "recent" integration of the *ter* gene cluster into a conjugative plasmid from either the *P. mirabilis* chromosome, or unknown common evolutionary ancestral microorganism. Additionally, tellurite resistance appears to be an integral feature of the genus *Proteus*. In the *P. mirabilis* strain used during this study the tellurite-resistance determinants are chromosomally encoded, while the other two known *ter* gene clusters of Gram-negative bacteria are plasmid-borne. This study is the first report of a chromosomal location of a *ter* locus. It has been postulated that at least some bacterial heavy-metal resistance determinants have moved from

plasmid to chromosome or in the reverse direction (Silver and Walderhaug, 1992). Therefore, it is more logical to imagine that the *ter* locus has moved from the *Proteus* chromosome to a conjugative plasmid, than that integration of a plasmid-derived locus occurred in the chromosome of all *Proteus* strains.

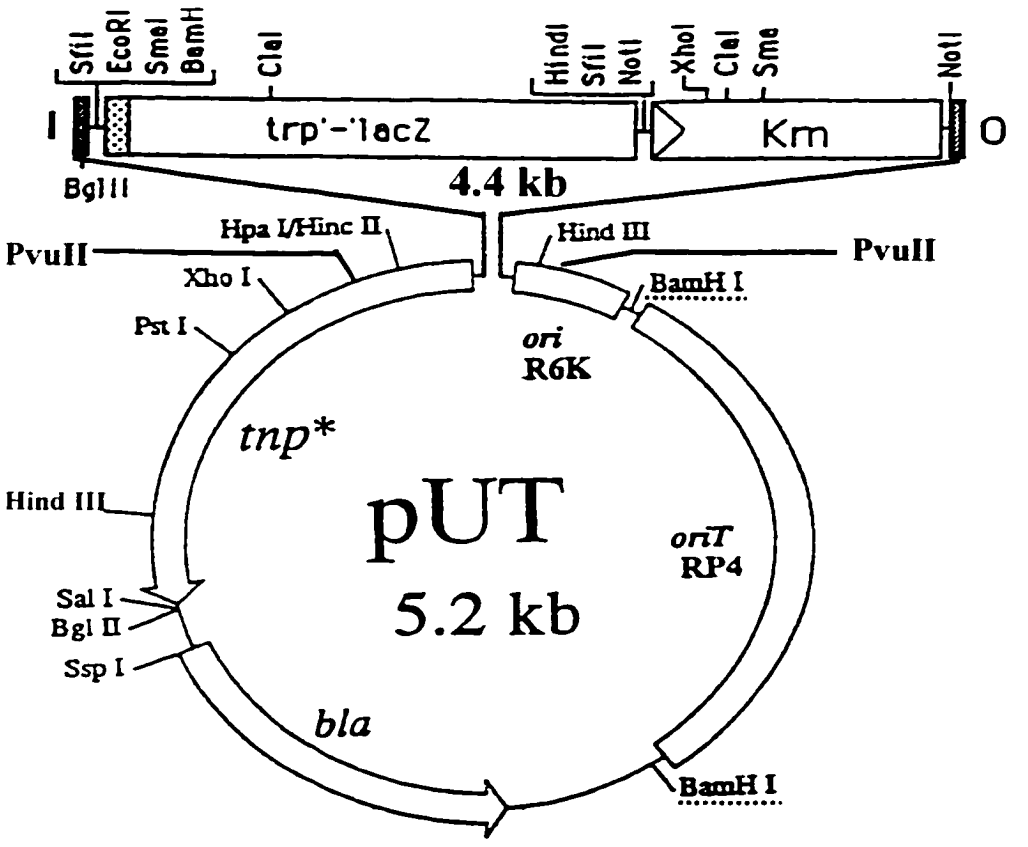
Future screens for tellurite resistance in the *Proteus* group bacteria (*Proteus*, *Morganella* and *Providenca* spp.) may prove this phenotype to be a distinguishing characteristic of these bacteria. In that case, tellurite resistance may serve as a basis for a fast and effective clinical test for urinary-tract pathogens of the *Proteus* group.

Two additional phenotypes (colicin resistance and phage resistance) encoded by the *ter* gene cluster were not investigated during this study, but present a worthwhile challenge for future research. With the rapid spread of antibiotic resistance among bacteria, such old and faithful weapons of biological warfare as colicins and phages may provide new possibilities for development of future antibacterial therapies (Alisky *et al.*, 1998).

I conclude with the quote that is, in my opinion, best suited to convey the overall significance of this work: "A typical scientific paper has never pretended to be more than another little piece in a larger jigsaw – not significant in itself itself but as an element in a grander scheme" (Ziman, 1969).

APPENDIX

Physical map of the mini-Tn*5* element and pUT delivery plasmid



The map is modified from De Lorenzo and Timmis (1992). *bla* - β-lactamase-encoding gene (ampicillin resistance determinant); tnp* - transposase gene; Km - kanamycin resistance cassette. *Bam*HI restriction sites used to generate a probe for Southern blot hybridization (see Figure 2) are underlined by dotted lines. *Pvu*II recognition sites mapped in this study are shown in bold.

BIBLIOGRAPHY

Alberti, L., and R. M. Harshey. 1990. Differentiation of *Serratia marcescens* 274 into swimmer and swarmer cells. *J. Bacteriol.* **172**:4322-4328.

Alisky, J., K. Iczkowski, A. Rapoport, and N. Troitsky. 1998. Bacteriophages show promise as antimicrobial agents. *J. Infect.* **36**:5-15.

Allison, C., N. Coleman, P. L. Jones, and C. Hughes. 1992. Ability of *Proteus mirabilis* to invade human urothelial cells is coupled to motility and swarming differentiation. *Infect. Immun.* **60**:4740-4746.

Allison, C., L. Emödy, N. Coleman, and C. Hughes. 1994. The role of swarm cell differentiation and multicellular migration in the uropathogenicity of *Proteus mirabilis*. *J. Infect. Dis.* **169**:1155-1158.

Allison, C., and C. Hughes. 1991. Bacterial swarming: an example of prokaryotic differentiation and multicellular behaviour. *Sci. Prog.* **75**:403-22.

Allison, C., H.-C. Lai, and C. Hughes. 1992a. Co-ordinate expression of virulence genes during swarm cell differentiation and population migration of *Proteus mirabilis*. *Mol. Microbiol.* **6**:1583-1591.

Allison, C., H. C. Lai, D. Gygi, and C. Hughes. 1993. Cell differentiation of *Proteus mirabilis* is initiated by glutamine, a specific chemoattractant for swarming cells. *Mol. Microbiol.* **8**:53-60.

Altschul, S. F., W. Gish, W. Miller, E. F. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403-410.

Andersson, J.O., and S. G. Andersson. 1997.Genomic rearrangements during evolution of the obligate intracellular parasite *Rickettsia prowazekii* as inferred from an analysis of 52015 bp nucleotide sequence. *Microbiology* **143**:2783-2795.

Araque, M., B. Nieves, O. Ruiz, and M. Dagert. 1997. Characterization of plasmids which mediate resistance to multiple antibiotics in gram-negative bacteria of nosocomial origin. *Enferm. Infec. Microbiol. Clin.* **15**:299-305.

Armitage, J. P. 1982. Changes in organization of the outer membrane of *Proteus mirabilis* during swarming: freeze-fracture structure and membrane fluidity analysis. *J. Bacteriol.* **150**:900-904.

Armitage, J. P., R. J. Rowbury, and D. G. Smith. 1975. Indirect evidence for cell wall and membrane differences between filamentous swarming cells and short non-swarming cells of *Proteus mirabilis*. *J. Gen. Microbiol.* **89**:199-202.

Armitage, J. P., and D. G. Smith. 1978. Flagella development during swarmer cell differentiation in *Proteus mirabilis*. *FEMS Microbiol. Lett.* **4**:163-165.

Armitage, J. P., D. G. Smith, and R. J. Rowbury. 1979. Alteration in cell envelope composition of *Proteus mirabilis* during the development of swarmer cells. *Biochim. Biophys. Acta* **584**:389-387.

Azeddoug, H., and G. Reysset. 1994. Cloning and sequencing of a chromosomal fragment from *Clostridium acetobutylicum* strain ABKn8 conferring chemical-damaging agents and UV resistance to *E. coli recA* strains. *Curr. Microbiol.* **29**:229-235.

Bahrani, F. K., D. E. Johnson, D. Robbins, and H. L. Mobley. 1991. *Proteus mirabilis* flagella and MR/P fimbriae: isolation, purification, N-terminal analysis, and serum antibody response following experimental urinary tract infection. *Infect. Immun.* **59**:3574-3580

Bain, G., C. E. Grant, and A. Tsang. 1991. Isolation and characterization of cDNA clones encoding polypeptides related to a *Dictyostelium discoideum* cyclic AMP binding protein. *J. Gen. Microbiol.* **137**:501-508.

Behmlander, R. M., and M. Dworkin. 1991. Extracellular fibril and contact-mediated cell interactions in *Myxococcus xanthus*. *J. Bacteriol.* **173**:7810-7821.

Belas, R. 1992. The swarming phenomenon of *Proteus mirabilis*. *ASM News* **58**:15-22.

Belas, R. 1994. Expression of multiple flagellin-encoding genes of *Proteus mirabilis*. *J. Bacteriol.* **176**:7169-7181.

Belas, R. 1996. *Proteus mirabilis* swarmer cell differentiation and urinary tract infection, p. 271-298. *In* H. L. T. Mobley and J. W. Warred (ed.), Urinary tract infections: Molecular pathogenesis and clinical management. ASM Press, Washington, D. C.

Belas, R. 1996a. Sensing, response, and adaptation to surfaces: swarmer cell differentiation and behavior, p. 281-331. *In* M. Fletcher (ed.), Bacterial adhesion: Molecular and Ecological Diversity. Willey-Liss, Inc., New York, N.Y.

Belas, R., D. Erskine, and D. Flaherty. 1991. Transposon mutagenesis in *Proteus mirabilis*. *J. Bacteriol.* **173**:6289-6293.

Belas, R., D. Erskine, and D. Flaherty. 1991a. *Proteus mirabilis* mutants defective in swarmer cell differentiation and multicellular behavior. *J. Bacteriol.* **173**:6279-6288.

Belas, R., and D. Flaherty. 1994. Sequence and genetic analysis of multiple flagellin-encoding genes from *Proteus mirabilis. Gene* **128**:33-41.

Belas, R., M. Goldman, and K. Ashliman. 1995. Genetic analysis of *Proteus mirabilis* mutants defective in swarmer cell elongation. *J. Bacteriol.* **177**:823-828.

Belas, R., Schneider, R., and M. Melch. 1998. Characterization of *Proteus mirabilis* precocious swarming mutant: identification of *rsbA*, encoding a regulator of swarming behavior. *J. Bacteriol.* **180**: 6126-6139.

Birnboim, H. C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res.* **7**:1513-1523.

Blattner, F. R., G. Plunkett III, G. F.Mayhew, N. T. Perna, and F. G. Glasner. 1997. Direct submission to the GenBank.

Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**:95-103.

Bose, R.N., B. S. Fonkeng, S. Moghaddas, and D. Stroup. 1998. Mechanisms of DNA damage by chromium (V) carcinogens. *Nucleic Acids Res.* **26**:1588-1596.

Bradley, D. E. 1980. Morphological and serological relationships of conjugative pili. *Plasmid* **4**:155-169.

Bradley, D. E. 1985. Detection of tellurite-resistance determinants in IncP plasmids. *J. Gen. Microbiol.* **131**:3135-3137.

Bradley. D. E., K. Grewal, D. E. Taylor, and J. Whelan. 1988. Characterisation of RP4 tellurite-resistance transposon Tn*521*. *J. Gen. Microbiol.* **134**:2009-2018.

Bradley, D. E., F. A. Sirgel, J. N. Coetzee, R. W. Hedges, and W. F. Coetzee. 1982. Phages C-2 and J: IncC and IncJ plasmid-dependent phages, respectively. *J. Gen. Microbiol.* **128**:2485-2498.

Branson, D., *Methods in Clinical Bacteriology*. Springfield, Ill.: Thomas. 1972.

Braun, V. 1997. Avoidance of iron toxicity through regulation of bacterial iron transport. *Biol. Chem.* **378**:779-786.

Braun, V., and C. Herman. 1993. Evolutionary relationships of uptake systems for biopolymers in *Escherichia coli*: cross-complementation between the TonB-ExbB-ExbD and the TolA-TolQ-TolR proteins. *Mol. Microbiol.* **8**:261-268.

Braun, V., H. Pilsl, and P. Gross. 1994. Colicins: structures, modes of action, transfer through membranes, and evolution. *Arch. Microbiol.***161**:199-206.

Brendel, V. and E. N. Trifonov. 1984. A computer algorithm for testing potential prokaryotic terminators. *Nucleic Acids Res.* **12**:4411-4427.

Brenner, D. J., J. J. Farmer III, G. R. Fanning, A. G. Steigerwalt, P. Klykken, H. G. Wathert, F. W. Hickman, and H. W. Ewing. 1978. Deoxyribonucleic acid relatedness of *Proteus* and *Providencia* species. *Int. J. Syst. Bacteriol.* **28**:269-282.

Brickman, T. J., and S. K. Armstrong. 1996. Colicin B and Ia as novel counterselective agents in interspecies conjugal DNA transfers from colicin-sensitive *Escherichia coli* donors to other Gram-negative recipient species. *Gene* **178**:39-42.

Brown, N. L., J. Camakaris, B. T. O. Lee, T. Williams, A. P. Morby, J. Parkhill, and D. A. Rouch. 1991. Bacterial resistances to mercury and copper. *J. Cell. Biochem.* **46**:1-9.

Brown, N. L., D. A. Rouch, and B. T. O. Lee. 1992. Copper resistance systems in bacteria. *Plasmid* **27**:41-51.

Browning, E. 1969. Tellurium, pp 310-316. *In* Toxicity of industrial metals. Butterworth, London.

Brunker, P., D. Rother, R. Sedlmeier, J. Klein, R. Mattes, and J. Altenbuchner. 1996. Regulation of the operon responsible for broad-spectrum mercury resistance in *Streptomyces lividans* 1326. *Mol. Gen. Genet.* **251**:307-315.

Burkart, M., A. Toguchi, and R. M. Harshey. 1998. The chemotaxis system, but not chemotaxis, is essential for swarming motility in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **95**:2568-2573.

Cao, J. G., and E. A. Meighen. 1989. Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. *J. Biol. Chem.* **264**:21670-21676.

Carpenter, P. B., and G. W. Ordal. 1993. *Bacillus subtilis* FlhA: a flagellar protein related to a new family of signal-transducing receptors *Mol. Microbiol.* **7**:735-743.

Chou P. Y., and G. D. Fasman. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol. Relat. Areas Mol. Biol.* **47**:45-148.

Chippendale, G. R., J. W. Warren, A. L. Trifillis, and H. L. T. Mobley. 1994. Internalization of *Proteus mirabilis* by human renal epithelial cells. *Infect. Immun.* **62**:3115-3121.

Chow, A. W., P. R. Taylor, T. T. Yoshkawa, and L. B. Guze. 1979. A nosocomial outbreak of infection due to multiply resistant *Proteus mirabilis*: role of intestinal colonization as a major reservoir. *J. Infect. Dis.* **130**:621-627.

Clapham, L. R., J. C. McLean, J. C. Nickel, J. Downey, and J. W. Costerton. 1990. The influence of bacteria on struvite crystal habit and its importance in urinary stone formation. *J. Crystal Growth* **104**:475-484.

Coetzee, J. N., N. Datta, and R. W. Hedges. 1972. R factors from *Proteus rettgeri*. *J. Gen. Microbiol.* **72**:543-552.

Cohen, S. N., A. C. Y. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. USA* **69**:2110-2116.

Cole, S.T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, S. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, S. Skelton, S. Squares, R. Sqares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell, 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**:537-544.

Cooper, J. F., J. Levin, and H. N. Wagner Jr. 1971. Quantitative comparison of *in vitro* and *in vivo* methods for the detection of endotoxin. *J. Lab. Clin. Med.* **78**:138-148.

Cosmina P., F. Rodriguez, F. de Ferra, G. Grandi, M. Perego, G. Venema, and D. van Sinderen. 1993. Sequence and analysis of the genetic locus responsible for surfactin synthesis in *Bacillus subtilis*. *Mol. Microbiol.* **8**:821-831.

Cox, G. B., D. Webb, J. Godovac-Zimmermann, and H. Rosenberg. 1988. Arg-220 of the PstA protein is required for phosphate transport through the phosphate-specific transport system of *Escherichia coli* but not for alkaline phosphatase repression. *J. Bacteriol.* **170**:2283-2286.

Cox, G. B., D. Webb, and H. Rosenberg. 1989. Specific amino acid residues in both the PstB and PstC proteins are required for phosphate transport by the *Escherichia coli* Pst system. *J. Bacteriol.* **171**:1531-1534.

Dana, J. R., and L. J. Shimkets. 1993. Regulation of cohesion-dependent cell interactions in *Myxococcus xanthus*. *J. Bacteriol.* **175**:3636-3647.

Deighton, C. H., J. W. Gray, A. M. Bint, and D. J. Walker. 1992. Anti-*Proteus* antibodies in rheumatoid arthritis same-sexed siblings. *Br. J. Rheumatol.* **31**:241-245.

De Lorenzo, V., M. Herrero, U. Jakkubzik, and K. N. Timmis. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative bacteria. *J. Bacteriol.* **172**:6568-6572.

Dennison, S., and R. W. Hedges. 1972. Host specificity of RNA phages. *J. Hyg.* **70**:55-61.

Dick, H., R. G. E. Murray, and S. Walmsley. 1985. Swarmer cell differentiation of *Proteus mirabilis* in liquid media. *Can. J. Microbiol.* **31**:1041-1050.

Dienes, L. 1946. Reproductive processes in *Proteus* cultures. *Proc. Soc. Exp. Biol. Med.* **63**:265-270.

Di Masi, D. R., J. C. White, C. A. Schnaitman, and C. Bradbeer. 1973. Transport of vitamin B_{12} in *Escherichia coli*: common receptor sites for vitamin B_{12} and the E colicins on the outer membrane of the cell envelope. *J. Bacteriol.* **115**:506-513.

Dinsmore, P. K., D. J. O'Sullivan, and T. R. Klaenhammer. 1998. A leucine repeat motif in AbiA is required for resistance of *Lactococcus lactis* to phages representing three species. *Gene* **212**:5-11.

Dobritsa, A. P., T. G. Mikhailova, and V. I. Dubovaia. 1985. Physical and genetic organization of the plasmid R15. *Genetica* **25**:748-755.

Donachie, W. D. 1993. The cell cycle of *Escherichia coli*. *Annu. Rev. Microbiol.* **47**:199-230.

D'Orazio, S. E. F., and C. M. Collins. 1995. UreR activates transcription at multiple promoters within the plasmid-encoded urease locus of the *Enterobacteriaceae. Mol. Microbiol.* **16:**145-155.

D'Orazio, S. E. F., V. Thomas, and C. M. Collins. 1996. Activation of transcription at divergent urea-dependent promoters by the urease gene regulator UreR. *Mol. Microbiol.* **21:**643-655.

Drechsel, H., A. Thieken, R. Reissbrodt, G. Jung, and G. Winkelmann. 1993. α-Keto acids are novel siderophores in the genera *Proteus*, *Providencia*, and *Morganella* and are produced by amino acid deaminases *J. Bacteriol.* **175:**2727-2733.

Dumanski, A. J., H. Hedelin, A. Edin-Liljegren, D. Beauchemin, and R. J. C. McLean. 1994. Unique ability of the *Proteus mirabilis* capsule to enhance mineral growth in infectious urinary calculi. *Infect. Immun.* **62:**2998-3003.

Ebringer, A., S. Khalafpour, and C. Wilson. 1989. Rheumatoid arthritis and *Proteus*: a possible etiological association. *Rheumatol. Int.* **9:**223-228.

Ewing, W. H. 1986. The tribe *Proteeae*, p. 443-459. *In* Edwards and Ewing's Identification of the *Enterobacteriaceae,* 4th ed. Elsevier Science Publishing Co., Inc., New York, NY.

Fairley, K. F., N. E. Carson, R. C. Gutch, P. Leighton, A. D. Grounds, E. C. Laird, P. H. McCallum, R. L. Sleeman, and C. M. O'Keefe. 1971. Site of infection in acute urinary-tract infection in general practice. *Lancet* **ii:**615-618.

Falkinham, J. O. I., and P. S. Hoffman. 1984. Unique developmental characteristics of the swarm and short cells of *Proteus vulgaris* and *Proteus mirabilis. J. Bacteriol.* **158:**1037-1040.

Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.***132:**6-13.

Feldgarden, M., S. Golden, H. Wilson, and M. A. Riley. 1995. Can phage defense maintain colicin plasmid in *Escherichia coli*? *Microbiology* **141:**2977-2984.

Fleming, A. 1932. On the specific antibacterial properties of penicillin and potassium tellurite. *J. Pathol. Bacteriol.* **35:**831-842.

Fleming, A., and M. Y. Young. 1940. The inhibitory action of potassium tellurite on coliform bacteria. *J. Pathol. Bacteriol.* **51:**29-35

Foster, T. J. 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiol. Rev.* **47**:361-409.

Furness, R. B., G. M. Fraser, N. A. Hay, and C. Hughes. 1997. Negative feedback from a *Proteus class* II flagellum export defect to the *flhDC* master operon controlling cell division and flagellum assembly. *J. Bacteriol.* **179**:5585-5588.

Gaisser, S., and C. Hughes. 1997. A locus coding for putative non-ribosomal peptide/polyketide synthase functions is mutated in a swarming-defective *Proteus mirabilis* strain. *Mol. Gen. Genet.* **253**:415-427.

Gao, Y., Y.-K. Wang, and T. R. Hoover. 1998. Mutational analysis of the phosphate-binding loop of *Rhizobium meliloti* DctD, a σ^{54}-dependent activator. *J. Bacteriol.* **180**:2792-2795.

Gerdes, H. H., and C. Kaether. 1996. Green fluorescent protein: applications in cell biology. *FEBS Lett.* **389**:44-47.

Goebel, W., and J. Kreft. 1974. Complex ColE1 DNA in *Escherichia coli* and *Proteus mirabilis*. *Mol. Gen. Genet.* **129**:149-166.

Gold, L., and M. Stormo. 1987. Translational initiation, p. 1302-1312. *In* J. L. Ingraham, K. B. Low, B. Magasanik, F. C. Neidhardt, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D. C.

Goncharoff, P., Saadi, S., Chang, C.-H., Saltzman, L. H., and D. H. Figurski. 1991. Structural, molecular, and genetic analysis of the *kilA* operon of broad-host-range plasmid RK2. *J. Bacteriol.* **173:** 3463-3477.

Grant, C. E., G. Bain, and A. Tsang. 1990. The molecular basis for alternative splicing of the CABP1 transcripts in *Dictyostelium discoideum*. *Nucleic Acids Res.* **18**:5457-5463.

Gygi, D., M. J. Bailey, C. Allison, and C. Hughes. 1995. Requirement for FlhA in flagella assembly and swarm-cell differentiation by *Proteus mirabilis*. *Mol. Microbiol.* **15**:761-769.

Gygi, D., G. Fraser, A. Dufour, and C. Hughes. 1997. A motile but non-swarming mutant of *Proteus mirabilis* lacks FlgN, a facilitator of flagella filament assembly. *Mol. Microbiol.* **25**:597-604.

Gygi, D., M. M. Rahman, H.-C. Lai, R. Carlson, J. Guard-Petter, and C. Hughes. 1992a. A cell-surface polysaccharide that facilitates rapid population

migration by differentiated swarm cells of *Proteus mirabilis*. *Mol. Microbiol.* **17**:1167-1175.

Haardt, M., and E. Bremer. 1996. Use of *phoA* and *lacZ* fusions to study the membrane topology of ProW, a component of the osmoregulated ProU transport system of *Escherichia coli*. *J. Bacteriol.* **178**:5370-5381.

Harshey, R. 1994. Bees aren't the only ones: swarming in Gram-negative bacteria. *Mol. Microbiol.* **13**:389-394.

Harshey, R. M. and T. Matsuyama. 1994. Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellated swarmer cells. *Proc. Natl. Acad. Sci. USA* **91**:8631-8635.

Hauser, G., 1885. Über Fäulnisbakterien und deren Beziehung zur Septicämia, p. 107.Vogel, Leipzig, Germany.

Hay, N. A., D. J. Tipper, D. Gygi, and C. Hughes. 1997. A nonswarming mutant of *Proteus mirabilis* lacks the Lrp global transcriptional regulator. *J. Bacteriol.* **179**:4741-4746.

Hedges, R. W. 1975. R factors from *Proteus mirabilis* and *P. vulgaris*. *J Gen. Microbiol.* **87**: 301-311.

Henriksen, J. 1972. Bacterial surface translocation: a survey and classification. *Bacteriol. Rev.* **36**:478-503.

Henikoff, S., J. G. Henikoff, W. J. Alford, and S. Pietrokovski. 1995. Automated construction and graphical presentation of protein blocks from unaligned sequences. *Gene-COMBIS, Gene* **163**:17-26.

Henrichsen, J. 1972. Bacterial surface translocation: a survey and a classification. *Bacteriol. Rev.* **36**:478-503.

Herrero, M., V. de Lorenzo, and K. N. Timmis. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J. Bacteriol.* **172**:6557-6567.

Herruzo-Cabrera, R., J. Garcia-Caballero, M. J. Medrano-Albero, F. Villar-Alvarez, J. J. Moreno-Moreno, and J. Rey-Calero. 1988. The Dienes effect as an epidemiological tool in a paraplegic unit. *Eur. J. Epidemiol.* **4**:212-215.

Hickman, F. W., and J. J. Farmer III. 1976. Differentiation *of Proteus mirabilis* by bacteriophage typing and the Dienes reaction. *J. Clin. Microbiol.* **3**:350-358.

Hill, C., K. J. Massey, and T. R. Klaenhammer. 1991. Rapid method to characterize lactococcal bacteriophage genomes. *Appl. Environ. Microbiol.* **57**:283-288.

Hill, S. M., M. G. Jobling, B. H. Lloyd, P. Strike, and D. A. Ritchie. 1993. Functional expression of the tellurite resistance determinant from the IncHI-2 plasmid pMER610. *Mol. Gen. Genet.* **241**:203-212.

Hodgkin, J., and D. Kaiser. 1979. Genetics of gliding motility in *Myxococcus xanthus* (Myxobacterales): genes controlling movement of single cells. *Mol. Gen. Genet.* **171**:167-176.

Hoeniger, J. F. M. 1964. Cellular changes accompanying the swarming of *Proteus mirabilis*. I. Observation of living cultures. *Can. J. Microbiol.* **10**:1-9.

Hoeniger, J. F. M. 1965. Development of flagella by *Proteus mirabilis*. *J. Gen. Microbiol.* **40**:29-42.

Hoeprich, P. D., G. F. Croft, and L. M. West. 1960. Tellurite reduction as an indicator of potentially pathogenic staphylococci. *J. Lab. Clin. Med.* **55**:120-128.

Hoffman, P. S. 1974. The swarming of *Proteus mirabilis*: formation of swarm cells and swarming. M. Sc. Thesis. Iowa State University.

Hoffman, P. S., and J. I. Falkinham. 1981. Induction of tryptophanase in short and swarm cells of *Proteus vulgaris*. *J. Bacteriol.* **148**:736-738.

Hoffman, P. S., M. Ripley, and R. Weeratna. 1992. Cloning and nucleotide sequence of a gene (*ompS*) encoding the major outer membrane protein of *Legionella pneumophila*. *J. Bacteriol.* **174**:914-920.

Hofmann, K., and W. Stoffel. 1993. TMBASE - A database of membrane spanning protein segments. *Biol. Chem. Hoppe-Seyler* **374**:166-173.

Hohn, B. and J. Collins. 1980. A small cosmid for efficient cloning of large DNA fragments. *Gene* **11**:291-298.

Hoopes, B. C. and W. R. McClure. 1987. Strategies in regulation of transcription initiation. p. 1231-1240. *In* J. L. Ingraham, K. B. Low, B. Magasanik, F. C. Neidhardt, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology. Washington, D. C.

Hou, Y., and D. E. Taylor. 1994. Incidence of tellurite resistance determinants among plasmids of different incompatibility gpoups. *Plasmid* **32**:306-311.

Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.* **62**:379-433.

Hughes, K. T., K. L. Gillen, M. J. Semon, and J. E. Karlinsey. 1993. Sensing structural intermediates in bacterial flagella assembly by export of a negative regulator. *Science* **262**:1277-1280.

Ito, Y., and T. Hirano. 1997. Carbenicillin-hydrolysing penicillinase mediated by a plasmid of *Proteus mirabilis* and its relationship to the PSE-type enzymes of *Pseudomonas aeruginosa*. *J. Appl. Microbiol.* **83**:175-180.

Jarbi, E., M. B. Carr, R. P. Hausinger, and P. A. Karpus. 1995. The crystal structure of urease from *Klebsiella aerogenes* at 2 Å resolution. *Science* **268**:998-1004.

Jesaitis, M. A. 1970. The nature of colicin K from *Proteus mirabilis*. *J. Exp. Med.* **131**:1016-1038.

Jobling, M. G., and D. A. Ritchie. 1987. Genetic and physical analysis of plasmid genes expressing inducible resistance of tellurite in *Escherichia coli*. *Mol. Gen. Genet.* **208**:288-293.

Jobling, M. G., and D. A. Ritchie. 1988. The nucleotide sequence of a plasmid determinant for resistance to tellurium anions. *Gene* **66**:245-258.

Johnson, D. E., R. G. Russel, C. V. Lackatell, J. C. Zulty, J. W. Warren, and H. L. T. Mobley. 1993. Contribution of *Proteus mirabilis* urease to persistence, urolithiasis, and renal pathology in a mouse model of ascending urinary tract infection. *Infect. Immun.* **61**:2748-2754.

Jones, B. D., C. V. Locatel, D. E. Johnson, J. W. Warren, and H. L. T. Mobley. 1990. Construction of a urease negative mutant of *Proteus mirabilis*: analysis of virulence in a mouse model of ascending urinary tract infection. *Infect. Immun.* **58**:1120-1123.

Jones, B. D., and H. L. T. Mobley. 1988. *Proteus mirabilis* urease: genetic organization, regulation, and expression of structural genes. *J. Bacteriol.* **170**: 3342-3349.

Jones, B. D., and H. L. T. Mobley. 1989. *Proteus mirabilis* urease: nucleotide sequence determination and comparison with jack bean urease. *J. Bacteriol.* **171**:6414-6422.

Jones, H. E., and R. W. A. Park. 1967. The short and long forms of *Proteus. J. Gen. Microbiol.* **47**:359-367.

Kaback, H. R. 1971. Specific organelles and extraction procedures. Bacterial membranes. *Methods Ezymol.* **22**:99-120.

Kadner, R. J., and P. J. Bassford, Jr. 1978. The role of the outer membrane in active transport, p. 413-455. *In* B. P. Rosen (ed.) Bacterial transport. Marcel Dekker, Inc., New York.

Kaneko,T.,S. Sato, H. Kotani,A. Tanaka, E. Asamizu, Y. Nakamura, N. Miyajima, M. Hirosawa, M. Sugiura, S. Sasamoto, T. Kimura, T. Hosouchi, A. Matsuno, A. Muraki, N. Nakazaki, K Naruo, S. Okumura, S. Shimpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda, and S. Tabata. 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res.* **3**:109-136.

Katz, L., and S. Donadio. 1993. Polyketide synthesis: prospects for hybrid antibiotics. *Annu. Rev. Microbiol.* **47**:875-912.

Kieny, M. P., R. Lathe, and J. P. Lecocq. 1983. New versatile cloning and sequencing vectors based on bacteriophage M13. *Gene* **26**:91-99.

Kim, S. K., D. Kaiser, and A. Kuspa. 1992. Control of cell density and pattern by intercellular signaling in *Myxococcus* development. *Annu. Rev. Microbiol.* **46**:117-139.

Klein, P. M. Kanehisa, and C. DeLisi. 1985. The detection and classification of membrane-spanning proteins. *Biochim. Biophys. Acta* **815**:468-476.

Kohnle, W., E. Vanek, K. Federlin, and H. E. Franz. 1975. Lokalisation eines Harnwegninfektes durch Nachweis von antikorperbesetzten Bakterien im Urin. *Dtsch. Med. Wochenschr.* **100**:2598-2602.

Konisky, J. 1982. Colicins and other bacteriocins with established modes of action. *Annu. Rev. Microbiol.* **36**:124-144.

Konkoly Thege, M., and S. Nikolnikov. 1988. Characterization of multiply resistant *Proteus mirabilis* isolates in Hungary. *Acta Microbiol. Hung.* **35**:423-428.

Kouzarides, T., and E. Ziff. 1989. Leucine zipper of fos, jun and GCN4 dictate dimerization specificity and thereby control DNA binding. *Nature* **340**:568-571.

Krizsanovich, K. 1973. Cryptic lysogeny of *Proteus mirabilis*. *J. Gen. Virol.* **19**:311-320.

Kroczek, R. A., and E. Siebert. 1990. Optimization of northern blot analysis by vacuum-blotting, RNA transfer, and ultraviolet fixation. *Anal. Biochem.* **184**:90-95.

Kumano, M., A. Tamakoshi, and K. Yamane. 1997. A 32 kb nucleotide sequence from the region of the lincomycin-resistance gene (22-25 degree) of the *Bacillus subtilis* chromosome and identification of the site of the lin-2 mutation. Unpublished, direct submission to the GenBank.

Kupfer, D. M., J. S. Downard, and B. A. Roe. 1996. Direct submission to the GenBank.

Kvittingen, J. 1949. Studies of the life-cycle of *Proteus* Hauser. Part II. *Acta Pathol. Microbiol. Scand.* **26**:855-878.

Lai, H.-C., D. Gygi, G. M. Farser, and C. Hughes. 1998. A swarming-defective mutant of *Proteus mirabilis* lacking a putative cation-transporting membrane P-type ATPase. *Microbiology* **144**:1957-1961.

Larsson, P., A. Fasth, U. Jodan, A. Sohl Akerlung, and C. Svanborg Eden. 1978. Urinary tract infections caused by *Proteus mirabilis* in children. The antibody response to O and H antigens and Tamm Horsfall *protein* and bacterial adherence to uroepithelium. *Acta Paediatr. Scand.* **67**: 591-596.

Lee, B. T. O., N. L. Brown, S. Rogers, A. Bergemann, J. Camakaris, and D. A. Rouch. 1990. Bacterial response to copper in the environment: copper resistance in *Escherichia coli* as a model system. *NATO ASI Ser. Ser. G.* **23**:625-632.

Liu, X., and P. Matsumura. 1994.The FlhC/FlhD complex, a transcriptional activator of the *Escherichia coli* flagellar class II operons. *J. Bacteriol.* **176**:7345-7451.

Lloyd-Jones, G., M. Osborne, D. A. Ritchie, P. Strike, J. L. Hobman, N. L. Brown, and D. Rouch. 1994. Accumulation and intracellular fate of tellurite in tellurite-resistant *Escherichia coli*: a model for the mechanism of resistance. *FEMS Microbiol. Lett.* **118**:113-120.

Lloyd-Jones, G., D. A. Ritchie, and P. Strike. 1991. Biochemical and biophysical analysis of plasmid pMJ600-encoded tellurite [TeO_3^{2-}] resistance. *FEMS Microbiol. Lett.* **81**:19-24.

Loomes, L. M., B. W. Senior, and M. A. Kerr. 1990. A proteolytic enzyme secreted by *Proteus mirabilis* degrades immunoglobulins of the immunoglobulin A1 (IgA1), IgA2 and IgG isotypes. *Infect. Immum.* **58**:1979-1985.

Luria, S. E., and J. L. Suit. 1987. Colicins and Col plasmids, p. 1615-1624. *In* J. L. Ingraham, K. B. Low, B. Magasanik, F. C. Neidhardt, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D. C.

Macnab, R. M. 1992. Genetics and biogenesis of bacterial flagella. *Annu. Rev. Genet.* **26**:131-158.

Magnuson, R., J. Solomon, and A. D. Grossman. 1994. Biochemical and genetic characterization of a competence pheromone from *B. subtilis. Cell* **77**:207-216.

Mandel, M., and A. Higa. 1970. Calcium-dependent bacteriophage DNA infection. *J. Mol. Biol.* **53**:159-163.

Mariotte, S., P. Nordmann, and M. H. Nicolas. 1994. Extended-spectrum beta-lactamase in *Proteus mirabilis. J. Antimicrob. Chemother.* **33**:925-935.

Massad, G., J. F. Fulkerson Jr., D. C. Watson, and H. L. T. Mobley. 1996. *Proteus mirabilis* ambient-temperature fimbriae: cloning and nucleotide sequence of the *atf* gene cluster. *Infect. Immun.* **64:** 4390-4395.

Massad, G., H. Zhao, and H. L. T. Mobley. 1995. *Proteus mirabilis* amino acid deaminase: cloning, nucleotide sequence, and characterization of *aad. J. Bacteriol.***177**:5878-5883.

Matthew, M., R. W. Hedges, and J. T. Smith. 1979. Types of β-lactamase determined by plasmids in gram-negative bacteria. *J. Bacteriol.* **138**:657-704.

McCarter, L., M. Hilmen, and M. Silverman. 1988. Flagellar dynamometer controls swarmer cell differentiation of *V. parahaemolyticus. Cell* **54**:345-351.

McCarter, L., and M. Silverman. 1989. Iron regulation of swarmer cell differentiation of *Vibrio parahaemolyticus. J. Bacteriol.* **171**:731-736.

McCarter, L. and M. Silverman. 1990. Surface-induced swarmer cell differentiation of *Vibrio parahaemolyticus. Mol. Microbiol.* **4**:1057-1062.

McLean. R. J., C. J. Nickel, K.-J. Cheng, and J. W. Costeron. 1988. The ecology and pathogenicity of urease-producing bacteria in the urinary tract. *Crit. Rev. Microbiol.* **16:**37-79.

Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Miller, V. L., and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170:** 2575-2583.

Mobley, H. L. T. 1996. Virulence of *Proteus mirabilis*, p. 245-270. *In* H. L. T. Mobley and J. W. Warren (ed.), Urinary tract infections: Molecular pathogenesis and clinical management. ASM Press. Washington. D. C.

Mobley, H. L. T., and R. Belas. 1995. Swarming and Pathogenicity of *Proteus mirabilis* in the urinary tract. *Trends Microbiol.* **3:**280-284.

Mobley, H. L. T., and R. P. Hausinger. 1989. Microbial ureases: significance, regulation, and molecular characterization. *Microbiol. Rev.* **53:**85-108.

Mobley, H. L. T., M. D. Island, and R. P. Hausinger. 1995. Molecular biology of microbial ureases. *Microbiol. Rev.* **59:**451-480.

Morrison, R. B., and A. Scott. 1966. Swarming of *Proteus* - a solution to an old problem? *Nature* **211:**255-257.

Nakai, K., and M. Kanehisa. 1991. Expert system for predicting protein localization sites in Gram-negative bacteria. *Prot.: Struct., Func., and Gen.* **11:**95-110.

Newman, E. B., and R. Lin. 1995. Leucine-responsive regulatory protein: a global regulator of gene expression in *E. coli*. *Annu. Rev. Microbiol.* **49:**747-775.

Nicholson, E. B., E. A. Concaugh, P. A. Foxall, M. D. Island, and H. L. T. Mobley. 1993. *Proteus mirabilis* urease: transcriptional regulation by UreR. *J. Bacteriol.* **175:**465-473.

Nugent, M. E. 1981. A conjugative 'plasmid' lacking autonomous replication. *J. Gen. Microbiol.* **126:**305-310.

Old, D. C., and R. Adegbola. 1982. Haemaglutinins and fimbriae of *Morganella*, *Proteus* and *Providencia*. *J. Med. Microbiol.* **15:**551-564.

Olukoya, D. K., S. I. Smith, and M. O. Ilori. 1997. Isolation and characterization of heavy metal resistant bacteria from Lagos Lagoon. *Folia Microbiol.* **42**:441-444.

Pearson, W. R. 1990. Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods Enzymol.* **183**:63-98.

Peerbooms, P. G. M., A. M. J. J. Verweij, and D. MacLaren. 1984. Vero cell invasiveness of *Proteus mirabilis*. *Infect. Immun.* **43**:1068-1071.

Peerbooms, P. G. M., A. M. J. J. Verweij, and D. MacLaren. 1985. Uropathogenic properties of *Proteus mirabilis* and *Proteus vulgaris*. *J. Med. Microbiol.* **19**:55-60.

Penner, J. L. 1989. Genus XI. *Proteus*, p. 491-494. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore, Md.

Penner, J. L. 1992. The genera *Proteus*, *Providencia* and *Morganella*, p. 2849-2853. *In* A. Balows, H. G. Trüper, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, vol. III. Springer-Verlag KG, Berlin, Germany.

Pillage, C. J., J.-J. Gordon, K. Jury, C. A. Shearman, and M. J. Gasson. 1994. Tellurium resistance carried on the *Lactococcus lactis sex* factor, abstr. P.21, p. 59. GIM 94 Satellite Workshop on Plasmid Diversity, Montreal, Quebec, Canada.

Pilsl, H., and V. Braun. 1995. Evidence that the immunity protein inactivates colicin 5 immediately prior the formation of the transmembrane channel. *J. Bacteriol.* **177**:6966-6972.

Postle, K. 1990. TonB and the Gram-negative dilemma. *Mol. Microbiol.* **4**:2019-2025.

Pretorius. G. H. J., and W. F. Coetzee. 1979. *Proteus mirabilis* phage 5006M: a physical characterization. *J. Gen. Microbiol.* **45**:389-395.

Pruß, B. M., and P. Matsumura. 1996. A regulator of the flagella regulon of *Escherichia coli*, *flhD*, also affects cell division. *J. Bacteriol.* **178**:668-674.

Pugsley, A. P. 1984. The ins and outs of colicins. I. Production and translocation across membranes. *Microbiol. Sci.* **1**:168-174.

Pugsley, A. P. 1984a. The ins and outs of colicins. II. Lethal action, immunity and ecological implications. *Microbiol. Sci.* **1**:203-207.

Ralston, D. M., and T. V. O'Halloran. 1990. Ultrasensitivity and heavy-metal selectivity of the allosterically modulated MerR transcriptional complex. *Proc. Natl. Acad. Sci. USA* **87**:3846-3850.

Rando, D., U. Steglitz, G. Mörsdof, and H. Kaltwasser. 1990. Nickel availability and urease expression in *Proteus mirabilis*. *Arch. Microbiol.* **154**:428-432.

Rauprich, O., M. Matsushita, C. J. Weijer, F. Siegert, S. E. Episov, and J. A. Shapiro. 1996. Periodic phenomenon in *Proteus mirabilis* swarm colony development. *J. Bacteriol.* **178**:6525-6538.

Revel, H. R., and C. P. Georgopoulos. 1969. Restriction of nonglucosylated T-even bacteriophages by prophage P1. *Virology* **29**:1-17.

Riley, M. A., and D. M. Gordon. 1992. A survey of Col plasmids in natural isolates of *Escherichia coli* and an investigation into the stability of Col plasmids lineages. *J. Gen. Microbiol.* **138**:1345-1352.

Robson, B., and J. Garnier. 1993. Protein structure prediction. *Nature* **361**:506-508.

Ròzalski, A., Z. Sidorczyk, and K. Kotelko. 1997. Potential virulence factors of *Proteus* bacilli. *Microbiol. Mol. Biol. Rev.* **61**:65-89.

Rubin, R. H., N. E. Tolkoff-Rubin, and R. S. Cortan. 1986. Urinary tract infection, pyelonephritis and reflux nephropathy, p. 1085-1143, *In* B. M. Brenner and F. C. Rector (ed.), The kidney. The W. B. Saunders Co., Philadelphia, Pa.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanger, F., S. Nicklen, and A. R. Coulsen. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.

Saraste, M., P. R. Shibbald, and A. Wittinghofer. 1990. The P-loop – a common motif in ATP and GTP binding proteins. *Trends Biochem. Sci.* **15**:430-434.

Scott, T. G. 1960. The bacteriology of urinary infections in paraplegia. *J. Clin. Pathol.* **35**:54-56.

Senior, B. W. 1977. Typing of *Proteus* strains by proticine production and sensitivity. *J. Med. Microbiol.* **10**:7-17.

Senior, B. W. 1977a. The Dienes phenomenon: identification of the determinants of compatibility. *J. Gen. Microbiol.* **102**:235-244.

Senior, B. W., L. M. Loomes, and M. A. Kerr. 1991. The production and activity *in vivo Proteus mirabilis* IgA protease in infections of the urinary tract. *J. Med. Microbiol.* **35**:203-207.

Senior, B. W., and C. Hughes. 1987. Production and properties of haemolysin from clinical isolates of the *Proteeae. J. Med. Microbiol.* **24**:17-25.

Shi, W., and D. R., Zusman. 1993. The two motility systems of *Myxococcus xanthus* show different selective advantages of various surfaces. *Proc. Natl. Acad. Sci. USA* **90**:3378-3382.

Shimkets, L. 1990. Social and developmental biology of the myxobacteria. *Microbiol. Rev.* **54**:473-501.

Shin, S., and C. Park. 1995. Modulation of flagella expression in *Escherichia coli* by acetyl phosphate and the osmoregulator OmpR. *J. Bacteriol.* **177**:4696-4702.

Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse. 1988. λ ZAP: A bacteriophage λ expression vector with *in vivo* excision properties. *Nucleic Acids Res.* **16**:7583-7590.

Sidorczyk, Z., and K. Zych. 1986. Lipopolysaccharides of flagellated and non-flagellated *Proteus vulgaris* strains. *Arch. Immunol. Ther. Exp.* **34**:461-469.

Silhavey, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Silver, S., and M. Walderhaug. 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. *Microbiol. Rev.* **56**:195-228.

Silverblatt, F. J. 1974. Host-parasite interractions in renal pelvis: a possible role of pili in the pathogenesis of pyelonephritis. *J. Exp. Med.* **140**:1696-1711.

Silverman, M., and M. Simon. 1974. Characterization of *Escherichia coli* flagellar mutants that are insensitive to catabolite repression. *J. Bacteriol.* **172**:1196-1203.

Skadhauge, K. 1950. Studies on enterococci with special reference to the serological properties. Monograph. Einar Munksgaards forlag, Copenhagen.

Skirrow, M. B. 1969. The Dienes (Mutual Inhibition) test in the investigation of *Proteus* infections. *J. Med. Microbiol.* **4**:471-477.

Smith, C. A., and I. Rayment. 1996. Active site comparisons highlight structural similarities between myosin and other P-loop proteins. *Biophys. J.* **70**:1590-1602.

Sogaard, H. C., K. Zimmermann-Neilsen, and K. Siboni. 1974. Antibiotic-resistant gram-negative bacilli in a urological ward for male patients during a nine-year period: relationship to antibiotic consumption. *J. Infect. Dis.* **130**:646-650.

Solovyov, V. V., A. A. Zharkikh, N. A. Kolchanov, and V. A. Ratner. 1984. The template RNAs of RNA polymerases can have compact secondary structure, formed by long double helices with partial violations of complementarity. *FEBS Lett.* **165**:72-78.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.

Sriwanthana, B., M. D. Island, and H. L. T. Mobley. 1993. Sequence of *Proteus mirabilis* urease accessory gene *ureG*. *Gene* **129**:103-106.

Sriwanthana, B., M. D. Island, D. Maneval, and H. L. T. Mobley. 1994. Single-step purification of *Proteus mirabilis* urease accessory protein UreE, a protein with naturally occurring histidine tail, by nickel chelate affinity chromotography. *J. Bacteriol.* **176**:6836-6841.

Stachelhaus, T., and M. A. Marahiel. 1995. Modular structure of genes encoding multifunctional peptide synthases required for non-ribosomal peptide synthesis. *FEMS Microbiol. Lett.* **125**:3-14.

Stephens, C., and L. Shapiro. 1993. An unusual promoter controls cell-cycle regulation and dependence on DNA replication of the *Caulobacter fliLM* early flagellar operon. *Mol. Microbiol.* **9**:1169-1179.

Stickler, D. J. 1998. Personal communication.

Stohs, S. J., and D. Bagehi. 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med.* **18**:321-336.

Story, P. 1954. *Proteus* infections in hospitals. *J. Pathol. Bacteriol.* **68**:55-62.

Sultan, W. W. 1969. Isolation of phenylalanine-negative *Proteus vulgaris*. *Appl. Microbiol.* **18**:688-672.

Summers, A. O., and G. A. Jacoby. 1977. Plasmid-determined resistance to tellurium compounds. *J. Bacteriol.* **129**:276-281.

Sun, X., A. L. Bognar, E. N. Baker, and C. A. Smith. 1998. Structural homologies with ATP- and folate-binding enzymes in the crystal structure of folylpolyglutamate synthetase. *Proc. Natl. Acad. Sci. USA* **95**:6647-6652.

Sutcliffe, J. G. 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. *Cold Spring Harbor Symp. Quant. Biol.* **43**:77-83.

Swihart, K. G., and R. A. Welch. 1990. The HpmA hemolysin is more common than HlyA among *Proteus* isolates. *Infect. Immun.* **58**:1853-1860.

Taylor, D. E., and R. B. Grant. 1977. Incompatibility and bacteriophage inhibition properties of N-1, a plasmid belonging to the H_2 incompatibility group. *Mol. Gen. Genet.* **153**:5-10.

Taylor, D. E., Y. Hou, R. J. Turber, and J. Weiner. 1994. Location of potassium tellurite resistance operon (*tehAtehB*) within the terminus of *Escherichia coli* K-12. *J. Bacteriol.* **176**:2740-2742.

Taylor, D. E., and A. O. Summers. 1979. Association of tellurium resistance and bacteriophage inhibition conferred by R plasmids. *J. Bacteriol.* **137**:1430-1433.

Taylor, D. E., E. G. Walter, R. Sherburne, and D. P. Bazett-Jones. 1988. Structure and location of tellurium deposited in *Escherichia coli* cells harboring tellurite resistance plasmids. *J. Ultrastruct. Mol. Struct. Res.* **99**:18-26.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673-4680.

Tomas, J. M., and W. W. Kay. 1986. Tellurite susceptibility and non-plasmid-mediated resistance in *Escherichia coli*. *Antimicrob. Agents Chemother.* **30**:127-131.

Toptchieva, A., Goodwin, A., Sisson, G., Landry, D., Cameron, B., and P. S. Hoffman. 1998. Regulation of urease expression in swarmer and swimmer cells of *Proteus mirabilis*. Submitted for publication.

Trevors, J. T. 1987. Copper resistance in bacteria. *Microbiol. Sci.* **4**:29-31.

Trevors, J. T., K. M. Oddie, and B. H. Belliveau. 1985. Metal resistance in bacteria. *FEMS Microbiol. Rev.* **32**:39-54.

Tsang, A. S., and M. Tasaka. 1986. Identification of multiple cyclic AMP-binding proteins in developing *Dictyostelium discoideum* cells. *J. Biol. Chem.* **261**:10753-10759.

Turner, R. J., Y. Hou, J. H. Weiner, and D. E. Taylor. 1992. The arsenical ATPase efflux pump mediates tellurite resistance. *J. Bacteriol.* **174**:3092-3094.

Turner, R. J., J. H. Wiener, and D. E. Taylor. 1995. The tellurite-resistance determinants *tehAtehB* and *klaAklaBtelB* have different biochemical requirements. *Microbiol.***141**:3133-3140.

Uphoff, T. S., and R. A. Welch. 1990. Nucleotide sequencing of the *Proteus mirabilis* calcium-independent hemolysin genes (*hpmA* and *hpmB*) reveals sequence similarity with the *Serratia marcescens* hemolysin genes (*shlA* and *shlB*). *J. Bacteriol.* **172**:1206-1216.

VanderMolen, G., and F. Williams. 1977. Observation of the swarming of *Proteus mirabilis* with scanning electron microscopy. *Can. J. Microbiol.* **23**:107-112.

von Heijne, G., and Y. Gavel. 1988. Topogenic signals in integral membrane proteins. *Eur. J. Biochem.* **174**:671-678.

Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay. 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1**:945-951.

Walter, E. G., and D. E. Taylor. 1992. Plasmid-mediated resistance to tellurite: expressed and cryptic. *Plasmid* **27**:52-64.

Walter, E. G., C. M. Thomas, J. P. Ibbotson, and D. E. Taylor. 1991. Transcriptional analysis, and sequence of the *kilA*-tellurite resistance region of plasmid RK2Te^r. *J. Bacteriol.* **173**:1111-1119.

Ward, M. J., and D. R. Zusman. 1997. Regulation of directed motility in *Myxococcus xanthus*. *Mol. Microbiol.* **24**:885-893

Warren, J. W. 1996. Clinical presentations and epidemiology of urinary tract infections, p. 3-27. *In* H. L. T. Mobley and J. W. Warren (ed.), Urinary tract infections: Molecular pathogenesis and clinical management. ASM Press, Washington, D. C.

Wassif, C., D. Cheek, and R. Belas. 1995. Molecular analysis of a metalloprotease from *Proteus mirabilis*. *J. Bacteriol.* **177**:5790-5798.

Watanabe, T., T. Takano, T. Arai, H. Nishida, and S. Sato. 1966. Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. X. Restriction and modification of phages by *fi* factors. *J. Bacteriol.* **92**:477-486.

Watanabe, Y., T. Yokota, Y. Higashi, Y. Wakai, and Y. Mine. 1991. *In vitro* and *in vivo* transferrable beta-lactam resistance due to a new plasmid-mediated oxyminocephalosporinase from a clinical isolate of *Proteus mirabilis*. *Microbiol. Immunol.* **35**:87-97.

Wenner, J. J., and L. F. Rettger. 1919. A systematic study of *Proteus* group of bacteria. *J. Bacteriol.* **4**: 331-353.

Whelan, K. F., M. Bekkering, M. and D. E. Taylor. 1996. Analysis of the region upstream of *terZ* in plasmid R478. Direct submission to GenBank.

Whelan, K. F., and E. Colleran. 1992. Restriction endonuclease mapping of the H12 incompatibility group plasmid R478. *J. Bacteriol.* **174**:1197-1204.

Whelan, K. F., Colleran, E. and D. E. Taylor. 1995. Phage inhibition, colicin resistance, and tellurite resistance are encoded by a single cluster of genes on the IncH12 plasmid R478. *J. Bacteriol.* **177**:5016-5027.

Whelan, K. F., R. K. Sherburne, and D. E. Taylor. 1997. Characterisation of a region of the IncH12 plasmid R478 which protects *Escherichia coli* from toxic effects specified by components of the tellurite, phage and colicin resistance cluster. *J. Bacteriol.* **179**:63-71.

Williams, F. D., D. M. Anderson, P. S. Hoffman, R. H. Schwarzhoff, and S. Leonard. 1976. Evidence against the involvement of chemotaxis in swarming of *Proteus mirabilis*. *J. Bacteriol.* **127**:237-248.

Williams, F. D. and R. H. Schwarzhoff. 1978. Nature of the swarming phenomenon in *Proteus*. *Annu. Rev. Microbiol.* **32**:101-122.

Wilson, C., M. Corbett, and A. Ebringer. 1990. Increased isolation of *Proteus mirabilis* species from rheumatoid arthritis patients and healthy controls. *Br. J. Reumatol.* **29** (Suppl. II): 99. (Abstract).

Wilson, D. R., and T. J. Beveridge. 1993. Bacterial flagellar filaments and their component flagellins *Can. J. Microbiol.* **39**:451-472.

Yager, T. D., and P. H. von Hippel. 1987. Transcript elongation and termination in *Escherichia coli* and *Salmonella typhimurium*, p. 1241-1275. *In* J. L. Ingraham, K. B. Low, B. Magasanik, F. C. Neidhardt, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Whachington, D. C.

Zharkikh, A., and W. H. Li. 1992. Statistical properties of bootstrap estimation of phylogenetic variability from nucleotide sequences. I. Four taxa with a molecular click. *Mol. Biol. Evol.* **9**:1119-111 47.

Ziman, J. M. 1969. Information, communication, knowledge. *Nature* **224**:318-324.