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Studies of Tellurite Resistance Genes from *Escherichia coli* and *Streptococcus pneumoniae*

by

Mingfu Liu ©

A thesis submited to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Master of Science

Department of Biological Sciences

Edmonton, Alberta

Spring 1999

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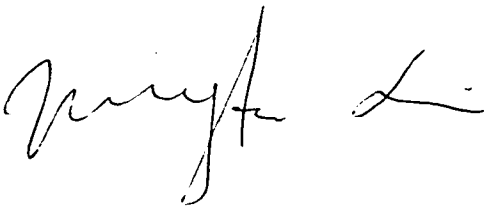

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Studies of Tellurite Resistance Genes from *Escherichia coli* and *Streptococcus pneumoniae*** submitted by **Mingfu Liu** in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

Tellurite resistance genes have been identified in a number of microorganisms; at least five different determinants have been characterized. The *Escherichia coli* chromosomal determinant for tellurite resistance consists of two genes (*tehAtehB*) which confer a resistance of 128 μg/ml K_2TeO_3 when expressed on a multi-copy plasmid, compared to the wild-type MIC of 2 μg/ml K_2TeO_3. TehA is a membrane protein with 10 predicted transmembrane segments, TehB is probably a cytoplasmic protein with three conserved motifs (I, II and III) that are found in many *S*-adenosyl-L-methionine (AdoMet)-dependent non-nucleic acid methyltransferases. Both TehA and TehB have three cysteine residues. Oligonucleotide-directed mutagenesis was carried out on all six cysteine residues, the results showed that cysteine residues in both TehA and TehB play a minor role in tellurite resistance: a single cysteine change had no effect, combinations of two or three cysteine changes had only minor effects (MICs of 16-64 μg/ml). The non-cysteine mutant, in which all six cysteine residues were replaced by alanine residues, maintained an MIC of 16 μg/ml K_2TeO_3. In contrast, substitution of the conserved aspartate residue in motif I by asparagine or alanine, or the conserved phenylalanine in motif II by tyrosine or alanine decreased resistance to background levels (2 μg/ml). These results are consistent with a role for motif I and II in tellurite resistance, suggesting that TehB may act as a methyltransferase.

The *Streptococcus pneumoniae* is a gram-positive bacterium which is naturally resistant to tellurite. A homologue of the *E. coli tehB* gene from *Streptococcus. pneumoniae* was cloned and sequenced. It encoded for a putative protein of 284 amino

acids which is 86 residues longer than the corresponding *Escherichia coli* TehB, but similar to the *Hemophilus influenzae* TehB homologue and the *Eikenella corrodens* hemagglutinin (Hag1) as well as homologues from *Actinobacillus actinomycetemcomiyans*, *Neisseria gonorrhoeae* and *Neisseria meningitidis*. The *S. pneumoniae* TehB shared 46-58% identity (52-68% similarity) and the similar hydropathy properties to these proteins. The results in this study showed that the *S. pneumoniae tehB* gene alone not only conferred *E. coli* high level resistance to tellurite (128 μg/ml), but also caused a filamentous morphology in *E. coli*. The filamentation may be related to its methyltransferase activity.

Acknowledgement

Upon the completion of my thesis, I would like to take this opportunity to acknowledge all those people who have helped me and encouraged me to continue my studies.

First I would like to thank my supervisors Dr. Diane E. Taylor and Dr. Kenneth L. Roy for their guidance, patience and helps during my research. I would also like to thank Dr. Brenda K. Leskiw and Dr. Glen D. Armstrong for serving as the committee members. My special thanks go to Dr. Dave Kelly and Dr. Raymond J. Turner for their suggestions, discussions and critical readings of my writings. I also thank Michelle Rooker for her skillful technical assistance, Richard Sherburne for the scanning electron micrograph and San Vinh for photographic assistance. I am deeply appreciative of Margo Diadio for generously providing the pneumococcus strain and Ann Progulske-Fox at the University of Florida for the hemagglutinin gene-containing plasmid.

My gratitude extends to Dr. Ross B. Hodgetts and Dr. Michael Durairaj for their help and encouragement. I also greatly appreciate the help and friendship from the past and present members in the lab, they are: Sean Cornell, Dr. Zhongmin Ge, Qin Jiang, Trevor Lawley, Dave Rasco, Craig Sherburne, Dr. Cathy Trieber, Dr. Joanne Simala-Grant and Dr. Ge Wang.

Finally I would like to express my extreme gratitude to my wife Hong and our son Xiangyu. Without their love, patience and understanding, this work would not be finished.

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1. Introduction

1.1 Properties of Tellurium and Its Compounds

Tellurium (Te) is an element in Group VIA of the Periodic Table, which was discovered in gold ores by the German scientist Franz Josoph Muller von Reichenstein in 1782, but was recognized and given its name 16 years later by the German chemist Martin Heinrich Klaproth. Te is grouped with oxygen (O), sulfur (S), selenium (Se) and polonium (Po). It is a metalloid and exhibits similar properties to other elements such as arsenic (As) and antimony (Sb).

Tellurium is most often found as the telluride of gold (calverite), and also combined with silver, copper, lead and nickel. Occasionally it is found in a pure form or in rocks as the ore tellurite (TeO_2). It is a by-product of copper refinery, and is recovered from its electrolytic anode muds. The world annual product is about 500 tons (Scansetti, 1992). Canada's production in 1996 was about 50 kilograms (Natural Resources Canada, 1996).

1.2 Applications

1.2.1 Industrial Applications

Tellurium compound is used for many industry purposes. They are used in the manufacture of electrical rectifiers, thermoelectric devices and semiconductors and as components of special alloys, where they improve their hardness and resistance to corrosion. They are employed as a vulcanizing agent in the processing of natural and

synthetic rubber, and in antiknock compounds for gasoline as well as in coloring agents for glass and ceramics (Hou, 1994; Walter, 1990).

1.2.2 Microbiological Applications

Tellurium compounds has been used as insecticides, germicides, and fungicides, and were once used to treat some human diseases, including syphilis, leprosy, tuberculosis and cystitis, based on the high toxicity of the compounds for the pathogens involved (Cooper, 1971). Tellurite (TeO_3^{2-}) has been found to be toxic to many microorganisms, particularly to gram-negative bacteria (Fleming, 1932; Scala and William, 1963). Many others, particularly gram-positive bacteria, for example, *Corynebacterium diphtheria* (Smith *et al.*, 1977), *Staphylococcus aureus* (Hoeprich *et al.*, 1960) and *Enterococus faecalis* (Appleman and Heinmiller, 1961), are naturally resistant to tellurite. This characteristic of differential resistance to tellurite has long been used to diagnose and identify certain bacteria. For example, it has been used for the laboratory determination of potential pathogenicity of *Staphylococcus* species isolated from clinical sources (Hoeprich *et al.*, 1960). Tellurite is also used for the isolation of *E. coli* O157 from meat or stool samples (Zadik *et al.*, 1993; Bolton *et al.*, 1996). In tellurite-containing media, these resistant bacteria form distinctive black or grey-black colonies, whereas the growth of tellurite-sensitive bacteria is inhibited (Hoeprich *et al.*, 1960; Johnson and Sneath, 1973; Smith, 1977; Dukta, 1977; O'Brien and Cowell, 1985; Bolton *et al.*, 1996).

1.2.3 Medical Applications

Unlike organosulfur and organoselenium compounds, only very few organotellurium compounds are present in natural sources. The reason could be that organotellurium compounds are very sensitive to light and oxidation. However a number of tellurium analogues of naturally occurring compounds have been successfully chemically synthesized, some of which promised to be useful.

An organotellurate, ammonium tri-chloro(dioxoethylene-o,o'-) tellurate (AS-101), has been found to have immunomodulating properties and minimal toxicity due to stimulation of cytokine production (Sredni *et al.*, 1987; Montero *et al.*, 1993). It also inhibits the production of human immunodeficiency virus (HIV) (Vonsover *et al.*, 1992) and has immunomodulatory effects on murine cytomegalovirus (MCMV)-mediated myelosuppression (Sredni *et al.*, 1994). Phase II clinical trials have been initiated on cancer patients in combination with Taxol (Vadhan-Raj *et al.*, 1995; Kalechman *et al.*, 1996). In addition, a series of amine platinum (II) tellurate complexes {(A)Pt(II)[$TeO_2(OH)_4$]} (Khokhar *et al.*, 1994) and a tellurium-containing cyanine (3-ethyl-3'-methyl-thiatelluracarbocyanine iodide) also showed good anti-tumor activities (Sun *et al.*, 1996).

Some tellurium-containing compounds have shown antimicrobial activities, these include tellurium sulphonamide Schiff base compounds, tellurium complexes with substituted chalcones, tellurium and selenium complexes with aromatic imine, 2-

substituted benzimidazoles and thiopicolinamide (Sadeh, 1987). For some telluroamino acids, anticarcinogenic and antileukaemic properties have been claimed (Sadeh, 1987).

Other organotellurium compounds include telluro-fatty acids and telluro-steroides which have been used in biomedical research (Sadeh, 1987; Knapp, 1981).

1.2.4 Other Applications

Since demonstration by Cowie and Cohen on the biosynthesis in *E. coli* that active proteins may contain selenomethionine instead of the conventional methionine, there have been numerous reports of the introduction of various amino acid analogues (Cowie and Cohen, 1957; Cowie, *et al.*, 1959; Bogisian, *et al.*, 1989). These seleno-containing proteins have been proved to be very useful for the elucidation of the protein synthesis mechanism, and recently for structural studies (Hendrickson, *et al.*, 1990; Hendrickson, 1991). Tellurium, heavier than selenium, is considered as a better substitution for the X-ray crystallographic analysis, because it does not require synchrotron radiation for the precise control of X-ray wavelengths (Boles, *et al.*, 1994). Telluromethionine has been successfully biosynthetically incorporated into many different proteins, including *E. coli* dihydrofolate reductase (Boles, *et al.*, 1994; Boles, *et al.*, 1995), human annexin (Budisa, *et al.*, 1998), human mitochondrial transferase (Humm, *et al.*, 1997), *Arabidopsis* glutathione-S-transferase, *Salmonella* tailspike adhesion protein (Steinbacher, *et al.*, 1997) and *Streptococcus aureus* pyrrolidone carboxyl peptidase (PYRase) (Boles, *et al.*, 1997). These telluromethionine-incorporated

proteins were found to have the same properties and to crystallize isomorphously compared to their native ones.

Telluroproteins were generally overexpressed in a methionine-auxothophic *E. coli* [for example *E. coli* DL41, *E. coli* B834(DE3)], exposed to telluromethionine added to the medium. Methionine, telluromethionine, as well as selenomethionine, were found to be activated and loaded with similar efficiencies on to methionyl tRNA (Boles *et al.*, 1994). Tellurocysteine can also be incorprated by a cysteine-auxotrophic *E. coli* strain (Besse, *et al.*, 1997).

Although these single-atom mutations are very useful for the study of protein structure and function, one difficulty which needs to be overcome is that telluromethionine is very sensitive to oxidation. This makes the synthesis of telluromethionine very difficult and the bio-incorporation of telluromethionine only occurs in buried positions within proteins (Boles, *et al.*, 1994; Bulisa, *et al.*, 1997).

1.3 Distribution of Tellurium Compounds

1.3.1 Environmental Distribution

Tellurium is a rare metalloid on earth, it is estimated to rank about seventy-fifth in natural abundance, *i.e.*, 0.002 ppm (Mason, 1958; Hampel, 1961), although the highlands on Venus might be covered by tellurium (Kerr, 1996). The element is, however, widely distributed in nature. In addition to the metal ores mentioned above, it has been detected from various soils (Hampel, 1967; D'Ulivo, 1997), plants (Schroeder, *at al.*, 1967;

Cowgill, 1988,), waters (Jinru *et al.*, 1983; Andreae, 1984) and some coals (Goldschmidt, 1958), although it was not detected by older analytical methods in soils (Vinogrador, 1959) and sea water (Mason, 1958; Goldschimidt, 1958). Schroeder's comprehensive survey indicated that tellurium (compounds) exists almost everywhere. The human body was also found to contain tellurium and there was about 600 mg of tellurium in a "standard" man, this level exceeds those of all other trace elements except iron, zinc and rubidium (Schroeder, *et al.*, 1967).

1.3.2 Tellurium in Biota

Tellurium in the human body is not evenly distributed. The greatest proportion is in bones with only a small amount in soft tissues (about 50 mg). This pattern of storage is different from that in the rat, the rabbit and the dog, in which the highest concentrations were found in kidney 24 hr post injection of radiolabeled sodium tellurite (De Meio and Henriques, 1947).

A relatively large amount of tellurium was also found in fresh garlic buds. When garlic (*Allium sativum*) is subjected to wet-ashed process, it contains about 70 ppm tellurium; but if garlic is dry ashed, tellurium could not be detected. Thus the tellurium in garlic is in a volatile form, presumably dimethyltelluride or another volatile telluride (Schroeder, *et al.*, 1967).

Vegetation and humus from remote mountain forests were found to have the same range of tellurium as that from industrial areas, suggesting that human influenced deposition is minimal (Schroeder, *et al.*, 1967).

The organic chemistry of tellurium, like sulfur and selenium, has been extensively studied (Irgolic, 1974), but an understanding of its biochemistry lags far behind that of sulfur and selenium. It is generally regarded as only just reaching a state of development comparable to selenium 30 years ago (Sadeh, 1987). There are very few reports on the incorporation of Te into proteins (Ramadan, *et al.*, 1989; Liangyao, *et al.*, 1993), however this does not mean that tellurium is not important in living systems. Very possibly its importance has not yet been appreciated because tellurium has received less attention than that of selenium; alternatively the methods used for its isolation have not been adequate. For example, chemists have found that organotellurium compounds, particularly of the aliphatic kind, are extremely sensitive to light and/or oxygen, making them very difficult to isolate (Sadeh, 1987; Boles, *et al.*, 1994).

The first attempt to detect naturally incorporated tellurium in protein dates back to the unsuccessful work of Kolar (Kolar, 1974). The first successful report appeared in 1989 (Ramadan, *et al.*, 1989). Using the tellurite-tolerant fungi *Aspergillus fumigatus*, *Aspergillus terreus* and *Penicillium chrysogenum*, tellurium was found incorporated into several types of low and high molecular weight proteins, the amino acid composition of these telluroprotein hydrolysates showed the presence of a high content of tellurocysteine, tellurocystine and telluromethionine (Ramadan, *et al.*, 1989). A

telluroprotein was also detected from *Saccharomyces cerevisiae* by gas chromotography (GC) and GC/mass spectrometry (MS) (Liangyau, *et al.,* 1993). Tellurium-associated non-proteinaceous compounds were found from *A. terreus*, which was interpreted as "metallothionein" or "tellurium chelator" (Abbass and Razak, 1991).

1.4 Microbial Resistance to Tellurite Compounds

Tellurite is reported to be toxic to most microorganisms, especially to gram-negative bacteria (Fleming, 1932; Scala and Williams, 1963). However tellurite-resistant gram-negative bacteria are frequently isolated from city sewage and hospital sludge. For example, Summers and Jacoby reported that 35% of gram-negative organisms isolated from hospital sewage were tellurite resistance, whereas 16% of bacteria isolated from city sludge were tellurite resistance (Summers and Jacoby, 1977). In another report, 12% of 326 clinical isolates of *Pseudomonas aeruginosa* were found to be resistant to tellurite (Cervantes-Vega, *et al.,* 1986). In 1977 the resistance to tellurite of both enteric bacteria and *Pseudomonas* was linked to the presence of plasmids (Summers and Jacoby, 1977). These tellurite-resistance plasmids mostly belong to the incompatibility (Inc) HI, HII and P groups (Summers and Jacoby, 1977; Taylor and Summers, 1979; Bradley, 1985). Since then, even more gram-negative bacteria have been found to be tellurite resistance, many of which have resistance encoded by chromosomal genes, for example, the *E. coli tehAtehB* operon (Taylor, *et al.,* 1994; Walter, *et al.,* 1991b) and *Haemophilus influenzae tehA* and *tehB* (Fleischmann, *et al.,* 1997), *Rhodobacter sphaeroides trgABcysK* and *telA* (O'Gara *et al.,* 1997), and *Pseudomonas syringae tpm* gene (Cournoyer *et al.,* 1998). Chromosomally-mediated tellurite resistance in some *Rhizobium* and the extremely

Table 1. Tellurite-resistance microorganisms

Microorganism	Gene	MIC [a]	Reference
Gram-negative bacteria			
Acinetobacter calcoaceticus	(chromosome)	175	Bradley *et al.*, 1988
A. calcoaceticus	(RP4Te^R)	300	Bradley *et al.*, 1988
Alcaligenes spp.	*TerZABCDEF* (MER610)	>1024	Jobling and Ritchie, 1987
Alcaligenes odorans	(chromosome)	30	Bradley *et al.*, 1988
Azotobacter vinelandii	N/D [c]	0.2 ppm [b]	Tchan and Webber, 1966
Bradyrhizobium spp.	N/D	2-8 mM	Kinkle *et al.*, 1966
Erythrobacter litoralis	N/D	1500	Yurkov *et al.*, 1996
Erythromicrobium spp	N/D	1200-2700	Yurkov *et al.*, 1996
Escherichia coli	*TehAtehB* (chromosome)	128	Walter *et al.*, 1991; Taylor *et al.*, 1994
Hemophilus influenzae	*TehA-tehB* (chromosome)	N/D	Fleischmann *et al.*, 1997
Paracoccus dentrificans	N/D	40	O'Gara *et al.*, 1997
Proteus vulgaris	N/D	N/D	Nermut, 1967
Pseudomonas aeruginosa	(chromosome)	75	Bradley *et al.*, 1988
P. aeruginosa	*KilAtelAtelB* (RP4Te^R)	256	Summers and Jacoby, 1977; Walter and Taylor, 1992
Pseudomonas fluorescens	N/D	N/D	Chasteen *et al.*, 1990
Pseudomonas syringae	*Tpm* (chromosome)	256	Cournoyer *et al.*, 1998
Pseudomonas putida	(chromosome)	10	Bradley et al., 1988
Pseudomonas putida	(RP4Te^R)	375	Bradley et al., 1988
Rhizobium spp.	(chromosome)	1-16 mM	Kinkle *et al.*, 1994
Rhodobacter capsulatus	N/D	800	Moore and Kaplan, 1992
Rhodobacter sphaeroides	*TrgABcysK-telA* (chromosome)	150	O'Gara *et al.*, 1997
Rhodocyclus gelatinous	N/D	10	Moore and Kaplan, 1992

Rhodospirillaceae	N/D	2-6 mM	Yamada *et al.*, 1997
Rhodospirillum rubrum	N/D	1-20	Moore and Kaplan, 1992
Rhodopseudomonas palustris	N/D	100-200	Moore and Kaplan, 1992
Rhodopseudomonas viridis	N/D	80	Moore and Kaplan, 1992
Roseococcus thiosulfatophilus	N/D	1200	Yurkov *et al.*, 1996
Serratia marcescens	*TerZABCDEF* (R478)	>1024	Whelan *et al.*, 1995
Salmonella ohio	(Mip233)	>1000	Vilchez *et al.*, 1997
Thermus spp.	(chromosome)	N/D	Chiong *et al.*, 1988b
Gram-positive bacteria			
Acholeplasma spp.	N/D	N/D	Vinther and Freundt, 1977
Bacillus anthracis	N/D	N/D	Klett, 1900
Bacillus stearothermophilus	N/D	N/D	Moscoso *et al.*, 1998
Bacillus subtilis	N/D	N/D	van Iterson and Leene, 1964
Corynebacterium diphtheriae	N/D	N/D	Morton and Anderson, 1941
Corynebacterium xerosis	N/D	N/D	Morton and Anderson, 1941
Mycobacteria	N/D	N/D	Corper, 1995
Mycobacterium avium	N/D	N/D	Terai *et al.*, 1958
Mycoplasma spp.	N/D	N/D	Vinther and freundt, 1977
Staphylococcus aureus	N/D	N/D	Hoeprich *et al.*, 1960
Staphylococcus faecalis	N/D	N/D	Appleman and Heinmiller, 1961; Tucker *et al.*, 1961
Streptococcus pneumoniae	*TehB* (chromosome)	128	This study
Yersinia malassezii	N/D	N/D	Brzin, 1968
Cyanobaterium			
Anabaena sp.	N/D	N/D	Bisalputra *et al.*, 1969
Nostoc sphaericum	N/D	0.1%	Bisalputra *et al.*, 1969
Fungi			
Acremonium falciforme	N/D	N/D	Chasteen *et al.*, 1990

Aspergillus fumigatus	N/D	N/D	Ramadan *et al.*, 1989
Aspergillus terreus	N/D	N/D	Abbass and Razak, 1991
Cadida albicans	N/D	N/D	Nickerson, 1954
Penicillium brevicaule	N/D	N/D	Bird and Challenger, 1939
Penicillium chrysogenum	N/D	N/D	Bird and Challenger, 1939
Penicillium citrinum	N/D	N/D	Chasteen *et al.*, 1990
Penicillium notatum	N/D	N/D	Bird and Challenger, 1939
Penicillium sp.	N/D	N/D	Huysmans and Frankenberger, 1939
Rhodotorula mucilaginosa	N/D	0.04-0.08% [b]	Corfield and Smith, 1970
Saccharomyces cerevisiae	N/D	N/D	Gharieb and Gadd, 1988; Nagai, 1965
Schizosaccharomyces pombe	N/D	10^{-3} M [b]	Smith, 1974

a. MIC measured in µg/ml, unless otherwise stated.

b. Concentrations used for growth, not MIC.

c. Not determined.

thermophilic *Thermus thermophilus* and *Thermus flavus* have also been reported (Chiong *et al.*, 1988a; Chiong *et al.*, 1988b; Kinkle *et al.*, 1994).

Unlike gram-negative bacteria, many gram-positive bacteria are found to be naturally resistant to tellurite, for example, *Corynebacterium diphtheriae* (Saragea *et al.*, 1979; Smith *et al.*, 1977), *Enterococcus faecalis* (Applemann and Heinmiller, 1961), *Staphylococcus aureus* (Hoeprich *et al.*, 1960) (Table 1). This characteristic of resistance has formed the basis of diagnostic techniques in clinical microbiology, for instance, high concentrations of K_2TeO_3 have been used in selective media since 1912 to isolate *C. diphtheriae* and distinguish its subtypes (Summers and Jacoby, 1977). However molecular studies of tellurite resistance in gram-positive bacteria have not been attempted, whereas resistance determined by plasmids or chromosomal genes in gram-negative bacteria remains poorly understood.

There has been a report of tellurite resistance in cyanobacteria. As in other microorganisms, the cyanobacteria *Nostoc sphaericum* and *Anabaena* sp., also turned blackish when grown in the presence of tellurite. The deposition of reduced tellurite was found to be associated with photosynthetic lamellae, but was not found in the cytoplasm or the cytoplasmic membrane (Bisalputra, *et al.*, 1969).

There have also been many observations on fungal tellurite resistance, most of which concern *Penicillium* strains, for example, *P. brevicaule, P. chrysogenum, P. notatum,* and *P. citrinum* (Birm and Challenger, 1939; Fleming and Alexander, 1972;

Chasteen, *et al.*, 1990). Other fungi include *Aspergillus* sp., (Ramadan, *et al.*, 1989; Abbass and Razak, 1991), *Acremonium falciforme* (Chasteen, 1990), *Rhodotorula mucilaginosa* (Corfield and Smith, 1970), *Schizosaccharomyces pombe* (Smith, 1974) and *Saccharomyces cerevisiae* (Corfield and Smith, 1970; Gharieb and Gadd, 1988; Liangyao, *et al.*, 1993) (Table 1). Many fungi have been found to produce a garlic-like odor when grown in the presence of tellurite. Dimethyltelluride and/or dimethylditelluride have been identified as the odorous ingredient (Birm and Challenger, 1939; Chasteen, *et al.*, 1990; Liangyao, *et al.*, 1993).

1.5 Te^R Determinants and Mechanisms

1.5.1 Phosphate Transport System Takes Up Tellurite in *E. coli*

Most, if not all, isolates of *E. coli* are highly susceptible to tellurium compounds, having an MIC of 0.25-1.0 μg/ml (Tomas and Kay, 1986; Taylor, *et al.*, 1988). However, spontaneous mutants resistant to up to 10 μg/ml tellurite and arsenate were readily obtained. These mutants were found to be defective in phosphate transport and were unable to grow on media containing low levels of phosphate. This low-level tellurite resistance could be eliminated by the introduction of a plasmid carrying the *pho*B region which involves phosphate regulation (Tomas and Kay, 1986). In addition phosphate transport was found to be competitively inhibited by tellurite. These results indicated that tellurite can be partially taken up by a phosphate transport system (Tomas and Kay, 1986).

1.5.2 Tellurite Resistance and Thiol Redox Coupling

It was suggested that tellurite toxicity stems from its strong oxidizing ability under physiological conditions, which may interfere with many cellular enzyme processes (Summers and Jacoby, 1977). An alternative suggestion is that Te (as well as Se) could replace sulfur in various cellular functions because they are chemically similar to sulfate, which would lead to catastrophic consequences (Summers and Jacoby, 1977).

This chemical similarity also led to another proposal that tellurite and selenite could be reduced (and thus detoxified) by the sulfate reduction pathway (Scala and Williams, 1963). This proposal was supported by the fact that the sensitivity of *E. coli* to tellurite was enhanced by adding L-methionine to the growth medium. The reason proposed for the increased sensitivity was that an exogenously reduced sulfur source represses this pathway, thus decreasing the rate of detoxification of selenite and tellurite (Scala and Williams, 1963). Alternatively, exogenous L-methionine could be used as a co-factor of the resistance determinant or could affect synthesis of cysteine which shares the sulfate reduction pathway (Hou, 1994). This latter hypothesis was supported by the fact that the addition of cysteine to growth medium ameliorated the toxic effects of tellurite and selenite in *E. coli* (Springer and Huber, 1973). It also agrees with the recent work on the Te^R determinant *tehAtehB* which was transformed into a series of *E. coli* mutants deficient in thiol redox coupling system or electron transport, such as *cys* (cysteine), *grx* (glutaredoxin), *gsh* (glutathione synthetase), *trx* (thioredoxin), *nad* (nicotinamide), *nar* (nitrate reductase), *sod* (superoxide dismutase) and *ubi* (ubiquinone).

All these mutations resulted in a decrease in the Te^R MIC encoded by *tehAtehB*. Therefore a reducing environment or electron-reducing equivalents were found to be required for the resistance of *tehAB*, although they were not required for *kilAtelAB* determinant from RK2 (Turner, *et al.*, 1995).

Exogenous L-methionine does not always affect tellurite resistance. It was reported that the addition of L-methionine to the growth medium of *T. thermus* and *R. sphaeroides* had no effect on their resistance (Chiong, *et al.*, 1988b; Moore and Kaplan, 1992).

These results were used to propose a model in which tellurite enters the cell by the phosphate uptake system and is reduced to elemental tellurium by nitrate reductase at the cytoplasmic membrane. Tellurite which evades this line of defense will be then acted on by glutathionine or other reduced thiols in the cytoplasm to give a further reduction. Superoxide dismutase would then act on O_2^- resulting from TeO_3^{2-} reduction. Elimination of any compounds of this cascade would shut down DNA synthesis, protein synthesis as well as most reductases (Turner, *et al.*, 1995)

An intact cysteine metabolic pathway is also important for the high-level tellurite resistance in *R. sphaeroides*. Exogenous cysteine resulted in a substantial decrease of resistance to tellurite or selenite (Moore and Kaplan, 1992). Recently the Te^R genes in *R. sphaeroides* have been sequenced. Two loci were identified: *trgABcysK* and *telA*. The

disruption of the *cysK* gene in the first locus resulted in a decreased resistance to half of that seen in wild type strains (75 vs. 150 μg/ml) (O'Gara, *et al.*, 1997).

1.5.3 Nitrate Reductase Is Responsible for the Basal Resistance to Tellurite in *E. coli*

Complete denitrification of nitrate to nitrogen requires 4 different reductases, *i.e.*, nitrate, nitrite, nitric oxide and nitrous oxide reductases. The first step in *E. coli* is carried out by two different membrane-bound nitrate reductases (NRs) A and Z. NR A is encoded by the *narGHIJ* operon, induced under anaerobic conditions and repressed by oxygen. NR Z is encoded by *narZTWV* operon and constitutively expressed at low levels, even in aerobic conditions (Blasco, *et al.*, 1989; Bonnefoy and Demoss, 1994).

Recently, NRs A and Z isolated from *E. coli* membrane were found to be able to reduce both tellurite and selenate. Reductases were absent from membrane extracts of mutants deleted in NRs A and Z (Avazeri, *et al.*, 1995, 1997). These NR mutants of *E. coli* were found to be hypersensitive to tellurite (MIC = 0.03-0.06 μg/ml vs. 2 μg/ml of wild type) under aerobic growth conditions (Avazeri, *et al.*, 1997). When *tehAB* and *kilAtelAB* were introduced into a NR mutant deleted in both NR A and NR Z, the MIC encoded by both Te^R determinants was reduced by 8-16 times (Turner *et al.*, 1995). In contrast, under anaerobic conditions, mutants deleted in NR activities did not show increased sensitivity and had the same level of resistance as the wild type *E. coli*. This lack of hypersensitivity may be due to an additional reductase activity (Avazeri, *et al.*,

1997). In addition, overexpression of NR A in wild type or NR A mutant *E. coli* resulted in elevated tellurite resistance (32 μg/ml in wild type, 8-16 μg/ml in NR A mutant) under both aerobic and anaerobic conditions (Avazeri, *et al.*, 1997).

Whether NR catalyses complete reduction of Te^{IV} to Te^0, or whether this reduction is the result of two enzymatic steps, is not known. NR mutants exposed to tellurite still turned black, suggesting that NR activity is not the only process responsible for the deposition of Te^0 in *E. coli* (Avazeri, *et al.*, 1997).

In addition to *E. coli* NRs, NRs of other bacteria like *Alcaligenes eutrophus*, *Paracoccus denitrificans* and *R. sphaeroides* spp. *denitrificans* also reduced tellurite and selenite (Avazeri, *et al.*, 1995, 1997). Selenite reductase activity of nitrite/nitrate reductases has also been reported in some other bacteria, including *Thauera selenatis*, *Alcaligenes xylosoxydans*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* (DeMoll-Decker and Macy, 1993; Macy, 1994). Whether enzymes from these bacteria have tellurite reductase activity has not been reported.

1.5.4 Reduction of Tellurite/Tellurate to Te^0

Almost all tellurite-resistance microorganisms turn black or dark-grey when grown in the presence of tellurite despite the difference in determinants. There are, however, a few exceptions to this rule. *Roseococcus thiosulfatophilus* did not turn black when grown in tellurite-containing minimal medium supplemented with L-glutamine, succinate, malate, tartrate or acetate (MIC = 1200 μg/ml); neither did *Erythromicrobium*

ezovicum when grown in minimal medium supplemented with acetate (MIC = 500 μg/ml), although they turned black when grown in rich medium (Yurkov, *et al.*, 1996).

The proposal that the black deposit produced by microorganisms growing in the presence of tellurite was metallic tellurium can be traced back to the work of Klett (Klett, 1900) and Scheurlen (Scheurlen, 1900). About 40 years later, the black needle-like precipitate from *Corynebacterium diphtheriae* was found to be soluble in bromine water and of high density in electron micrographs (Morton and Anderson, 1941), whereas the black precipitate from *Proteus vulgaris* was soluble in both hot sulfuric acid and bromine water, but not soluble in water and organic solvents (Nermut, 1963). By using X-ray diffraction analysis the black precipitate from *S. faecalis* and *C. diphtheriae* was found to be consistent with the metallic tellurium (Tucker, *et al.*, 1962). The electron spectroscopic imaging (Taylor, *et al.*, 1988) and x-ray energy-dispersive spectrogram (EDS) (Yamada, *et al.*, 1997) led to similar conclusions. In the work on *R. sphaeroides*, 93 mg of refined powder, which showed similar physical properties to elemental tellurium (black, insoluble in aqueous and organic solvents), was obtained from 275 mg K_2TeO_3-containing growth medium (equivalent to 138 mg of Te^{IV}), indicating a 67% reduction rate of Te^{IV} to Te^0 (Moore and Kaplan, 1992).

Different sites of Te^0 deposition have been observed. For most of the microorganisms reported, tellurite was reduced in close proximicity to the cell membrane (Bradley, *et al.*, 1988; Lloyd-Jones, *et al.*,1994; Suzina, *et al.*, 1995; Taylor, *et al.*, 1988), or randomly distributed in the cytoplasm (Tchan and Webber, 1966; Yamada, *et al.*,

1997). In the work of Moore and Kaplan, they purified the black tellurium from the membrane fraction of *R. sphaeroides*. No tellurium was found in either cytoplasmic or periplasmic fractions (Moore and Kaplan, 1992). For some bacteria, for example *Erythromicrobium ursincola*, the deposition is very abundant and black crystals occupied 20-30% of the cell volume (Yurkov, *et al.*, 1996). In *Bacillus subtilis*, the reduction site was associated with the point of flagella insertion (van Iterson, *et al.*, 1964), while in the cyanobacterium, *Nostoc sphaericum*, the reduction product was associated with the photosynthetic lamellae, and there was no evidence of reduction on either the plasma membrane or in any of the cytoplasmic granules (Bisalputra, *et al.*, 1969).

In eukaryotic cells, the reduction seems to be associated with intracytoplasmic membrane systems. For example, the black product was confined to particulate loci visible under light microscopy in *Candida albicans* (Nickerson, 1974), while in *S. cerevisiae* and *Rhodotorula mucilaginosa*, tellurium is deposited mainly on a specialized area of the endoplasmic reticulum (Corfield and Smith, 1970). In *S. pombe*, the black precipitate was found on a localized system of membranous vesicles forming an area about 500 nm in diameter, which possibly resulted from the degradation of some of the mitochondria (Smith 1974). In mammalian heart tissue, Te was also deposited in the mitochondria (Barnett and Palade, 1957; Barnett and Palade, 1958)

These observations are consistent with recent work on *S. cerevisiae*. Mutants devoid of vacuolar-like structure or deficient in specific protein subunits of the vacuolar (V)-H^+-ATPase showed increased sensitivity to tellurite (as well as to chromate) and a

much lighter color despite the higher tellurium content compared to the parental strains, indicating that the functional vacuole plays a role in resistance to metalloids in yeast (Gharieb and Gadd, 1998). The role of yeast vacuoles in the compartmentalization, homeostasis, or detoxification of a wide range of toxic metal ions is already well established (Gadd, 1993; Klionski, *et al.,* 1990; Ramsay and Gadd, 1997; White and Gadd, 1986).

1.5.5 Other Mechanisms of Tellurite Resistance

A protein fraction from *Mycobacterium avium* was found to be able to reduce tellurite in the presence of NADH or malate or malic dehydrogenase and the protein was referred to as tellurite reductase (Terai, *et al.,* 1958). Similar NADH-dependent tellurite-reducing proteins are also found in *T. thermophilus, T. flavus* (Chiong, *et al.,* 1988b), *R. sphaeroides* (Moore and Kaplan, 1992) and *B. stearothermophilus* (Moscoso *et al.,* 1998), while extracts of *Micrococcus lactilyticus* reduced tellurite at the expense of molecular hydrogen (Woolfolk and Whiteley, 1962).

It was reported that *arsABC* from the IncFI plasmid R773, which was originally found to be resistant to arsenic and antimony compounds, had an intermediate cross-resistance to tellurite (64 μg/ml) mediated by the efflux pump mechanism (Turner, *et al.,* 1992).

1.5.6 Variety of Te^R Determinants

Tellurite-resistance genes are very diverse; at least five Te^R determinants have been identified, two of them have plasmid origins, whereas three are chromosomal. They all appear to be unrelated to each other at both DNA and protein levels (Jobling and Ritchie, 1987; Whelan *et al.*, 1995; Walter *et al.*, 1991a; Walter *et al.*, 1991b; Taylor *et al.*, 1994; O'Gara *et al.*, 1997; Cournoyer *et al.*, 1998).

1.5.6.1 The *terZABCDEF* Determinant from IncH Plasmids

Te^R is a characteristic marker carried by many incompatibility (Inc) HI2, IncHI3 and IncHII (Summers and Jacoby, 1977; Walter and Taylor, 1992; Hou and Taylor, 1994). This high-level resistance has been extensively studied in the IncHI2 plasmids, pMER610 and R478, and shown to depend on the *ter* operon which comprises 7 genes: *terZ, -A, -B, -C, -D, -E, -F*. Both determinants have the same gene order and their encoded products share 78-95% identity. In addition, the amino acid sequences of TerD, TerE and TerZ were found to be highly related (for example, TerD and TerE of pMER610 are 66.3% identical). Similarity was also observed between TerA and TerF, and highly related amino-acid domain were noted among various subsets of the five latter proteins. Both determinants confer a high-level resistance to tellurite (256 μg/ml), and resistance to bacteriophage infection (Phi) and pore-forming colicins (PacB) (Jobling and Ritchie, 1987; Whelan *et al.*, 1995).

It has been difficult to clone individual genes or to make internal deletions in the *ter* operon because they appear to have lethal effects on *E. coli* cells, therefore, it is not

clear exactly which genes are required for any of the three traits (Te^R, Phi and PacB). In addition, the operon fragment of R478 was clonable only in cells containing a second part which is about 6-kb upstream of the *ter* operon (Whelan *et al.*, 1995). This second part was sequenced, and shown to contain three genes, termed *terWYX*, oriented in a opposite direction from the *ter* operon (Whelan *et al.*, 1997). The single insert clone of this second part did not encode for genes responsible for any of the three phenotypes (Te^R, Phi and PacB). Complementation analysis indicated that only *terW* protects cells from toxic effects of the *ter* cluster, whereas *terY* and *terX* had no effect (Whelan *et al.*, 1997). However, the *terX* gene product showed high similarity to TerE, TerD and TerZ. More particularly, there is a conserved motif consisting of 13 residues, GDN(R/L)TG(E/A)GDGDDE, in all three proteins (Whelan *et al.*, 1997).

Overexpression of the *ter* operon in *E. coli* resulted in a filamentous morphology (exceeding 200 μm in length) in which nuclei were found to be evenly distributed along the filaments, indicating that cell division was inhibited but chromosomal DNA replication was not. This filamentous phenotype could not be eliminated by *terW*. Tn1000 transposition into *terZ, -A, -B* and *-C* inhibited or partially inhibited filamentation, whereas insertion into *terD* and *terF* did not (no insertion in *terE* was found) (Whelan *et al.*, 1997).

The genes showing some degree of amino acid similarity to *ter* genes exist in at least four other systems. These include the slime mold *Dictyostellium discoideum* and the gram-positive bacteria *Clostridium acetobutylicum*, *Bacillus subtilis* and *Lactococcus*

lactis. All *ter* gene products except TerC were found to be related to both subunits of the cyclic AMP-binding protein of *D. discoideum* (Hill *et al.*, 1993; Tsang and Tanaka, 1986; Whelan *et al.*, 1995), and to ORFb and ORFc of *Clostridium acetobutylicum* (Azeddoug and Reysset, 1994; Whelan *et al.*, 1995), especially TerD and TerE which had 45-50% amino acid identity to these proteins (Whelan *et al.*, 1995). Genes for ORFb and ORFc confer methyl methane sulfonate (MMS), mitomycin C (MC) and UV resistance to *recA* strains of *E. coli* when cloned on pUC19 plasmid, but they did not confer tellurite resistance to *E. coli*. Moreover, *C. acetobutylicum*, with or without a plasmid carrying the *orfabc* operon, also did not appear to be resistant to tellurite (Azeddoug and Reysset, 1994). Some of the *L. lactis* sex factor polypeptides displayed up to 55% amino acid identity with some pMER610 polypeptides (Pillage *et al.*, 1994), but there function is known.

In the 25-degree region of *Bacillus subtilis* chromosome, there are many genes which confer resistance to drugs and metals. Seven of these genes appear to be in a operon. The products of four genes (YceC, YceD, YceE and YceF) showed some similarity to TerZ, TerD, TerE and TerC of R478 (38%, 57%, 54% and 23% identity, respectively), but the products of the three other genes (YceG, YceH and YceI) had no similarity with that of three other *ter* genes (*terA*, *terB* and *terF*). However YceH showed 24% (39%) identity (similarity) with TelA of RK2 (Kumano *et al.*, 1997). *B. subtilis* was reported to be tellurite resistant (van Iterson and Leene, 1964). It is not clear if these *ter* gene homologues are involved in the resistance.

MIP233 is the only member of IncHI3, it also confers a high level of resistance to tellurite (MIC > 1000 μg/ml). DNA hybridization showed that pMER610 or R478 *ter* genes is closely related to that of MIP233 (Hou, 1994). However, in another report, a 2.2-kb fragment from MIP233 encoding the resistance to tellurite and channel-forming colicins did not hybridize to R478 and pMER610 nor to many other IncH plasmids (Vilchez *et al.*, 1997). Recently, this 2.2-kb fragment was partially sequenced and revealed an ORF which displayed strong homology with the enzyme O-acetylserine sulfhydrylase (CysK) for the terminal step of cysteine synthesis in many organisms, suggesting CysK activity may involve tellurite resistance in this plasmid (Rodriguez-Lemoine *et al.*, 1998).

1.5.6.2 The *kilAtelAtelB* from IncP Plasmid

RK2 (sometimes called RP1, RP4, R18 or R68) is a 60-kb plasmid which is able to transfer among and be maintained in many bacteria. It contains a complicated *kilA-korA* regulon (Figurski *et al.*, 1982). Within this regulon is a tellurite-resistant determinant that is normally cryptic or expressed at a very low level in wild type RK2 (Walter and Taylor, 1992). Tellurite-resistance variants, $RK2Te^R$, were readily obtained by plating cells harboring RK2 on tellurite-containing media (Bradley, 1985). It was found that the Te^R determinant is comprised of an operon of three genes: *kilA, telA* and *telB* (referred to as *klaA, klaB* and *klaC* in $RK2Te^S$) (Goncharoff *et al.*, 1991; Walter *et al.*, 1991). The *kilA* gene, named for its lethal effect (Figurski *et al.*, 1982), encodes a 257-aa hydrophilic protein. TelA is also a hydrophilic protein, whereas TelB is a membrane protein (Walter, 1990). The only difference between the nucleotide sequences

of RK2Te^R *kilAtelAtelB* and RK2Te^S *klaAklaBklaC* was a single base change of A to T in the *telB* gene, which gives rise to a single amino acid change of serine to cysteine at position 125 in the TelB protein (Goncharoff *et al.*, 1991; Walter *et al.*, 1991). The importance of this cysteine in RK2Te^R has been demonstrated by replacing it with other residues. The replacements resulted in drastic decreases of tellurite resistance (256 vs. 8-16 μg/ml) (Turner *et al.*, 1994). In TelB, there is a second cysteine at position 132 which was also found to be involved in tellurite resistance, some changes (*e.g.*, Cys to Ala or Ser) had greater effect (256 vs. 8 μg/ml), whereas others (*e.g.*, Cys to Ile or Arg) had less effect (256 vs. 64-128 μg/ml) (Turner *et al.*, 1993; Turner *et al.*, 1994).

Each of the three genes is required for the full resistance in RK2Te^R, and can be complemented in vivo. There seems to be a delicate balance of the gene products, the more products produced, the lower tellurite resistance the operon encodes (Goncharoff *et al.*, 1991; Turner *et al.*, 1994). In addition, cells harboring the *kilA* gene alone or the three genes of the *kilAtelAtelB* operon expressed either in *trans* or in *cis* behind a *tac* promoter were found to form non-sepatated filaments up to 10-30 times the length of control cells. This filamentation effect was amplified when cells were grown in minimal medium (Turner *et al.*, 1994)

Homologues of *telA* have been found in both gram-positive and gram-negative bacterial chromosomes. In *R. sphaeroides*, a single *telA* homologue was responsible for 67% of the tellurite resistance (O'Gara *et al.*, 1997). In *B. subtilis*, it is not known whether

YceH, which is 39% similar to RK2 TelA, is involved in tellurite resistance (Kumano *et al.*, 1997).

1.5.6.3 The *tehAtehB* from *E. coli* Chromosome

The *tehAtehB* operon, originally believed to have been cloned from IncHII plasmid pHH1508a, was subsequently found to be located at 32.3 min. on the *E. coli* chromosome (Berlyn, 1998; Taylor *et al.*, 1994; Walter *et al.*, 1991b). It appears to be phenotypically silent (with an MIC of about 2 μg/ml), but confers high level tellurite resistance (128 μg/ml) upon overexpression on a high-copy number plasmid or behind a strong promoter (Taylor *et al.*, 1994). The *tehA* encodes a 330-aa membrane protein with about 10 transmembrane segments. The *tehB*, on the other hand, encodes a 197-aa polypeptide displaying a very weak membrane association and is very likely a soluble protein (Walter, 1990).

Recently, homologues of *tehA* and *tehB* have been sequenced from the *Haemophilus influenzae* chromosome and the deduced proteins show 45% and 55% identity to *E. coli* TehA and TehB, respectively, however they are not in the same operon but are widely separated on the chromosome (Fleischmann *et al.*, 1997). *Eikenella corrodens* hemagglutinin (Hag1) also showed significant homology (51% identity) to *E. coli* TehB (Rao *et al.*, 1993). The *H. influenzae* TehB and *E. corrodens* Hag1 are 90-aa and 103-aa longer than *E. coli* TehB, respectively.

In both *E. coli* TehA and TehB, there are three cysteine residues. The cysteines in TehA were predicted to be located on the periplasmic side of the cytoplasmic membrane (Turner *et al.*, 1997; Walter *et al.*, 1991). One of them was conserved in *H. influenzae* TehA but shifted one position, whereas two of the three cysteines in TehB were conserved in *H. influenzae* TehB and *E. corrodens* Hagl (Liu *et al.*, 1998).

A region of *E. coli* TehA, localized between transmembrane segments (TMS) II and TMS V (residues 49 to 193), was found to be homologous to the members of the small multidrug resistance (SMR) family which are involved in the efflux of lipophilic drugs. It has 53% (23%) similarity (identity) with Qac Kae of *Klebsiella aerogenes* plasmid R751, 55% (22%) with Emr Eco of *E. coli*, but no homology was found between *E. coli* TehA and other bacterial or eukaryotic multidrug resistance transporters such as QacA or P glycoprotein. The *H. influenzae* TehA has the same degree of homology to the SMR proteins as the *E. coli* TehA does, but it does not contain any similarity to the SMR signature sequence (Turner *et al.*, 1997).

The expression of *E. coli tehA* with or without *tehB* was studied. Overexpression of *tehA* resulted in hypersensitivity to dequalinium Cl and methylviologen (paraquat) and resistance to tetraphenylarsenium Cl, ethidium bromide, crystal violet and proflavin. Co-expression of *tehA* and *tehB* decreased ethidium resistance and transport, and also gave hypersensitivity to dequalinium Cl and methylviologen (paraquat). In contrast, the overexpression of *H. influenzae tehA* did not have any effect on these drugs. The

presence of SMR signature sequence in *E. coli tehA* suggests that it may play a role in transport activity (Turner *et al.*, 1997).

By transforming *tehAB* into *E. coli* mutants deficient in electron transport process and/or thiol redox coupling (e.g., *ubi, nad, cys, nar, trx, grx, gsh,* and *sod*), it was found that the MICs of these mutants ranged from 1-16 μg/ml tellurite, compared to 128 (g/ml for wild type strain containing *tehAB*, or 0.03-2 μg/ml for strains containing a control plasmid. On the other hand, *kilAtelAB* showed little or no dependency on the host genotypes. These results suggest that these two determinants possess very different biochemical mechanisms of resistance (Turner *et al.*, 1995).

1.5.6.4 The *trgABcysK* and *telA* from *Rhodobacter sphaeroides*

R. sphaeroides is not only resistant to tellurite and selenite, but also to at least 15 other rare-earth oxides and oxyanions (Moore and Kaplan, 1992). It seems several different mechanisms are involved in resistance to these materials. Resistance to the tellurite class (*e.g.*, tellurate, tellurite, selenate, selenite and rhodium sesquioxide) resulted in evolution of gas and accumulation of intracellular deposits, and was not affected by exogenous L-methionine or phosphate but was reduced 40-fold by addition of cysteine to the growth media (Moore and Kaplan, 1992). In contrast, resistance to the periodate class oxyanions (*e.g.*, periodate, siliconate and siliconite) did not result in metal deposition and gas evolution but was inhibited by exogenous phosphate. Resistance to arsenate class oxyanions (*e.g.*, arsenate, molybdate and tungstate, etc.) may involve a third distinct mechanism, as evidenced by the lack of intracellular metal deposition and

gas release and insensitivity to extracellular phosphate or cysteine (Moore and Kaplan, 1992).

The gas from the headspace of a *R. sphaeroides* culture in the presence of tellurite has been identified to be H_2 by mass spectrometry (MS) (Moore and Kaplan, 1992). The black deposit was purified from the cytoplasmic membranes of *R. sphaeroides* and showed similar physical properties to metallic tellurium. Te^0 was not found in the photosynthetic or intracytoplasmic membrane (Moore and Kaplan, 1992). This was consistent with the finding that $FADH_2$-dependent TeO_3^{2-} reductase activity was only found in the cytoplasmic membrane fraction of *R. sphaeroides*, not in the periplasm or cytoplasm (Moore and Kaplan, 1992).

Recently, two loci involved in tellurite resistance in *R. sphaeroides* have been identified and characterized (O'Gara *et al.*, 1997). The first locus contains 4 genes: *trgA, trgB, cysK* and *orf323*. TrgA and TrgB are likely membrane-associated proteins and have no homology to known proteins in databases. About 300-bp downstream of *trgB* is an ORF whose product is strongly homologous with cysteine synthetase (CysK) in many other organisms. The *orf323* is an ORF of 323 amino acids. Both *trgABcysKorf323* and *trgAB* were found to confer a similar level of increased tellurite resistance (40 vs. 150 μg/ml) when introduced into a related bacterium, *Paracoccus denitrificans*, indicating that *cysK-orf323* is not necessary. However, when either *trgB* or *trgAB* were interrupted by mutation in the *R. sphaeroides* chromosome, the resistance was not affected compared to that of wild type *R. sphaeroides* (150 vs. 150 μg/ml). In contrast, the resistance of the

cysK null mutant was reduced to approximately half that of the wild type strain (75 vs. 150 μg/ml) (O'Gara *et al.*, 1997), which is consistent with the earlier report that cysteine metabolism plays some role in tellurite resisitance in *R. sphaeroides* (Moore and Kaplan, 1992). In both systems, the *orf323* appears not to be required (O'Gara *et al.*, 1997).

The second locus is represented by the *telA* gene, which is separated by 115 kb from the first locus. The *telA* gene product is 65% similar to that of TelA of *kilAtelAB* of RK2, and the sequences immediately downstream have no similarity to other components of *kilAtelAB*. Inactivation of *telA* from the *R. sphaeroides* chromosome reduced resistance to 50 μg/ml (from 150 μg/ml), and it could be restored by *telA* in *trans* (O'Gara *et al.*, 1997). Although the *R. sphaeroides telA* is homologous to that of *kilAtelAB*, the gene products could not functionally substitute for each other, indicating a substantial functional divergence between these two gene products (O'Gara *et al.*, 1997). The fact that *kilAtelAB*-mediated tellurite resistance is independent of cysteine metabolism (Turner *et al.*, 1995) also suggests that these two TelAs are unlikely to be related.

By using various *R. sphaeroides* mutants and different growth conditions, respiration, photosynthesis and CO_2 fixation were found to be important for high-level tellurite resistance. These processes involve electron transport and are believed to affect the cellular capacity to reduce tellurite (Moore and Kaplan, 1992). However, these two loci, *trgAtrgBcysKorf323* and *telA*, do not appear to be directly involved in electron transport but contribute significantly to tellurite resistance. This suggests that the

phenomenon of tellurite resistance in *R. sphaeroides* is complex and involves multiple mechanisms (O'Gara *et al.*, 1997).

1.5.6.5 The *tpm* Gene from *Pseudomonas syringae*

Most recently, a single gene responsible for tellurite resistance in the pea blight pathogen, *Pseudomonas synringae* pathovar *pisi* (*P. s. pisi*), has been identified on its chromosome. The gene encodes a protein of 218 aa which is 30% (55%) identical (similar) to human thiopurine methyltransferase (hTPMP) and has been called bacterial thiopurine methyltransferase gene (*tpm*). It also has some similarity to a methyltransferase from *Synchocystis* (18% identical, 43% similar), but no apparent homology to any other Te^R proteins reported previously (Cournoyer *et al.*, 1998).

The hTPMT is a cytoplasmic enzyme that catalyses the *S*-adenosyl-L-methionine (AdoMet) methylation of sulphydryl compounds (Honchel *et al.*, 1993), The known substrates for hTPMT include the thiopurine drugs 6-mercaptopurine (6-MP) and azathiopurine. The thiopurine transferase activity of *P. synringae tpm* gene has been confirmed by assaying a cell-free extract of *E. coli* harboring *tpm*, which was found to catalyze AdoMet methylation of 6-MP, this activity can be inhibited by *S*-adenosyl-L-homocysteine (AdoHcy), a product after transfer of methyl group from AdoMet. Whether tellurite is a substrate for the *tpm* product is not reported, but has been implied (Cournoyer *et al.*, 1998).

The distribution of the *tpm* gene among *P. synringae* and related bacteria was studied with PCR and DNA probing. It was found that *tpm* exists in all *P. s. pisi*, *P. s. syringae* and three *P. s. phaseolicola* isolates, but not in the *P. s. maculicola*, *P. s. morsprunorum*, *P. s. tomato*, *P. viridiflava*, *P. putida* as well as six *P. s. phaseolicola* isolates (Cournoyer *et al.*, 1998).

1.6 Objectives of This Study

The above sections demonstrated that the tellurite resistance determinants in microorganisms are very diverse and are not completely understood, additional experiments are required therefore to elucidate the mechanism of tellurite resistance. In the present study, the *tehAB* tellurite resistance determinant from the *E. coli* chromosome was investigated by using the site-directed mutagenesis method. The second part of this research on the *Streptococcus pneumoniae* tellurite resistance gene represents the first attempt to study the molecular biology of tellurite resistance in a gram-positive bacterium.

2. Materials and Methods

2.1 Bacterial Strains and Plasmids

Bacterial strains and the plasmids used in this study are summarized in Table 2. The *E. coli* strain JM109 was used for cloning and in MIC assays of different mutant clones. *E. coli* strain BL21(DE3)/pLysS was used for overexpression of TehB proteins and the comparison of MIC's of different bacterial tellurite resistance genes. The culture of *S. pneumoniae* type 1 was provided by M. Diadio, Department of Medical Microbiology and Immunology, University of Alberta. The plasmid pML204 is a construct containing the *Eikenella corrodens* hemagglutinin (*hag1*) gene recloned from the original plasmid pVKR204 provided by A. Progulske-Fox, University of Florida (Rao *et al.*, 1993). The plasmid pTWT101 is a pTZ19R-based phagemid construct in which a 3.3-kb fragment containing *E. coli* chromosomal *tehAtehB* genes was recloned from the original clone pDT1364 (Taylor *et al.*, 1994; Turner *et al.*, 1995).

2.2 Media and Growth Conditions

The *E. coli* strains were cultured at 37°C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) with or without 1.5% agar (Sambrook *et al.*, 1989). *S. pneumoniae* were cultured in Todd-Hewitt (Oxoid, Hampshire, England) broth supplemented with 0.5% yeast extract (THY) or grown on THY-blood agar containing 5% defibrinated sheep blood (Dalynn, Calgary), in a 5% CO_2 atmosphere (Berry *et al.*, 1989). Where appropriate, ampicillin was added to the medium at a concentration of 50 μg /ml, chloramphenicol at 30 μg /ml.

Table 2. Bacterial strains and plasmids used in this study

Strain/plasmid	Description[a]	Reference
E. coli		
JM109	*endA1 recA1 gyrA96 thi hsdR17 relA1 supE44* Δ(*lac-proAB*)*mcrA*[*F' traD36 proAB lacI*^{q}*Z* ΔM15]	Yanisch-Perron *et al.*, 1985
BL21(DE3)/pLysS [b]	F$^-$*ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (cI*ts*857 *indl* *Sam7nin5 lacUV5*-T7gene1) pLysS (Cm^r)	Novagen, Madison, WN.
S. pneumoniae		
S. pneumoniae type1		M. Diadio
Plasmids		
pTZ18U	A phagemid vectors with *lacZ* and T7 promoters, Ap^r	Bio-Rad, Hercules, CA
pTZ19R	A phagemid vectors with *lacZ* and T7 promoters, Ap^r	Bio-Rad, Hercules, CA
pTSPtehB-2	*S. pneumoniae tehB* fragment in pTZ18U, Ap^r	This study
pTWT101	*E. coli tehAB* fragment in pTZ19R, Ap^r	Turner *et al.*, 1995
pML111	*E. coli tehB* in pTZ18U, Ap^r	This study
pTWT134	*H. hemophilus tehB* homologue in pTZ18R, Ap^r	This lab
pML204	*E. corrodens hag1* fragment in pTZ18U, Ap^r	Rao *et al.*, 1993 This study

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; *hag1*, *E. corrodens* hemogglutinin gene; *tehB*, tellurite resistance gene B.

[b] pLysS is a lysozyme gene-containing plasmid.

2.3 Minimal Inhibitory Concentration (MIC)

Different methods were used to determine the tellurite MICs for *E. coli* and *S. pneumoniae.* For *E. coli,* the agar dilution method was used as described previously (Turner *et al.,* 1997). Briefly, overnight cultures of JM109 were diluted 10^2-10^4 fold in LB broth to give appox. 10^4-10^6 cells/ml. A 5 µl volume was spotted onto LB plates containing serial twofold dilutions of potassium tellurite (K_2TeO_2). The plates were then incubated as described above. The lowest concentration of tellurite without growth was defined as MIC.

The MIC for *S. pneumoniae* was determined by the broth dilution method (Hindler, 1998). An overnight culture of *S. pneumoniae* was inoculated into THY tubes containing serial twofold dilutions of potassium tellurite, which were then kept overnight in a 5% CO_2 incubator. The growth was measured by optical density at 600 nm. The lowest concentration of tellurite without growth was defined as the MIC. All the MICs were determined at least three times.

2.4 DNA Techniques

Plasmid DNA was isolated from *E. coli* by the alkaline lysis method as described by Birnboim and Doly (Birnboim and Doly, 1979). Briefly, overnight cultures of plasmid-containing *E. coli* were first suspended in Solution I (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH8.0), and subsequently lysed by treatment with Solution II (1% SDS, 0.2 M NaCl), then neutralized by the addition of Solution III (potassium acetate and acetic acid). The chromosomal DNA was removed by centrifugation. After

phenol extraction, the reannealed plasmid DNA was recovered by the ethanol precipitation. Plasmid DNA was dissolved in TE buffer containing RNase.

Restriction digestion of DNA was carried out according to the manufacturer's recommendations, and each enzyme was incubated in its own buffer. All the restriction enzymes were obtained from Gibco-BRL (Burlington, ON). The digestion reactions were terminated by the addition of 1/5 volume of 6 x loading buffer (0.25% bromophenol blue, 40% sucrose), and the samples were then subjected to horizontal agarose gel electrophoresis (0.7%) in 1 x TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). One-kb DNA ladder (Gibco-BRL) was used as the standard marker. After electrophoresis, the gels were stained with ethidium bromide and visualized under ultraviolet (UV) light. Spin-X centrifuge tube filters for DNA recovery from agarose gels were purchased from Corning Costar (Cambridge, MA).

DNA ligation was performed based on the manufacturer's suggestion. T4 DNA ligase was obtained from Gibco-BRL. DNA transformations were carried out using the classical $CaCl_2$ method, 50 mM $CaCl_2$ used to prepare competent cells (Sambrook *et al.*, 1989). Competent cells were prepared in bulk and stored for future use at -80°C in the presence of 20% glycerol (Sambrook *et al.*, 1989).

2.5 Oligonucleotide Primers

The oligonucleotide primers used in this study are listed in Table 3. ML53 and ML54 were used to amplify the *tehB* gene from *Streptococcus pneumoniae* type 1 strain,

Table 3. Oligonucleotide primers for site-directed mutagenesis

Mutation[a]	Mutagenic oligonucleotide [b]	Codon change
C109A	5'-CTGGCGGTGGCCCTGTTCAG-3'	TGC → GCC
C156A	5'-GCAATGGCCGCTGGTGCGTT-3'	TGT → GCT
C212A	5'-CTGGTGGCTGCTAGTGCCTG-3'	TGT → GCT
C39A	5'-GATCTGGGCGCTGGCAATGG-3'	TGT → GCT
C123A	5'-ATGCAACGTGCCACTAAACC-3'	TGC → GCC
C143A	5'-GATTATCCAGCTACCGTCGG3-3'	TGT → GCT
D36A	5'-AAACGCTGGCTCTGGGCTGT-3'	GGA → GGC
D36N	5'-AAACGCTGAATCTGGGCTGT-3'	GGA → GAA
D58A	5'-ACGCATGGGCTAAAAATGCC-3'	GGA → GGC
Y96A	5'-TGATAGACAGGCCGATTTTAT-3	TAC → GCC
D96E	5'-ACAGTACGAATTTATTCTTTC-3'	GAT→ GAA
D96N	5'-ATAGACAGTACAATTTTATTCT-3	GAT→ AAT
F97Y	5'-AGTACGATTATATTCTTTCGA-3	TTT→ TAT
F97A	5'-ACAGTACGATGCTATTCTTTC-3'	TTT→ GCT
ML10[c]	5'-CTGCGGCTCTGGCTATGCT-3'	
Universal[c]	5'-GTTTTCCCAGTCACGACGACGTTGTA-3'	
ML53[d]	5'-ATA*CTCGAG*ACTTCTTTTAGGACTTGCCA AA-3'	
ML54[d]	5'-ATA*CTGCAG*GATCCTCTAACACATTTACCAA-3'	

[a] Mutation was designated by one-letter symbol of the amino acid residue being changed, followed by the sequence number and the substituted residue.

[b] Nucleotide change is underlined. Only the coding-strand primers are listed.

[c] Flanking primers were common in all of the mutagenic reactions.

[d] Primers for amplification of the *S. pneumoniae tehB* gene. The italicized sequences indicate extensions introduced to create convenient restriction sites *Xho*I in ML53 and *Pst*I in ML54.

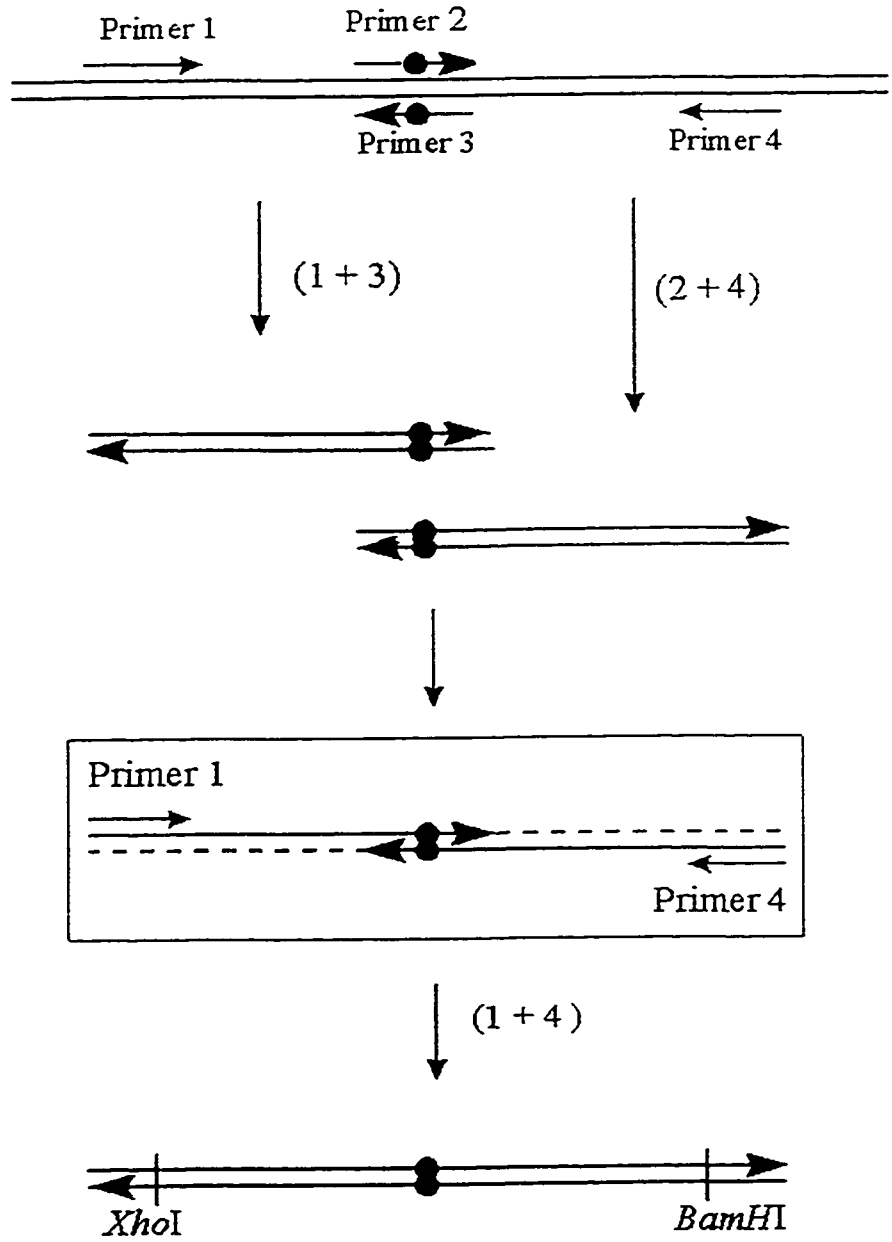
and all other primers, with the exception of ML10 and Universal which were used as flanking primers, were used to make site-directed mutagenesis on the *E. coli tehA* and *tehB* genes. ML10 was complementary to a region 70-bp upstream of the *E. coli tehA* start codon, while "Universal" matches the flanking region of polylinker in the vector. All primers in this study were synthesized by GIBCO-BRL (Burlington, ON).

2.6 Site-Directed Mutagenesis

The two-stage, PCR-based overlapping extension method (Ho *et al.*, 1989) was used. The *Taq* DNA polymerase was purchased from GIBCO-BRL. For each mutation, four primers were used; two internal primers which were complementary and contained mutating nucleotides, and two flanking primers were common in all the mutagenic reactions. DNA fragments with overlapping ends obtained from the first-stage PCR were gel-purified and used as templates in the second-stage PCR (Fig. 1). The final products (about 2 kb) were then digested with *Xho*I/*Bam*HI and used to replace the corresponding wild-type fragment in pTWT101.

To create multiple mutations, the existing mutant DNA was used as template. For example, to make a triple mutant C109A/C156A/C212A, C109A and C212A DNA were used as templates for the first-stage PCR to produce two overlapping fragments with the overlapping region containing the third mutation C156A. Clones containing the desired mutations were identified by DNA sequencing across the mutation site. The complete gene was sequenced to verify that no other, unwanted, changes had occurred.

Fig. 1. Schematic diagram of site-directed mutagenesis by the two-stage overlapping method. The oligonucleotide primers and the newly-synthesized double-stranded (ds) DNA are represented by lines with arrows indicating the 5'→3' direction. The site of mutagenesis is indicated by the black dots. The boxed portion represents the proposed intermediate steps during the course of reaction where the denatured fragments (recovered from first-stage PCR) anneal at the overlap and are extended by DNA polymerase (dotted line) to form the mutant product. By adding additional "primer 1" and "primer 4", the mutant product is further amplified (Ho *et al.*, 1989).



Mutant product

2.7 The *S. pneumoniae tehB* Gene Cloning

Primers ML53 (5'-ATA*CTCGAG*ACTTCTTTTAGGACTTGCCA AA-3') and ML54 (5'-ATA*CTGCAG*GATCCTCTAACACATTTACCAA-3') (italicized sequences indicate extensions introduced to create restriction sites *Xho*I and *Pst*I, respectively) were designed based on the unfinished genome sequence of *S. pneumoniae* in National Center for Biotechnology Information (NCBI) database. The *S. pneumoniae tehB* gene fragment (about 1.14 kb) was amplified by a modified colony PCR using primers ML53 and ML54 (Ge and Taylor, 1997). After purification of the amplified DNA from an agarose gel and digestion with the restriction enzymes *Xho*I and *Pst*I, the *tehB* fragment was then cloned into the vector pTZ18U which had been digested with the same enzymes. The *S. pneumoniae tehB*-containing plasmid was termed pTSPtehB-2.

2.8 DNA Sequencing

DNA sequencing was performed on double-stranded templates using the Thermo-Sequenase Radiolabeled Terminator Cycle Sequencing kit, based on the dideoxy chain termination method (Sanger *et al.*, 1977), purchased from Amersham (Cleveland, OH). The kit includes [α-^{33}P]-labeled ddNTPs and the thermostable Thermo Sequenase DNA polymerase which combines the advantages of both T7 Sequenase and *Taq* DNA polymerase. The sequencing reactions were carried out according to the manual with 30 cycles: 95°C, 30 seconds; 50-55°C, 30 seconds; 72°C, 1 minute. In addition to ML53 and ML54, the mutagenic primers listed in Table 3 were also used as sequencing primers. After cycling, the products were subjected to electrophoresis on a 5-6% urea-polyacrylamide gel at 50-55°C. The gels were vacuum-dried at 80°C for 2 hrs and

exposed to X-ray film. A Perkin-Elmer DNA thermal cycler (Model 480) (Norwalk, CT) was used for the cycle sequencing, the PCR for site-directed mutagenesis, and the *S. pneumoniae tehB* amplification.

2.9 Protein Expression

Expression of the *S. pneumoniae tehB* gene, as well as the *Haemophilus influenzae tehB* and *Eikenella corrodens* haemagglutinin genes, was carried out as described previously (Ge and Taylor, 1996). BL21(DE3)/pLysS cells harboring various plasmids (pTSPtehB-2, pML111, pTWT134 and pML204) (Table 2) were grown in LB broth to an optical density of 0.5 at 600 nm, and then washed and suspended in M9 medium supplemented with arginine, threonine, leucine, proline and thiamine. The cells were then incubated at 37°C for 1 hr with agitation and induced by the addition of 0.1 mM IPTG for 30 min. Rifampicin was subsequently added to a final concentration of 200 μg/ml for 30 min. to stop *E. coli* RNA transcription. [^{35}S]-methionine was then added and incubation was continued for 1 hr. Cells were pelleted and resuspended in cracking buffer (50 mM Tris-HCl (pH 6.8), 1% SDS, 20 mM EDTA, 1% mercaptoethanol and 10% glycerol). A 5-μl sample was loaded onto a 15% SDS-polyacrylamide gel. BenchMark Prestained Protein Ladder (Gibco-BRL) was used as a standard marker.

2.10 Computer Protocols

DNA and protein databases were searched at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) by using the BLAST program (Altschul *et al.*, 1990). Sequence analysis was carried out with the Genetics

Computer Group (GCG) software package (University of Wisconsin) (Devereux *et al.*, 1984). The alignment of methyltransferase motifs was combined with some manual analysis. Hydropathy plots and amino acid compositions were obtained from the DNAsis program (Hitachi Software) by the method of Kyte and Doolittle (Kyte and Doolittle, 1982).

2.11 The Accession Number of the Nucleotide Sequence

The nucleotide sequence of the *S. pneumoniae tehB homologue* has been deposited in GenBank with accession number AF 079807.

3. Results

Part I. Mutagenesis of Putative Methyltransferase Motifs in *E. coli* TehB Eliminates Tellurite Resistance.

3.1 Conservation of Cysteine Residues in TehA, TehB, and Their Homologous Proteins

Cysteine residues are involved in resistance to many metal(loid)s, such as Hg^{2-} (Helmann *et al.*, 1990; Morby *et al.*, 1995), AsO_2^-, AsO_4^{3-} (Shi *et al.*, 1996; Xu *et al.*, 1998), Cu^{2+} (Cha and Cooksey, 1991), and Cd^{2+} (Silver and Walderhaug, 1992). In the tellurite resistance systems, the two cysteine residues in TelB of *kilAtelAtelB* have already been demonstrated to be essential for the resistance (Turner *et al.*, 1994). In the *E. coli tehAB* system, *tehA* and *tehB* genes are in one operon. TehA is a membrane protein predicted to have 10 transmembrane segments, whereas TehB is a soluble protein. In both TehA and TehB, there are three cysteine residues (Walter *et al.*, 1991). Recently, homologues of these two genes have been sequenced in the *Haemophilus influenzae* chromosome, however they are not in the same operon but are widely separated (Fleischmann *et al.*, 1997). The *Eikenella corrodens* hemagglutinin (Rao *et al.*, 1993) also shows a significant homology (53-58% similarity) to these TehB proteins. From amino acid sequence comparisons, two cysteine residues in *E. coli* TehB were found to be conserved in *H. influenzae* TehB and *E. corrodens* hemagglutinin (Fig. 2). One cysteine residue was conserved in *E. coli* and *H. influenzae* TehA proteins but was shifted one position (data not shown).

Fig. 2. Comparison of *E. coli* TehB and its homologues. There are three cysteine residues in *E. coli* TehB, two of them are conserved in the *H. influenzae* TehB and the *Eikenella corrodens* hemagglutinin. The conserved cysteine residues are highlighted in black; the unconserved cysteine is labeled with an asterisk (*). EcTehB, *E. coli* TehB; HitehB, *H. influenzae* TehB; EcrHag1, *E. corrodens* hemagglutinin. Gaps were introduced to make the best alignment.

```
          1                                                  50
EcTehB    ---------- ---------- ---------- ---------- ----------
HiTehB    ---------- ---MKNELIC YKQMPVWTKD NLPQMFQEKH NTKVGTWGKL
EcrHag1   MVSALSCTHE RRCYAIRTHL LQNYAGMGLS HYSGSSFVAA QHTGWYLRQA

          51                                                 100
EcTehB    ---------- ---------- ---------- ---------- ----------
HiTehB    TVLKGKLKFY ELTENGDVIA EHIFTPESHI PFVEPQAWHR VEALSDDLEC
EcrHag1   ARIARAAEVF ELQEDGTVLA EHILQPDSGV WTLYPQAQHK VEPLDDDFAV

          101                                                150
EcTehB    ---MIIRDEN YFTDKYELTR THSEVLEAVK VVKPGKTLDL GCGNGRNSLY
HiTehB    TLGFYCKKED YFSKKYNTTA IHGDVVDAAK IISPCKVLDL GCGQGRNSLY
EcrHag1   QLEFHCEKAD YFHKKHGMTT THSAIREAVQ TVAPCKTLDL GCGQGHNALF

          151                                                200
EcTehB    LAANGYDVDA WDKNAMSIAN VERIKSIENL DNLHTRVVDL NNLTFDRQYD
HiTehB    LSLLGYDVTS WDHNENSIAF LNETKEKENL -NISTALYDI NAANIQENYD
EcrHag1   LSLAGYDVRA VDHSPAAVAS VLDMAAREQL P-LRADAYDI NAAALNEDYD

          201                        *                       250
EcTehB    FILSTVVLMF LEAKTIPGLI ANMQRCTKPG GYNLIVAAMD TADYPCTVGF
HiTehB    FIVSTVVFMF LNRERVPSII KNMKEHTNVG GYNLIVAAMS TDDVPCPLPF
EcrHag1   FIFATVVFIF LQAGRVPEII ADMQAHTRPG GYNLIVSAMD TADYPCHMPF

          251                                                301
EcTehB    PFAFKEGELR RYYEGWERVK YNEDVGELHR TDANGNRIKL RFATMLARKK
HiTehB    SFTFAENELK EYYKDWEFLE YNENMGELHK TDENGNRIKM KFATMLARKK
EcrHag1   SFTFKEDELR QYYADWELLK YEEAVGLMHA TDAQGRPIQL KFVTMLAKKPG
```

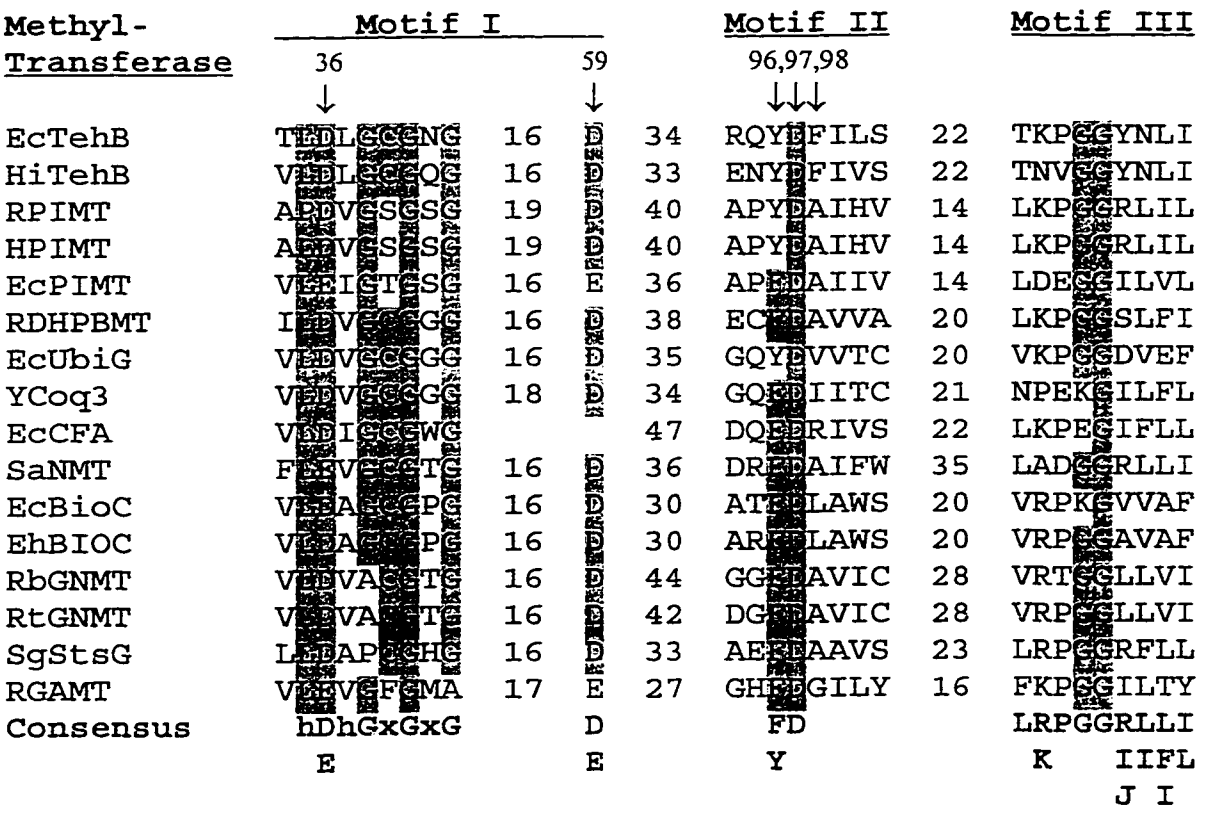
3.2 Identification of *S*-Adenosylmethionine-Dependent Methyltransferase Motifs in TehB

By computer search, it was noted that TehB displayed obvious similarity in amino acid sequence to many AdoMet-dependent non-nucleic acid methyltransferases within the regions of motifs I, II and III, with comparable intervals (Fig. 3; R. Turner, personal communication). Motif I is a glycine-rich region, and the *E. coli* TehB contains a sequence agreeing well with the consensus h(D/E)hGxGxG, where 'h' represents a hydrophobic residue, and 'x' is any residue (Kagan and Clarke, 1994; Wu *et al.*, 1992). Motif I is generally followed by an acidic amino acid (aspartate or glutamate) on the C-terminal side 17-19 residues apart (Kagan and Clark, 1994). In TehB, there are 16 residues before this aspartate (Fig. 3)

Thirty-four residues downstream from motif I lies motif II, which comprises 8 residues. Motif II is unusually rich in aromatic amino acids (tyrosine, tryptophan and phenylalanine) around the central invariant aspartate (Kagan and Clarke, 1994). Aromatic rings are considered to be involved in binding AdoMet through cation-π interaction with the sulphonium moiety of AdoMet (Dougherty and Stauffer, 1990; McCurdy *et al.*, 1992). The importance of tyrosine in motif II of rat guanidinoacetate methyltransferase (RGAMT) has already been demonstrated (Hamahata *et al.*, 1996). Cation-π interaction may also be involved in the catalysis of *N*-specific DNA methyltransferases (Schuluckebier *et al.*, 1998). In TehB, four aromatic residues were found in this region, they are phenylalanine (-5 position with respect to aspartate), tyrosine (-1), phenylalanine (+1) and phenylalanine (+10).

Fig. 3. Alignment of sequences of *E. coli* TehB, *H. influenzae* TehB and some non-nucleic acid methyltransferases to demonstrate the three conserved motifs. The mutated residues in motifs I and II are indicated by arrows. The sequences are from the NCBI database. The names are abbreviated and their accession numbers are in parenthesis.

EcTehB, *E. coli* tellurite resistance TehB (M74072);
HiTehB, *Haemophilus influenzae* tellurite resistance TehB homologue (U32807);
RPIMT, rat protein L-isoaspartyl carboxyl methyltransferase (D11475);
HPIMT, human protein L-isoaspartyl carboxyl methyltransferase (P22061);
EcPIMT, *E. coli* protein L-isoaspartyl carboxyl methyltransferase (P24206);
RDHPBMT, rat dihydroxypolyprenylbenzoate methyltransferase (L20427);
EcUbiG, *E. coli* ubiquinone biosynthesis-related peorein (M87509);
YCoq3, *S. cerevisiae* 3, 4-dihydroxy-5-hexaprenylbenzoate methyltransferase (M73270);
EcCFA, *E. coli* cyclopropane fatty acid synthetase (M98330);
SaNMT, *Streptomyces anulatus* N-methyltransferase (X92429);
EcBioC, *E. coli* biotin synthesis protein (P12999);
EhBioC, *Erwinia herbicola* biotin synthesis protein;
RbGNMT, rabbit glycine N-methyltransferase (D13307);
RtGNMT, rat glycine N-methyltransferase (X06150);
SgStsG, *Streptomyces griseus* methyltransferase involved in N-methyl-L-glucosamine pathway (Y08763).
RGAMT, rat guanidinoacetate methyltransferase (J03588).


Methyl-
Transferase
Motif I
Motif II
Motif III
36
59
96,97,98
EcTehB 16 34 22
HiTehB 16 33 22
RPIMT 19 40 14
HPIMT 19 40 14
EcPIMT 16 36 14
RDHPBMT 16 38 20
EcUbiG 16 35 20
YCoq3 18 34 21
EcCFA 47 22
SaNMT 16 36 35
EcBioC 16 30 20
EhBIOC 16 30 20
RbGNMT 16 44 28
RtGNMT 16 42 28
SgStsG 16 33 23
RGAMT 17 27 16
Consensus
hDhGxGxG
D
FD
LRPGGRLLI
E
E
Y
K
IIFL
J I

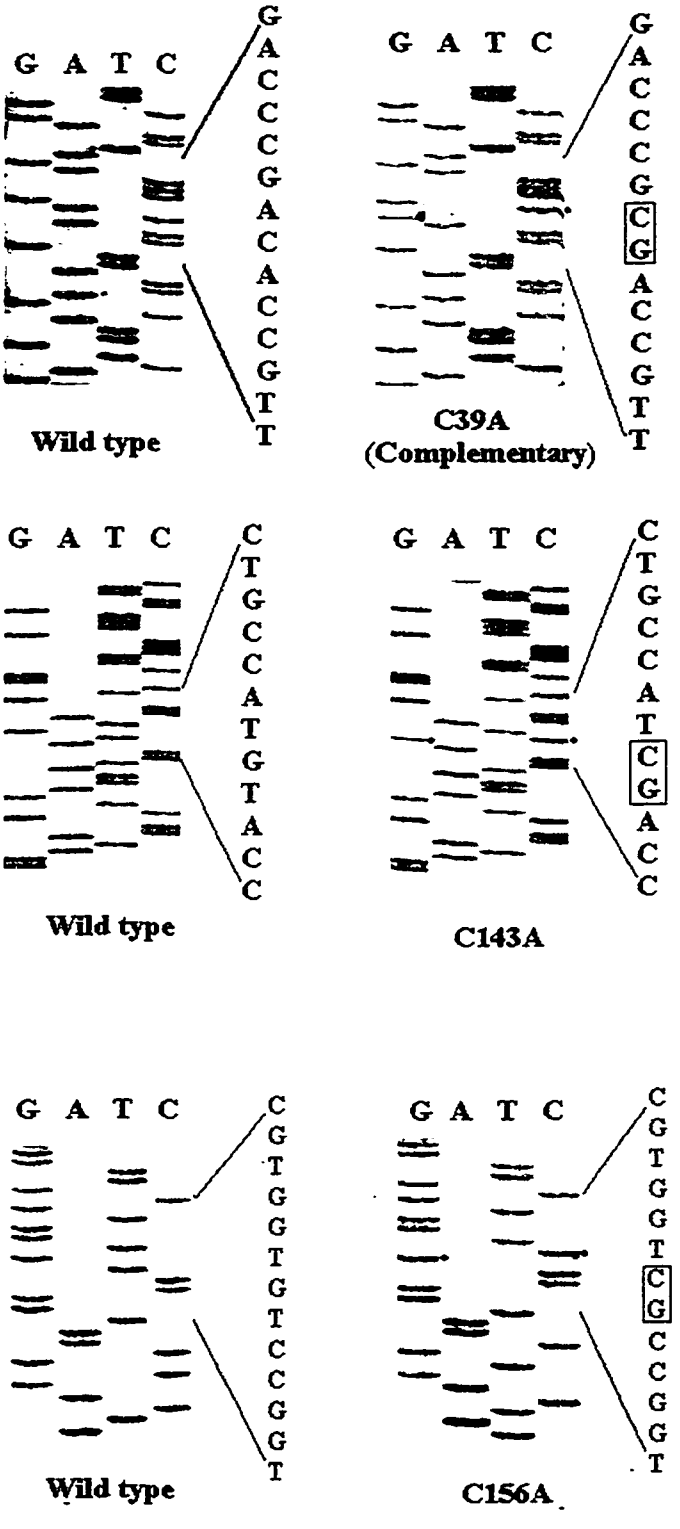
Motif III is 22 residues away from motif II and consists of a 9-residue block. In the middle of the block are two highly conserved glycine residues. Motif III of TehB is consistent with the consensus: L(R/K)PGG(R/I/J)(L/I)(L/F/I)(I/L) (Ingrosso *et al.*, 1989; Kagan and Clarke, 1994). However the significance of motif III in methyltransferases has been questioned, because site-directed mutagenesis of several residues of motif III of RGAMT did not markedly change its enzymatic activity (Gomi, *et al.*, 1992).

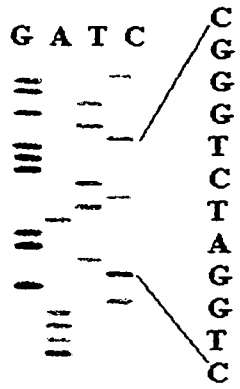
3.3 Mutagenesis of the Cysteine Residues in *E. coli* TehA and TehB, and the Conserved Residues in the AdoMet Motifs I and II

To determine if these conserved as well unconserved cysteines were involved in tellurite resistance, each of the six cysteine residues in both TehA and TehB were replaced with an alanine. Many combinations of these mutations were also created. Each mutant was confirmed by DNA sequencing. A PCR-based site-directed mutagenesis method was used to create these mutations (see section 2.6, site-directed mutagenesis). Representative sequencing results are displayed in Fig. 4.

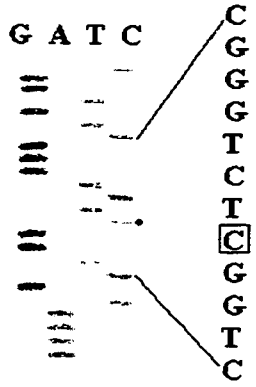
To determine if the AdoMet-binding motifs in TehB are involved in tellurite resistance, site-directed mutagenesis was performed on two aspartate residues (Asp36 and Asp59) in motif I, and three residues (Tyr96, Asp97 and Phe98) in motif II (see Fig. 3). The aspartate residues in motif I were substituted to alanine and/or asparagine (Asp36Ala, Asp59Ala and Asp59Asn), while the mutations in motif II were Tyr96Ala, Asp97Glu, Asp97Asn, Phe98Tyr and Phe98Ala. All of these mutations were confirmed by DNA sequencing. Some of the sequencing results are shown in Fig. 4.

Fig. 4. Some of the DNA sequencing showing the regions of the site-directed mutagenesis. The thermo-sequenase radiolabeled terminator cycle sequencing kit was used. The ddNTPs were labeled with [α-^{33}p]. For each panel, the sequence for the wild type gene is on the left, and the mutant sequence is on the right with changed bases labeled with black dots/boxes. The sequence here for C39A is complementary to the primer sequence list in Table 3.

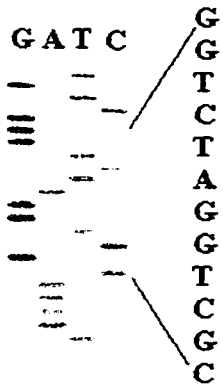

G A T C
GACCCCGACACCGTT
Wild type
G A T C
GACCCCGCGACCGTT
C39A
(Complementary)
G A T C
CTGCCATGTACC
Wild type
G A T C
CTGCCATCGACC
C143A
G A T C
CGTGGTGTCCGGT
Wild type
G A T C
CGTGGTCGCCGGT
C156A



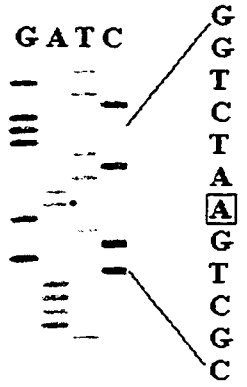
Wild type



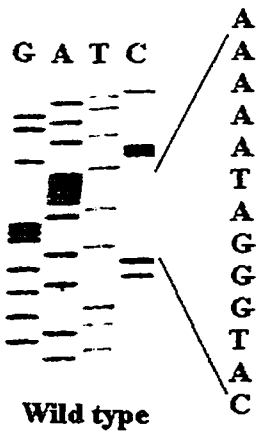
D36A



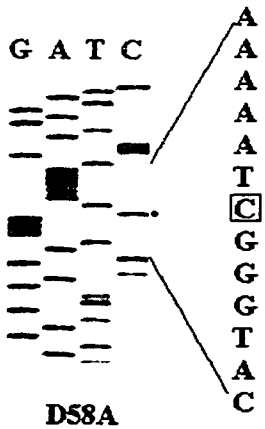
Wild type



D36N



Wild type



D58A

3.4 Effect of the Mutagenesis of Cysteine Residues on Tellurite Resistance

The effect of substitution of cysteines with alanines on tellurite resistance was evaluated by measuring the ability of clones to encode resistance to tellurite, *E. coli* JM109 containing the cloning vector was used as the negetive control (background level of tellurite resistance is ~ 2 μg/ml) and the original plasmid pTWT101 containing the wild type *tehAB* fragment as the positive control, as shown in Table 4. The resistance for all these mutants was consistent and determined at least three times. The resistance (MIC) for all six single Cys mutants showed no change with respect to the wild type, indicating that no single cysteine was essential for tellurite resistance. The same result was obtained with the TehA triple mutant. Various combinations of cysteine substitutions in TehB did cause a decrease in tellurite resistance, with different combinations having different degrees of susceptibility. The TehB triple mutant C39A/C123A/C143A and double mutant C123A/C143A (Table 4) had a greater effect on resistance with MICs reduced to 16 μg/ml K_2TeO_3. In contrast, the TehB double mutant C39A/C123A specified a smaller effect (MIC = 32 μg/ml), the MIC encoded by the TehB double mutant C39A/C143A was only slightly decreased (64 μg/ml) compared to the wild type.

The addition of TehA cysteine mutations to the TehB double mutants gave distinct results, some mutants, C109A(TehA)/C39A/C143A(TehB), C212A(TehA)/ C39A/C143A (TehB), specified a lower resistance than the corresponding TehB double mutant C39A/C143A alone, while other mutants encoded higher resistance than the corresponding TehB mutants alone. For example, the MIC for C109A(TehA)/C123A/ C143A(TehB) is 64 μg/ml, although with TehB double mutant C123A/C143A, the MIC

Table 4. Tellurite resistance of cysteine mutants in TehATehB

TehA			TehB			MIC
109	156	212[a]	39[b]	123	143[b]	(μg/ml)
-	-	-	-	-	-	2[c]
C[d]	C	C	C	C	C	128[e]
A[f]	C	C	C	C	C	128
C	A	C	C	C	C	128
C	C	A	C	C	C	128
C	C	C	A	C	C	128
C	C	C	C	A	C	128
C	C	C	C	C	A	128
A	A	A	C	C	C	128
A	A	A	A	A	A	16
C	C	C	A	A	A	16
C	C	C	C	A	A	16
A	C	C	A	C	A	16
C	C	A	A	C	A	16
C	C	C	A	C	A	32
C	C	A	C	A	A	32
C	A	C	C	A	A	32
C	C	C	A	A	C	64
C	A	C	A	C	A	64
A	C	C	C	A	A	64

[a] Residue is conserved but shifted one position.

[b] Residue is conserved.

[c] Negative control containing plasmid lacking *tehAtehB* genes.

[d] Cysteine residue unchanged.

[e] Positive control with wild type *tehAtehB* genes.

[f] Substitution of cysteines by alanines are bold.

is 16 μg/ml. The global replacement of all six cysteines with alanines produced a non-cysteine mutant which has an MIC similar to the TehB triple and some double mutants (16 μg/ml) (Table 4).

3.5 The Effect of Mutagenesis of Conserved Residues in Motifs I and II of TehB on Tellurite Resistance

Tellurite resistance of all three motif I mutants (Asp36Ala, Asp59Ala and Asp59Asn) and two motif II mutants (Phe98Tyr, and Phe98Ala) were reduced to 2 μg/ml, equivalent to that of wild type *E. coli*. Substitutions Tyr96Ala and Asp97Asn had only a partial effect on tellurite resistance, whereas Asp97Glu had no effect (Table 5). The drop in MIC was not due to decreased expression as SDS-polyacrylamide gel electrophoresis showed that expression of the mutant TehB proteins was similar to the wild type (data not shown). These results suggest that motifs I and II of *E. coli* TehB, like those which play a direct role in AdoMet binding in other methyltransferases, are directly involved in Te^R.

Table 5. Tellurite resistance of mutants in TehB methyltransferase motifs

Mutants	MIC (μg/ml)
pTZ19R	2[a]
pTWT101	128[b]
Asp36Ala	2
Asp36Asn	2
Asp59Ala	2
Tyr96Ala	16
Asp97Glu	128
Asp97Asn	64
Phe98Tyr	2
Phe98Ala	2

[a] Negative control with vector lacking *tehAtehB* genes.

[b] Positive control with wild type *tehAtehB* genes

Part II. Characterization of the Gram-Positive *Streptococcus pneumoniae* Tellurite Resistance Gene

3.6 The *S. pneumoniae* Is Resistant to Tellurite

A broth dilution method is used to determine if *S. pneumoniae* is resistant to tellurite. An overnight *S. pneumoniae* culture was inoculated into different concentrations of tellurite-containing media and incubated at 37°C in the presence of CO_2. The *S. pneumoniae* cultures grew at all tellurite concentrations ranging from 0.5 μg/ml to 64 μg/ml but not at 128 μg/ml where no obvious OD change was observed. The MIC of *S. pneumoniae* to tellurite is 128 μg/ml.

3.7 Nucleotide Sequence of *S. pneumoniae tehB* Gene

The putative *S. pneumoniae tehB* gene was amplified by PCR using primers MF53 and MF54 (see Table 3) and cloned into pTZ18U (see Table 2). Four clones randomly selected from three independent PCR reactions were sequenced and found to have the same sequences as those that appeared in the NCBI unfinished genome database (http://www.ncbi.nlm.nih.gov), except for one nucleotide change at base 475 in the *tehB* open reading frame (ORF) which resulted in an alteration of glutamine to glutamic acid in the deduced TehB protein sequence. The ORF could encode a protein of 284 amino acids with molecular mass of 32,444 daltons. The ribosome-binding site (RBS) GAGG identified by Shine and Dalgarno (Shine and Dalgarno, 1974) is located 9 bp upstream from the proposed translational start codon (ATG). An hexanucleotide (TTGCCA) has 5

Fig. 5. Nucleotide and the deduced amino acid sequences of the clone pTSPtehB-2. The hexanucleotides corresponding to the *E. coli* promoter regions are indicated by "-35" and "-10". The possible ribosome-binding sequence (Shine-Dalgarno) is labeled "SD". A region downstream of the stop codon which could form a stem-loop structure in mRNA is marked as "stem-loop". Some restriction enzymes are underlined and labeled. The nucleotides are numbered on the left. The sequences corresponding to primers ML53 and ML54 are bold at both ends.

```
                .    -35  .          .   -10  .          .          .
  1 CTTCTTTTAGGACTTGCCAAAGGGAAGCATGACTATGACAAACGGGAGTCTATCAAACGT
                .     EcoR V        .          .          .         SD
 61 CGTGAGCAAAATCGAGATATCGCGCGTGTGATGAAAGCTGTTAATCAGCGATAAAAAGAG
                .          .          .          .          .          .
121 GAATTGAAAATGGAAAAATTAGTTGCCTATAAACGCATGCCTTTGTGGAATAAACAAACA
              M  E  K  L  V  A  Y  K  R  M  P  L  W  N  K  Q  T
                .          .          .          .          .          .
181 ATGCCTGAAGCTGTTCAGCAAAAGCACAATACAAAAGTTGGGACTTGGGGGAAAATTACT
    M  P  E  A  V  Q  Q  K  H  N  T  K  V  G  T  W  G  K  I  T
                .          .          .          .          .          .
241 GTCTTGAAGGGAGCTCTCAAGTTTATTGAATTGACAGAAGAAGGGGAAGTTCTAGCTGAA
    V  L  K  G  A  L  K  F  I  E  L  T  E  E  G  E  V  L  A  E
                .          .          .          .          .          .
301 CACCTCTTTGAAGCAGGGGCAGACAATCCAATGGCCCAACCTCAAGCCTGGCACCGAGTG
    H  L  F  E  A  G  A  D  N  P  M  A  Q  P  Q  A  W  H  R  V
                .          .          .          .          .          .
361 GAAGCTGCCACAGATGATGTGGAATGGTACTTGGAATTTTATTGTAAACCTGAGGATTAT
    E  A  A  T  D  D  V  E  W  Y  L  E  F  Y  C  K  P  E  D  Y
                .          .          .     Eco0109I      .          .
421 TTTGCTAAAAAATACAATACCAATCCTGTTCATTCAGAGGTCCTAGAGGCCATGgAGACA
    F  A  K  K  Y  N  T  N  P  V  H  S  E  V  L  E  A  M  E  T
              . HindIII.          .          .          .          .
481 GTGAAACAAGGGAAAGCTTTGGATTTGGGTTGTGGTCAGGGGCGTAATTCTCTTTTTCTA
    V  K  Q  G  K  A  L  D  L  G  C  G  Q  G  R  N  S  L  F  L
                .          .          .          .          .          .
541 GCCCAGCAAGATTTTGATGTGACGGCTGTAGATCAAAATGGACTAGCTCTTGAAATCTTG
    A  Q  Q  D  F  D  V  T  A  V  D  Q  N  G  L  A  L  E  I  L
                .          .          .          .     EcoR V     .
601 CAAAGCATTGTGGAGCAGGAAGATTTGGACATGCCTGTTGGCCTTTACGATATCAATTCA
    Q  S  I  V  E  Q  E  D  L  D  M  P  V  G  L  Y  D  I  N  S
                .          .          .          .          .          .
661 GCTAGCATTGAACAAGAATATGATTTTATCGTTTCAACAGTTGTTCTCATGTTTCTACAA
    A  S  I  E  Q  E  Y  D  F  I  V  S  T  V  V  L  M  F  L  Q
                .          .          .          .          .          .
721 GCGGACCGCATTCCAGCTATTATTCAAAATATGCAGGAGAAAACCAGTGTTGGTGGTTAC
    A  D  R  I  P  A  I  I  Q  N  M  Q  E  K  T  S  V  G  G  Y
                .          .          .          .     Hpa I      .
781 AACCTTATCGTTTGTGCCATGGACACGGAGGATTATCCTTGCTCGGTTAACTTCCCATTC
    N  L  I  V  C  A  M  D  T  E  D  Y  P  C  S  V  N  F  P  F
                .          .          .          .          .          .
841 ACCTTTAAAGAAGGAGAACTGGCAGACTATTACAAGGATTGGGAATTGGTTAAGTACAAT
    T  F  K  E  G  E  L  A  D  Y  Y  K  D  W  E  L  V  K  Y  N
                .          .          .          .          .          .
901 GAAAATCCAGGCCATTTGCACCGTCGCGATGAGAATGGCAATCGTATTCAACTACGCTTT
    E  N  P  G  H  L  H  R  R  D  E  N  G  N  R  I  Q  L  R  F
                .          .          .          .     Stem-loop  .
961 GCGACCTTACTAGCTAAGAAAATCAAGTAAACACACATGAAGATTAGGAATTTTCCTGAT
    A  T  L  L  A  K  K  I  K  *
                .          .          .     EcoRI      .          .
1021 CTTTTTTCTTTTTTACGAATGATATAGAAAAGGAGGGAATTCATGTTTGTTGCGAGAGAT
                .          .          .
1081 GCTAGGGGAGAATTGGTAAATGTGTTAGAGGAT 1114
```

out of 6 residues identical to the *E. coli* promoter -35 consensus sequences (TTGACA). A second region (TATGAC) starts at base 34 and roughly matches the *E. coli* potential -10 portion (TATAAT) (Rosenberg and Court, 1979). A possible stem-loop structure consisting of an 11-bp-long inverted repeat (AAGATTAGGAA ... TTCCTGATCTT) was identified 11 bp downstream of the stop codon, which was followed by a string of T's. This may represent a rho-independent transcription terminator (Platt, 1986) (Fig. 5).

3.8 Comparison of *S. pneumoniae* TehB and Other Homologous Proteins

The deduced amino acid sequence of *S. pneumoniae* TehB was used to search for homologous proteins in NCBI databases. In addition to significant similarity to the *E. coli* (Taylor *et al.*, 1994; Walter *et al.*, 1991b) and *Haemophilus influenzae* TehB proteins (Fleischmann *et al.*, 1997), it also showed striking apparent homologies with the *Eikenella corrodens* haemagglutinin (Hag1) (Rao *et al.*, 1993) and similar proteins from *Actinobacillus actinomycetemcomitans*, *Neisseria gonorrhoeae and Neisseria meningitidis*. Among these proteins, the *E. coli* TehB is the shortest one, consisting of only 197 amino acids; all other proteins are 90-100 residues longer than *E. coli* TehB at the N-terminus. The homology is distributed throughout the complete sequences, but the N-terminal extra part (with respect to *E. coli* TehB) showed a relatively lower degree of similarity (Fig. 6). Motifs I, II and III which were found in many AdoMet-dependent non-nucleic acid methyltransferases are also present in all of these sequences (Fig. 6) (Kagan and Clarke, 1994). These homologies, determined as the percentage of identity and similarity between the amino acid sequences of the different proteins, are summarized in Table 6. The *S. pneumoniae* TehB typically exhibits 46-58% identity (52-

Fig. 6. Sequence comparison of *S. pneumoniae* TehB and homologues from seven other bacteria. Sequences were aligned using the Pileup program of GCG software package with GapWeight of 12 and GapLengthWeight of 4 (University of Wisconsin, Madison). Identical residues across all sequences are highlighted in black. The putative motifs of non-DNA methyltransferase for AdoMet-binding is indicated. Gaps (dots) are introduced to give the best alignment. The names are abbreviated and their accession numbers are as follows:

SpTehB, *S. pneumoniae* TehB homologue (accession number: AF079807; this study);

HiTehB, *H. influenzae* tellurite resistance TehB homologue (U32827; Fleschmann *et al.*, 1997);

EcrHag1, *E. corrodens* hemogglutinin (Hag1) (P35647; Rao *et al.*, 1993);

EcTehB, *E. coli* tellurite resistance TehB (M74072; Walter *et al.*, 1991b).

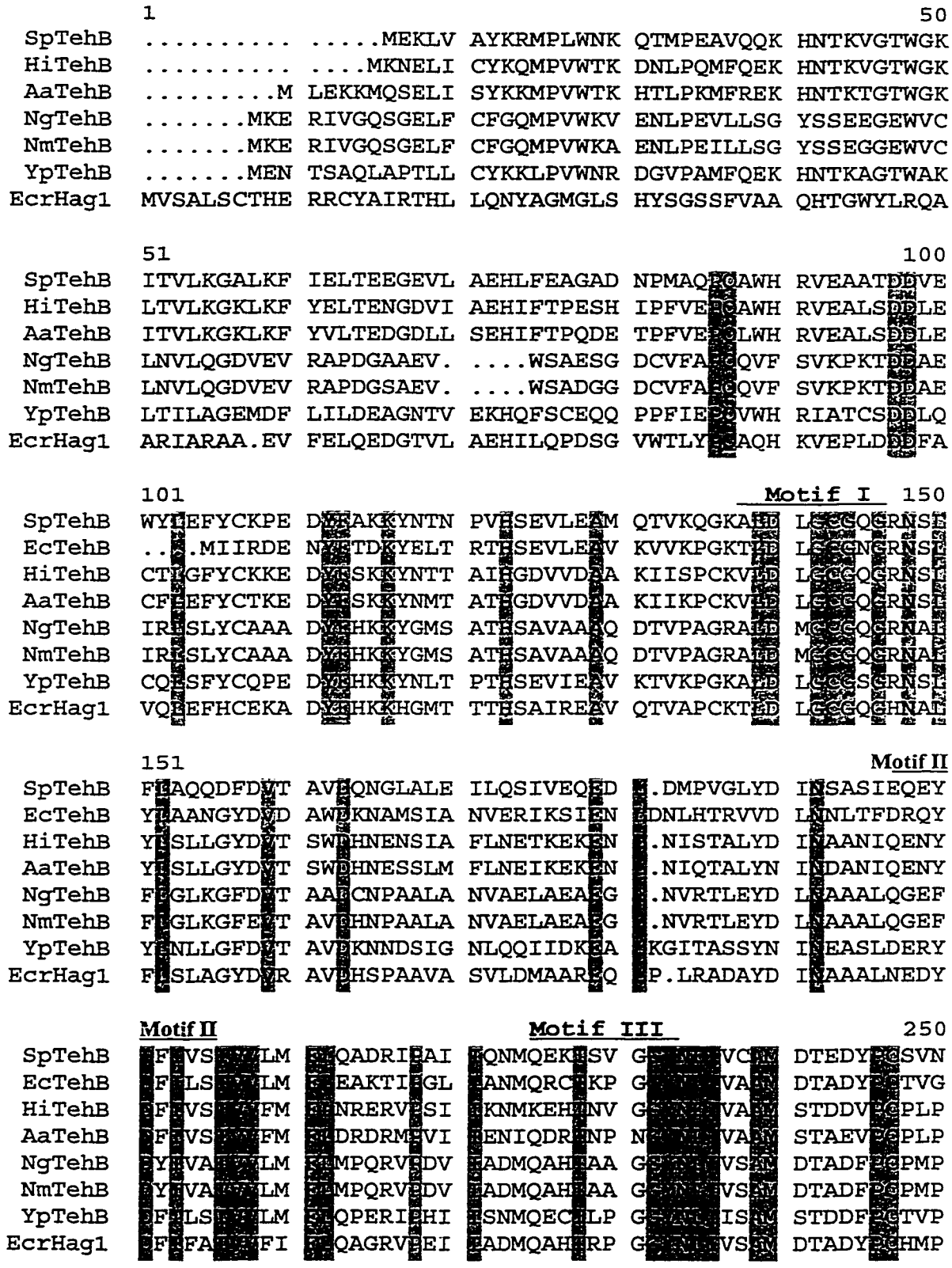
The following sequences were from the NCBI database of unfinished genome sequences:

AaTehB, *A. actinomycetemcomiyans* TehB homologue;

NgTehB, *N. gonorrhoeae* TehB homologue;

NmTehB, *N. meningitis* TehB homologue;

YpTehB, *Y. pestis* TehB homologue.


1 50
SpTehB ....MEKLV AYKRMPLWNK QTMPEAVQQK HNTKVGTWGK
HiTehB ....MKNELI CYKQMPVWTK DNLPQMFQEK HNTKVGTWGK
AaTehB .....M LEKKMQSELI SYKKMPVWTK HTLPKMFREK HNTKTGTWGK
NgTehB .....MKE RIVGQSGELF CFGQMPVWKV ENLPEVLLSG YSSEEGEWVC
NmTehB .....MKE RIVGQSGELF CFGQMPVWKA ENLPEILLSG YSSEGGEWVC
YpTehB .....MEN TSAQLAPTLL CYKKLPVWNR DGVPAMFQEK HNTKAGTWAK
EcrHag1 MVSALSCTHE RRCYAIRTHL LQNYAGMGLS HYSGSSFVAA QHTGWYLRQA
51 100
SpTehB ITVLKGALKF IELTEEGEVL AEHLFEAGAD NPMAQPQAWH RVEAATDDVE
HiTehB LTVLKGKLKF YELTENGDVI AEHIFTPESH IPFVEPQAWH RVEALSDDLE
AaTehB ITVLKGKLKF YVLTEDGDLL SEHIFTPQDE TPFVEPQLWH RVEALSDDLE
NgTehB LNVLQGDVEV RAPDGAAEV. ....WSAESG DCVFAPQQVF SVKPKTDDAE
NmTehB LNVLQGDVEV RAPDGSAEV. ....WSADGG DCVFAPQQVF SVKPKTDDAE
YpTehB LTILAGEMDF LILDEAGNTV EKHQFSCEQQ PPFIEPQVWH RIATCSDDLQ
EcrHag1 ARIARAA.EV FELQEDGTVL AEHILQPDSG VWTLYPQAQH KVEPLDDDFA
101 Motif I 150
SpTehB WYIEFYCKPE DWEAKKYNTN PVHSEVLEAM QTVKQGKAHD LGCGQGRNSL
EcTehB ..A.MIIRDE NYETDKYELT RTHSEVLEAV KVVKPGKTLD LGCGNGRNSL
HiTehB CTIGFYCKKE DWSKKYNTT AIHGDVVDAA KIISPCKVLD LGCGQGRNSL
AaTehB CFIEFYCTKE DWSKKYNMT ATHGDVVDAA KIIKPCKVLD LGCGQGRNSL
NgTehB IRISLYCAAA DWHHKKYGMS ATHSAVAAAQ DTVPAGRALD MGCGQGRNAL
NmTehB IRISLYCAAA DWHHKKYGMS ATHSAVAAAQ DTVPAGRALD MGCGQGRNAL
YpTehB CQISFYCQPE DWHHKKYNLT PTHSEVIEAV KTVKPGKALD LGCGSGRNSL
EcrHag1 VQIEFHCEKA DWHHKKHGMT TTHSAIREAV QTVAPCKTLD LGCGQGHNAL
151 Motif II
SpTehB FIAQQDFDVT AVHQNGLALE ILQSIVEQED .DMPVGLYD INSASIEQEY
EcTehB YIAANGYDVD AWDKNAMSIA NVERIKSIEN GDNLHTRVVD LNNLTFDRQY
HiTehB YISLLGYDVT SWDHNENSIA FLNETKEKEN .NISTALYD INAANIQENY
AaTehB YISLLGYDVT SWDHNESSLM FLNEIKEKEN .NIQTALYN INDANIQENY
NgTehB FIGLKGFDVT AADCNPAALA NVAELAEAEG .NVRTLEYD LNAAALQGEF
NmTehB FIGLKGFEVT AVDHNPAALA NVAELAEAEG .NVRTLEYD LNAAALQGEF
YpTehB YINLLGFDVT AVDKNNDSIG NLQQIIDKEA KGITASSYN INEASLDERY
EcrHag1 FISLAGYDVR AVDHSPAAVA SVLDMAAREQ LP.LRADAYD INAAALNEDY
Motif II Motif III 250
SpTehB IFIVSWVLM EQADRIEAI FQNMQEKESV GSVCM DTEDYPCSVN
EcTehB IFILSWVLM GEAKTIEGL IANMQRCEKP GSVAM DTADYPCTVG
HiTehB IFIVSWIFM EDNRERVPSI EKNMKEHINV GSVAM STDDVPCPLP
AaTehB IFIVSWIFM GEDRDRMPVI EENIQDRINP NSVAM STAEVPCPLP
NgTehB IYIVAWVLM KEMPQRVEDV EADMQAHIAA GSVSM DTADFPCPMP
NmTehB IYIVAWVLM KEMPQRVEDV EADMQAHIAA GSVSM DTADFPCPMP
YpTehB IFILSWVLM SEQPERIEHI ESNMQECILP GSISM STDDFPCTVP
EcrHag1 IFIFAWIFI KEQAGRVEEI EADMQAHIRP GSVSM DTADYPCHMP

	301
SpTehB	IK
EcTehB	K.
HiTehB	K.
AaTehB	K.
NgTehB	PG
NmTehB	PE
YpTehB	..
EcrHag1	PG

Table 6. Homologies of tellurite resistance TehB homologues from various bacteria

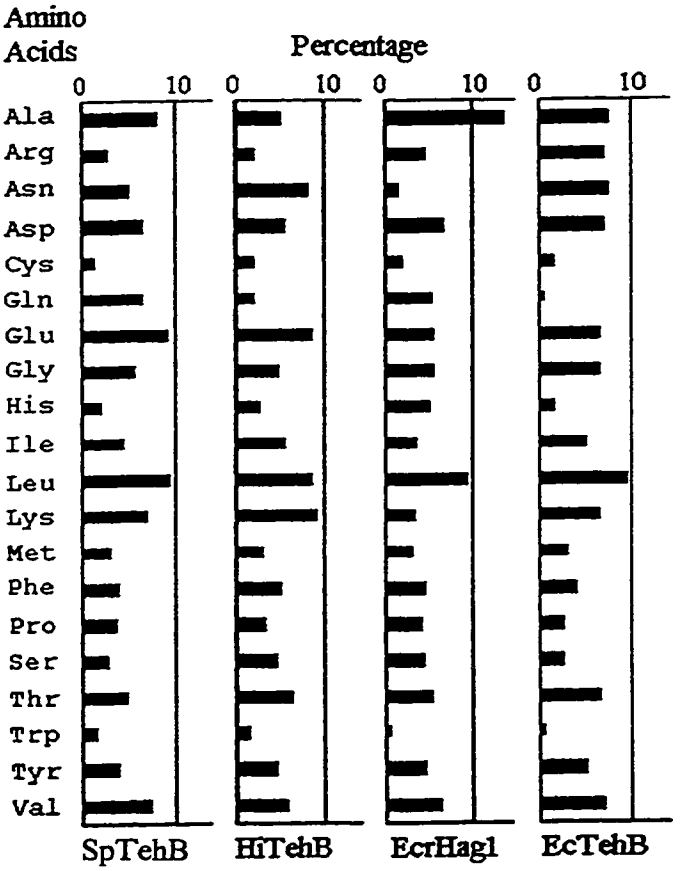
Bacterium	Identity (similarity) %						
	A. a	E. c	E. cor	H. i	N. g	N. m	Yp
S. pneumoniae	56 (65)	57 (65)	46 (52)	58 (68)	47 (54)	47 (54)	56 (65)
A. actinomyce-temcomiyans		55 (62)	44 (51)	83 (95)	44 (52)	44 (53)	55 (67)
E. coli			51 (58)	55 (61)	49 (59)	48 (59)	60 (68)
E. corrodens				45 (53)	46 (56)	46 (55)	42 (51)
H. influenzae					47 (56)	47 (56)	58 (67)
N. gonorrhoeae						97 (98)	42 (54)
N. meningitidis							41 (54)

68% similarity) to these homologous proteins. Homologies among the other proteins are generally higher than 44% identity (51% similarity). Proteins from *N. gonorroheae* and *N. meningitidis* have the highest similarity (97% identity, 98% similarity) (Table 6). The high degree of similarity between *S. pneumoniae* TehB and the other proteins suggest that they have a common evolutionary origin, *i.e.* they are homologous.

The amino acid composition of *S. pneumoniae* TehB and the homologous proteins was analyzed using the DNAsis program. The compositions of these proteins are basically very similar. The *S. pneumoniae* TehB has 15.4% acidic residues (Asp, Glu) and 11.9% basic residues (Arg, His and Lys), *H. influenzae* TehB has 14.3% acidic and 14.0% basic, while *E. corrodens* Hag1 and *E. coli* TehB appear to be slightly basic, having 12.3% and 13.7% acidic residues, and 13.0% and 15.2% basic residues, respectively. Neither acidic nor basic residues predominate in these proteins, indicating their pI's are probably close to neutral overall.

The composition of other residues were also calculated. Most residues have strikingly similar percentages in these proteins, with only a few exceptions. For example, *E. corrodens* has only 1.7% Asn, while other proteins have 5.2-8.0% Asp (Fig 7). The similar amino acid composition in these homologous proteins indicates that they probably evolved from a common ancestor and that most of the amino acids remained unchanged over time.

Fig. 7. The amino acid composition of *S. pneumoniae* TehB and the homologous proteins from *H. influenzae*, *E. corrodens* and *E. coli*. The DNAsis program was used for the calculation. The percentage of each amino acid in each protein is indicated by a horizontal bar. SpTehB, *S. pneumoniae* TehB; HiTehB, *H. influenzae* TehB; EcrHagl, *E. corrodens* Hagl; EcTehB, *E. coli* TehB.

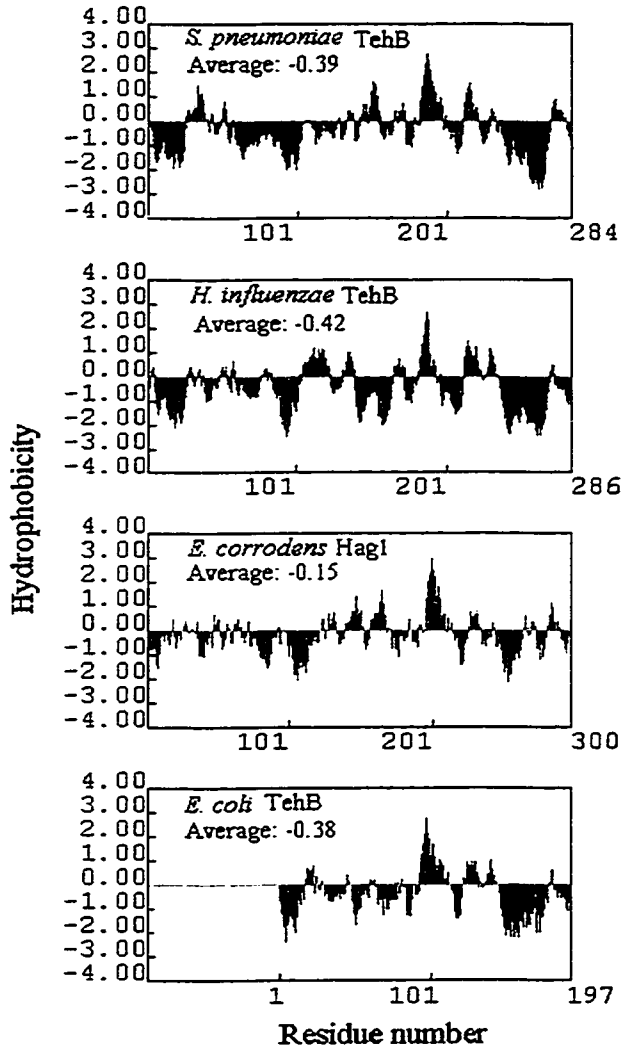

Amino
Acids
Percentage
0
10
Ala
Arg
Asn
Asp
Cys
Gln
Glu
Gly
His
Ile
Leu
Lys
Met
Phe
Pro
Ser
Thr
Trp
Tyr
Val
SpTehB
HiTehB
EcrHag1
EcTehB

The hydrophobicity of these proteins was analyzed using the algorithm of Kyte and Doolittle (Kyte and Doolittle, 1982) with a window of 11 amino acids. In this method, isoleucine (I) is considered as the most hydrophobic amino acid, with the other six hydrophobic amino acids being: valine (V), leucine (L), phenylalanine (F), cysteine (C), and methionine (M)/alanine (A) (in decreasing order of hydrophobicity). The hydrophobicity at each amino acid position was estimated by averaging the hydrophobicity values of each amino acid within a surrounding window (in this study, the window is 11). These calculated values were then plotted on a graph versus the amino acid residue number. The hydropathy plot of these four proteins displayed striking similarity, with all of they appearing to be predominantly hydrophilic, especially the N-terminal portion with only a few small regions extending above the zero line. The middle region is relatively hydrophobic. The short-form *E. coli* TehB lacks the N-terminal 90-100 amino acids, but it has a similar hydrophobicity pattern as the corresponding parts of the longer-form proteins (Fig.8).

3.9 The *S. pneumoniae tehB* Gene Confers Tellurite Resistance on *E. coli*

The *S. pneumoniae tehB* was cloned into pTZ18U under the control of both the *lacZ* and T7 RNA polymerase promoters (pTSPtehB-2). When this construct was first transformed into JM109, which does not have a T7 RNA polymerase gene (in the absence of IPTG), JM109(pTSPtehB-2) showed a high level resistance to tellurite (128 μg/ml), possibly due to leaky expression from the *lacZ* promoter. Transformations of pTSPtehB-2 into BL21(DE3), which has a p*lac*-controlled T7 RNA polymerase gene,

Fig. 8. Hydropathy plot analysis of *S. pneumoniae* TehB and the homologous proteins from *H. influenzae*, *E. corrodens* and *E. coli*. The DNAsis program was used with the algorithm of Kyte and Doolittle and a window of 11 amino acid residues (Kyte and Doolittle, 1982). The positive and negative values indicate hydrophobicity and hydrophilicity respectively. The average values are indicated on each plot.


Hydrophobicity
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
S. pneumoniae TehB
Average: -0.39
101
201
284
H. influenzae TehB
Average: -0.42
286
E. corrodens HagI
Average: -0.15
300
E. coli TehB
Average: -0.38
1
197
Residue number

were unsuccessful (even in the absence of IPTG), while other control plasmids were easily transformed. This indicated that *S. pneumoniae* TehB is toxic to *E. coli* cells; too much of this protein could result in lethality of BL21(DE3). This lethality could be circumvented by using a modified host BL21(DE3)/pLysS containing a lysozyme-producing plasmid (lysozyme is a natural inhibitor of T7 RNA polymerase) as described below.

To study *S. pneumoniae tehB* toxicity and the tellurite resistance, we compared *S. pneumoniae tehB* and homologues from *E. coli* and *H. influenzae* as well as the *E. corrodens hag1* gene. All of these genes were cloned into the same vector (pTZ18U) and transformed into BL21(DE3)/pLysS. When these transformants were not induced, or were induced with 0.1 mM of IPTG, BL21(DE3)/pLysS(pTSPtehB-2) had an MIC of 128-256 μg/ml, similar to that in JM109. The other *tehB* transformants had an MIC of 64 μg/ml. However, when induced with a higher concentration of IPTG (1 mM), all of them showed a 2-4 fold decrease in tellurite resistance, except for host carrying pTWT101 which contains the complete *tehAB* operon (including the promoter region) (Table 7). The reason may be that pTWT101 uses its own promoter, the T7 RNA promoter did not work on it effectively.

3.10 Filamentous Morphology of *E. coli* Caused by *S. pneumoniae tehB* Gene

The *E. coli* JM109 harboring pTSPtehB-2 (under the *lacZ* promoter) was found to grow much more slowly than it did without the plasmid, and small colonies were

Table 7. Tellurite resistance in various TehB constructs

Construct	Gene	MIC ($\mu g\ ml^{-1}$)		
		0	0.1	1.0 (mM)[a]
pTZ18U	- (vector)	2	2	2
pTSPtehB-2	*S. pneumoniae tehB*	128	256	32-64
pTWT101[b]	*E. coli tehAB*	128	128-256	256
pML111	*E. coli tehB*	64	64	32
pTWT134	*H. influenzae tehB*	64	64	16-32
pML204	*E. corrodens hag1*	64	64	16

[a] IPTG concentration for induction in BL21(DE3)/pLysS.

[b] Complete *tehAB* operon, including the promoter region.

Fig. 9. Scanning electron micrograph of *E. coli* JM109 containing the plasmid pTSPtehB-2. The cells were freshly transformed and grown on an LB-ampicillin plate overnight at 37°C. The agar plug containing whole colonies was used for scanning as described previously (Whelan *et al.*, 1997). Bar (ruler) = 12.0 μm.

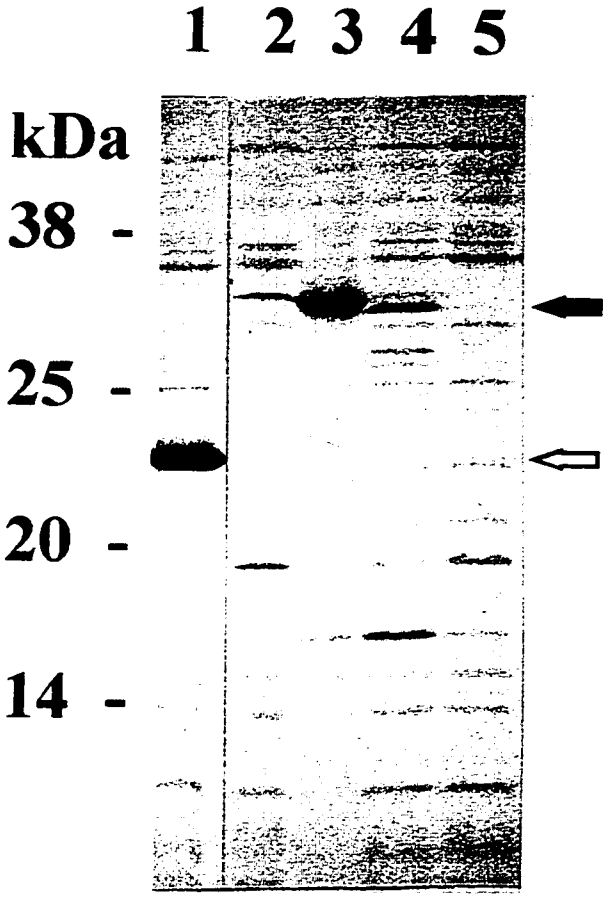
produced. We examined the cell morphology by light microscopy and found that the cells were elongated. Scanning electron microscopy showed that these colonies consisted of long filaments. These filaments were typically over 5-10 times longer than the normal wild type *E. coli* cells. Shorter filaments (2-3 times longer) were rare (Fig. 9). JM109 containing *H. influenzae tehB* or *E. corrodens hag1* also showed elongated morphology, but the average length was a little shorter than that caused by pTSPtehB (2-5 times longer) (data not shown).

3.11 Protein Expression

The *tehB* genes were cloned under the control of the T7 promoter. A BL21(DE3)/pLysS strain, which carries a p*lac*-controlled T7 polymerase gene on the chromosome was used to overexpress the *tehB* genes of *S. pneumoniae*, *E. coli*, and *H. influenzae* as well as *E. corrodens hag1* gene. The expression results are shown in Fig. 10. The BL21(DE3)/pLysS(pTSPtehB-2) produced a specific protein band of 31 kDa, which is in agreement with the predicted molecular mass of the deduced *S. pneumoniae* TehB (~ 32.6 kDa). The *H. influenzae* TehB and *E. corrodens* Hag1, which have a length similar to that of *S. pneumoniae* TehB showed similar protein bands (Fig. 10, closed arrowhead), whereas the *tehB* in pTWT101, which encodes the short-form *E. coli* TehB, displayed a band of ~23 kDa (Fig. 10, open arrowhead), that is consistent with previous expression results (Walter *et al.*, 1991b). The control plasmid pTZ18U had no such bands (Fig. 10).

The proteins were synthesized and labeled with [^{35}S]-methionine (see section 2.9). The *E. coli* TehB has six methionine residues, all three other proteins have 9 methionines (see Fig. 7). However they had weaker bands than that of *E. coli*, especially the *S. pneumoniae* and *H. influenzae* TehB proteins (Fig. 10), indicating that these genes had different levels of expression in this system.

Fig. 10. Expression of *S. pneumoniae* TehB, *E. coli* TehB, *H. influenzae* TehB and *E. corrodens* Hag1. The proteins were synthesized and labeled with [^{35}S] as described in Materials and Methods. The proteins were separated on a 15% SDS-polyacrylamide gel and exposed to Kodak BioMax X-ray film. Lanes: 1, *E. coli* TehB; 2, *E. corrodens* Hag1; 3, *H. influenzae* TehB; 4, *S. pneumoniae* TehB; 5, plasmid pTZ18U only. TehBs of *S. pneumoniae* and *H. influenzae* and Hag1 of *E. corrodens* were indicated by a closed arrowhead, and *E. coli* TehB by an open arrowhead. Molecular mass markers are indicated on the left.


1 2 3 4 5
kDa
38 -
25 -
20 -
14 -

4. Discussion

In this study, we constructed a series of mutations in the *E. coli* tellurite resistance proteins TehA and TehB. Substitutions included cysteines and other residues conserved in putative AdoMet-binding motifs I and II. To date, crystal structures of six methyltransferases have been elucidated; four of them are DNA methyltransferases: *Hha* I DNA methyltransferase (M. *Hha* I) (Cheng *et al.*, 1993), M. *Taq* I (Labahn *et al.*, 1994), M. *Hae*III (Reinish *et al.*, 1995) and M. *Pvu*II (Gong *et al.*, 1997); two of them are small molecule methyltransferases: catechol *O*-methyltransferase (Vidgren *et al.*, 1994) and glycine *N*-methyltransferase (GNMT) (Fu *et al.*, 1996). Generally DNA/RNA methyltransferases lack motifs II and III of the non-nucleic acid methyltransferases, but possess a glycine-rich sequence which shows a weak resemblance to motif I (Ingrosso *et al.*, 1989; Kagan and Clarke, 1994). Despite low sequence similarity, the tertiary structure of AdoMet-binding domains of these six enzymes are strikingly similar to each other, suggesting that many (if not all) methyltransferases may have a common structure for AdoMet-binding (Cheng, 1995; Fu *et al.*, 1996; Schluckebier *et al.*, 1995). *E. coli* TehB shares similarities with all three motifs of GNMT which have been found to be involved in AdoMet-binding (Fu *et al.*, 1996) (Fig 1), thus it is likely that TehB has a similar AdoMet-binding site. In our study, the aspartate mutations in motif I and the phenylalanine mutations in motif II reduced the resistance to the background 2 μg/ml K_2TeO_3, suggesting that *tehB* may encode a methyltransferase which mediates tellurite resistance. Such a mechanism is supported by tellurite accumulation experiments where the *tehAB* determinant was found to remove tellurite continuously from the growth media. The filter-sterilized supernatant from broth cultures of bacteria carrying the *tehAB*

plasmid reduced the toxicity by 128-fold, whereas other determinants (for example *kilAtelAB*) reduced toxicity only by 50% (Walter, 1990). The *tpm* gene from *P. syringae*, which catalyses a methylation mechanism, also mediates tellurite uptake and elimination from the medium (Cournoyer *et al.*, 1998).

Cysteine residues have been found to be involved in resistance to many metals or metalloids (Cha and Cooksey, 1991; Helmann *et al.*, 1990; Morby *et al.*, 1995; Shi *et al.*, 1996; Silver and Walderhaug, 1992; Xu *et al.*, 1998). However, with *tehAtehB*, direct thiol biochemistry may not be involved, since each single mutant was fully active with resistance to tellurite identical to that of the wild type. The fact that combinations of cysteine mutations showed a decrease in tellurite resistance suggests that additional factors play a role. This is especially true in the case of Cys143. Whenever it is missing in the double TehB mutants, there is a greater reduction in tellurite resistance.

The Pro142Cys143 sequence located at the end of motif III (C-terminal side) is highly conserved in all known *E. coli* TehB homologues, including the *H. influenzae* homologue, *S. pneumoniae* homologue, *E. corrodens* haemagglutinin (Hag1) as well as homologous proteins from *A. actinomycetemcomitans*, *N. gonorrhoeae and N. meningitidis* (Fig. 6). This is reminiscent of a catalytic cysteine in the DNA methyltransferase M.*Hha*I, in which a massive conformational change upon DNA binding brings Pro80Cys81, Glu119 and Arg165 together to form an active site. In M.*Hha*I, the Cys81 sulfhydryl group is very close to the target cytosine and makes a nucleophilic attack on carbon-6 of this cytosine (Cheng, *et al.*, 1993; Cheng, 1995). In

Schizosaccharomyces pombe, the inert pseudo DNA methyltransferase was activated by deletion of the serine from the Pro-Ser-Cys motif which corresponds to the Pro-Cys motif of DNA methyltransferases (Pinarbasi *et al.*, 1996).

In this research, the single Cys143A mutant had no tellurite resistance. Combinations of C143A and other cysteine mutations have only partial effects, suggesting that Cys143 in TehB does not have as important a function as that seen in M.*Hha* I. The reason for the partial effects of combinations of cysteine mutations may be that these multiple substitutions result in conformational changes affecting protein folding or stability. However if thiol groups within the cysteines of Teh proteins are involved in tellurite before methylation occurs, then a somewhat different mechanism might occur. Two cysteines could react with tellurite to give Cys-S-Te-S-Cys, or a Te atom could be coordinated using 4 thiols (4 cysteines or 3 cysteines + the sulphur from the AdoMet), which may involve one or more proteins. For example, cysteines coordinate the arsenic (As) and antimony (Sb) atoms within the ArsA protein dimer of the arsenate resistance efflux transporter (Xu *et al.*, 1998). In that case, two cysteines from one subunit of the homodimer and one from the second, react with the arsenite to form soft metal-thiol bonds. Further structural information on TehB is required to resolve what is occurring at the active site.

Microbial methylation of metalloides to yield volatile derivatives is a well-known phenomenon. Many bacteria, fungi and protozoa have been found to methylate arsenic compounds (Huysmans and Frankenberger, 1991; Tamaki and Frankenberger, 1992),

selenium compounds (Gharieb *et al.*, 1995; Thompson-Eaglem and Frankenberger, 1989), antimony compounds (Gurleyuk *et al.*, 1997; Jenkins *et al.*, 1998) and sulphide (Drotar *et al.*, 1987). While much less work has been done on tellurium methylation, there have been some observations that both fungi and bacteria are capable of methylating tellurium compounds. For example, several *Penicillium* strains (*P. brevicaule*, *P. chrysogenum* and *P. notatum*) and the bacterium *Pseudomonas fluorescens* were found to produce dimethyltelluride (CH_3TeCH_3) (Bird and Challenger, 1939, Fleming and Alexander, 1972; Chasteen *et al.*, 1990), and, in addition the fungi *Acremonium falciforme* and *P. citrinum* produced CH_3TeCH_3 and dimethylditelluride ($CH_3TeTeCH_3$) and another unknown compound (Chasteen *et al.*, 1990). A *Penicillium* sp. isolated from evaporation pond water was also capable of yielding CH_3TeCH_3 and $CH_3TeTeCH_3$ (Huysmans and Frankenberger, 1991).

A recent study on the tellurite resistance gene (*tpm*) from *P. syringae* pathovar *pisi* provided more direct evidence of how methylation was involved in tellurite resistance. The *tpm* gene appears to encode a methyltransferase which can catalyse AdoMet methylation of 6-mercaptopurine (a substrate for human thiopurine methyltransferase) (Cournoyer *et al.*, 1998), and the *tpm* gene was detected in most *P. syringae* legume pathogens. The *tpm* product is 55% similar to human thiopurine methyltransferase and 43% similar to a methyltransferase homologue from *Synechocystis* (Cournoyer *et al.*, 1998). In contrast, there is no homology with the *E. coli* TehB investigated in this research, suggesting an evolutionary convergence on methylation of

tellurite. Methylation of tellurium compounds may be a mechanism responsible for tellurite resistance in both prokaryotic and eukaryotic organisms.

There were other reports that *P. aeruginosa* (Summers and Jacoby, 1977), *Thermus* spp. (Chiong *et al.*, 1988a) and *B. stearothermophilus* (Moscoso *et al.*, 1998) gave a garlic-like odor when grown in the presence of tellurite. Garlic-like odor breath was also noted in industrial workers who were exposed to Te dust, TeO_2 fumes and gaseous H_2Te_2 (Cerwenka and Cooper, 1961; Blackadder and Manderson, 1975), or after administration of tellurium compounds to rats and dogs (Karlson and Frankenberger, 1993), guinea pigs (Amdur, 1958) and ducks (Carlton and Kelly, 1967). In these reports, CH_3TeCH_3 was considered to be the odorous ingredient in breath, although other compounds of Te also have this property. Additional work is required to demonstrate methyltransferase activity directly, the forms of Te produced, and to explain the role of this aspect of metabolism in tellurite resistance.

For a long time, many gram-positive bacteria have been found to be naturally resistant to tellurite, for example, *C. diphtheriae* (Saragea *et al.*, 1979; Smith *et al.*, 1977), *E. faecalis* (Applemann and Heinmiller, 1961), and *Staphylococcus aureus* (Hoeprich *et al.*, 1960). This characteristic of resistance has formed the basis of diagnostic techniques in clinical microbiology to isolate a pathogen and its subtypes (Summers and Jacoby, 1977). However, molecular studies of tellurite resistance in gram-positive bacteria have not been reported. The work on the *S. pneumoniae tehB* gene in this research represents the first recorded attempt. The *S. pneumoniae tehB* gene appears

to encode a protein of 284 amino acids, which has a high level of similarity to TehB homologues from other bacteria (Table 6 and Fig. 6). The high-level of similarity among these bacteria is surprising when it is considered that *E. corrodens* Hag1 is a hemagglutinin (Rao *et al.*, 1993). Whether *S. pneumoniae tehB* also has erythrocyte-agglutinating activity is not known, however *E. corrodens hag1* has been demonstrated to confer tellurite resistance on *E. coli* in this study. This result presents an interesting question: how is tellurite resistance associated hemagglutination? It has been assumed that tellurite resistance might not be the main function for some of the Te^R determinants. It may also confer resistance to other toxic metal ions which have not yet been identified (Walter and Taylor, 1992). The fact that tellurite resistance was frequently found to be linked with such phenotypes as bacteriophage inhibition (Phi) and resistance to colicins (PacB) inhibition supported this hypothesis (Jobling *et al.*, 1987; Taylor and Summers, 1979; Whelan *et al.*, 1997). In this study, *E. coli tehA*, which is located in the same operon as *tehB*, was found to play only a minor role in tellurite resistance (Table 7), suggesting that *tehAtehB* may have other main function(s). The distribution of *tehB* homologues in different bacteria with a high-level homology may also indicate that TehB has other functions and is important for the survival of these bacteria.

We compared all the TehB proteins and the TehB homologues available up to a recent date. There are two types of TehB: the short form and the long form. It is surprising to find that only the *E. coli* TehB is a short protein, all other homologous proteins are longer. The extra N-terminal part of these proteins appears to be unique. We searched all the databases and found no homologues except for a hypothetical protein of

Fig. 11. Sequence comparison of *S. pneumoniae* TehB N-terminal part with **(A)** *E. coli* hypothetical protein of 119 amino acids (fl19) (accession number: AE000274) whose gene is located in the GAPA-RND intergenic region; and **(B)** *S. pombe* tryptophan synthase (accession number: P00931). Vertical lines indicate identical residues; ":" represents conservative changes. Gaps (dots) are introduced to make the best alignment. SpTehB, *S. pneumoniae* TehB; fl19, *E. coli* hypothetical protein; TrpSyn, *S. pombe* tryptophan synthase.

A

```
SpTehB        MEKLVAYKRMPLWNKQTMPEAVQQKH...NTKVGTWGKITVLKGAL 43
                :  :  :  | |||||  |  : ::|    |: | : :: |: ||
  f119    MLQIPQNYIHTRSTPFWNKQTAPAGIFERHLDKGTRPGVYPRLSVMHGAV 50

SpTehB    KFIELTEEGEVLAEHLFEAGADN.PMAQPQAWHRVEAATDDVEWYLEFYC 92
          |::   :|     :       |        |  || :|| |||    ::|:
  f119    KYLGYADEHSAEPDQVILIEAGQFAVFPPEKWHNIEAMTDDTYFNIDFFV 100

SpTehB    KPEDYFAKKYNTNPVHSEV 111
           ||           :|
  f119    APEVLMEGAQQRKVIHNGK 119
```

B

```
SpTehB: 25  KHNTKVGTWGKITVLKGALKFIELTEEGEVLAEHLFEAGADNPMAQPQ 72
              ||:  : | ||: |  |   ::   |:|::   |   || | |   |:
TrpSyn: 301 KHSATL.TMGKVGVFHGVRTYVLQREDGQIQDTHSISAGLDYPGVGPE 347
```

119 amino acids (f119), located in the so-called "GAPA-RND" intergenic region of the *E. coli* chromosome (from glyceroldehyde-3-phosphate dehydrogenase A gene to RNase D gene) (~ 40 min.), with molecular mass of 13.6 kDa which shares 25% identity (44% similarity) with the N-terminus of *S. pneumoniae* TehB (Fig. 11). Its function is unknown (Berlyn, 1998). Considering that the N-terminal part of these long-form proteins have a much lower degree of similarity than the rest of the proteins (for example, the N-terminal 100 amino acids of *S. pneumoniae* TehB is only 32% identical [39% similar] to that of *E. corrodens* Hag1), it is likely that this hypothetical protein is evolutionarily related to these proteins. In addition, this N-terminal part shares a very limited similarity to the tryptophan synthases from many different organisms. A representative comparison with a region of *S. pombe* tryptophan synthase is shown in Fig. 11b; other tryptophan synthases have similar region of similarity.

In *E. coli*, the *tehA* and *tehB* genes are in one operon at 32.3 min on the chromosome (Berlyn, 1998), while they are widely separated in the *H. influenzae* chromosome (Fleischmann *et al.*, 1997). Since the whole genome sequence of *S. pneumoniae* is not available yet, the existence of *tehA* in *S. pneumoniae* is unknown. The sequences upstream and downstream of the *S. pneumoniae tehB* have been searched from the NCBI database of unfinished genome sequences, and no *tehA* was found. *S. pneumoniae* may contain a *tehA* homologue widely separated from *tehB* like that in *H. influenzae*. However extensive PCR experiments using primers based on the homology of *E. coli* and *H. influenzae* TehA proteins did not locate a *tehA* gene (data not shown).

Filamentation of *E. coli* caused by overexpression of *S. pneumoniae tehB* (as well as *H. influenzae tehB* and *E. corrodens hagI*) raised an interesting question, because other two plasmid-encoded Te^R determinants, *i.e.*, the *ter* operon from plasmid R478 and the *kil* locus from RK2, also caused similar filamentation (Turner *et al.*, 1994; Whelan *et al.*, 1997). The *ter* determinant contains 7 genes (Jobling *et al.*, 1988; Whelan *et al.*, 1995), while *kil* has 3 genes (Walter *et al.*, 1991a). No homologies were found among these Te^R proteins, or between these Te^R proteins and known elements involved in cell division in *E. coli* (Bramhill, 1997), it was not possible to relate Te^R and cell division. In addition to cell division elements, it has been noted that some other factors, for example, the *E. coli* *S*-adenosyl-L-methionine (AdoMet) synthetase gene (*metK*) mutation (Newman *et al.*, 1998), the *E. coli* elongation factor Tu (EF-Tu) alteration (Zeef *et al.*, 1995), the *E. coli* Urf74.3 overexpression (Lyngstadaas *et al.*, 1995) and the *Salmonella typhimurium dam* gene mutation (Brawer *et al.*, 1998), also resulted in filamentation of the cells. The reason that these mutations result in filamentations is not known. In the case of the *metK* mutation in *E. coli*, it was speculated that a low level of AdoMet affects cell morphology either by influencing methylation of EF-Tu, which associates with the cell membrane and could be involved in regulating cell division, or by influencing some particular steps in cell division which need to be activated by methylation (Newman *et al.*, 1998). Like *E. coli* TehB, *S. pneumoniae* TehB as well as other homologues also have the three conserved motifs of AdoMet-dependent non-nucleic acid methyltransferases (Fig. 6). *E. coli* TehB might be a methyltransferase as evidenced by the site-directed mutagenesis on the conserved residues of motifs I and II. *S. pneumoniae* TehB and other homologues share similar high homologies with *E. coli* TehB (although they are much

longer), they may also have methyltransferase activities. It is possible that overexpression of these proteins in *E. coli* exhausts the AdoMet pool to below a certain critical threshold, which would then affect the methylation of some cell division-related proteins. This may be the reason for filamentation by these proteins. Future work is required to determine if this hypothesis is correct.

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Appendix I

Abbreviations

aa	Amino acids
AdoHcy	*S*-adenosyl-L-homocysteine
AdoMet	*S*-adenosyl-L-methionine
As	Arsenic
bp	Basepairs
DNA	Deoxyribonucleic acid
ds	Double-stranded
EDTA	Disodium ethylene diamine tetraacetate
FAD	Flavin adenine dinucleotide
GC	Gas chromotography
GNMT	Glycine *N*-methyltransferase
Hg	Mercury
hr	Hour
Inc	Incompatibility group
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobases
K_2TeO_3	Potassium tellurite
Kd	Kilodalton
lacZ	Gene for β-galactosidase
LB	Luria-Bertani broth

M	Molar
M.	DNA methyltransferase
mg	Milligram
min	Minute(s)
MIC	Minimal inhibitory concentration
ml	Milliliter
mM	Milllimolar
MS	Mass spectrometry
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide
nm	Nanometer
NTP	Nucleoside triphosphate
ORF	Open reading frame
PacB	Resistance to pore-forming colicins
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Phi	Bacteriophage inhibition
RBS	Ribosome binding site
RGAMT	Rat guanidinoacetate methyltransferase
RNase	Ribonuclease
Sb	Antimony
SD	Shine-Dalgarno sequence
SDS	Sodium dodecyl sulphate

Te	Tellurium
tehAtehB (*tehAB*)	Tellurite resistance genes A and B which are in a single operon
TehATehB	Proteins encoded by *tehAtehB*
Te^R	Tellurite resistance
THY	Todd-Hewitt broth supplemented with yeast extract
tpm	*Pseudomonas syringae* tellurite resistance gene
Tris	Tris (hydroxymethyl) aminomethane
μg	Microgram

Appendix II

Amino Acid Abbreviations

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine